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Generation of Free Radicals during Decomposition of Hydroperoxide in the Presence of Myeloperoxidase or Activated Neutrophils

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Abstract—It was shown with the spin trap α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone that myeloperoxidase (MPO) in the presence of its substrates H_2O_2 and Cl^- as well as activated neutrophils destroy *tert*-butyl hydroperoxide producing two adducts of O-centered radicals which were identified as peroxyl and alcoxyl radicals. Inhibitory analysis performed with traps of hypochlorite (taurine and methionine), free radical scavengers (2,6-di-*tret*-butyl-4-methylphenol and mannitol), and MPO inhibitors (salicylhydroxamic acid and 4-aminobenzoic acid hydrazide) revealed that the destruction of the hydroperoxide group in the presence of isolated MPO or activated neutrophils was directly caused by the activity of MPO: some radical intermediates appeared as a result of the chlorination cycle of MPO at the stage of hypochlorite generation, whereas the other radicals were produced independently of hypochlorite, presumably with involvement of the peroxidase cycle of MPO. The data suggest that the activated neutrophils located in the inflammatory foci and secreting MPO into the extracellular space can convert hydroperoxides into free radicals initiating lipid peroxidation and other free radical reactions and, thus, promoting destruction of protein–lipid complexes (biological membranes, blood lipoproteins, etc.).

Key words: myeloperoxidase, neutrophils, hypochlorite, *tret*-butyl hydroperoxide, free radicals, peroxyl radical, spin traps

Reactions of lipid peroxidation (LPO) often accompany the development of pathological processes in the human body and in some cases can induce them [1-3]. In particular, LPO can be initiated with hypochlorous acid HOCl (the ionized form is hypochlorite OCl^-) [4, 5], which is generated in the organism as a result of two-electron oxidation of chloride in the reaction

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O, \qquad (1)$$

catalyzed by myeloperoxidase (MPO) [1, 6, 7]. This enzyme is abundant in monocytes and especially in human blood neutrophils and is released into the extracellular space on activation of these cells [7, 8]. We have recently found that LPO can be initiated by the hypochlorite reaction with organic hydroperoxides, which are, as a rule, accumulated in unsaturated lipids during their autooxidation. In particular, the interaction of hypochlorite with *tert*-butyl hydroperoxide results in a free radical peroxyl intermediate, such as *tert*-butylperoxyl radical [9, 10].

To approximate natural conditions, in the present work we complicated the model, and the decomposition of *tert*-butyl hydroperoxide ($(CH_3)_3COOH$) was studied in the presence of the so-called HOCl-producing systems, such as MPO (under conditions providing catalysis by this enzyme of HOCl production) and of stimulated neutrophils which contain MPO and on activation secret it into the extracellular space. Electron paramagnetic resonance (EPR) of spin traps was used which allowed us to directly record generation of free radicals in the systems under study [11].

Abbreviations: LPO) lipid peroxidation; 4-POBN) α-(4-pyridyl-1-oxide)-N-*tret*-butylnitrone; EPR) electron paramagnetic resonance; BHT) 2,6-di-*tret*-butyl-4-methylphenol; MPO) myeloperoxidase.

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MATERIALS AND METHODS

Chemical reagents. We used α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (4-POBN), hypochlorite, 2,6-di-*tert*-butyl-4-methylphenol (BHT), D-mannitol, and zymosan A (Sigma, USA); salicylhydroxamic acid and 4-aminobenzoic acid hydrazide (Aldrich, USA); H_2O_2 (Merck, Germany); urographin (Schering, Germany); *tert*-butyl hydroperoxide, L-methionine, and taurine (Fluka, Switzerland); MPO from human polymorphonuclear leukocytes (Planta Natural Products, Austria). Other reagents were of domestic production.

Neutrophils were isolated from human blood as described in [12, 13]. Blood was collected into a plastic vessel containing heparin (10 U/ml) and maintained for 1 h at room temperature that resulted in sedimentation of most of the erythrocytes. The supernatant (2 ml) containing leukocytes and platelets was taken and carefully deposited onto 3 ml of the Ficoll–urographin mixture with the specific density of 1.078 g/cm^3. Then the specimens were centrifuged for 25 min at 400*g*, the precipitate was supplemented with 1 ml of distilled water, mixed for 40 sec to provide lysis of erythrocytes in the precipitate. The remaining cells were washed twice in 10 ml of Hanks' solution (pH 7.4), and the suspension was centrifuged for 10 min at 400*g*. Then the precipitate was carefully resuspended in 1 ml of Hanks' solution (pH 7.4), and neutrophils were counted in a Goryaev's chamber. All procedures were performed in plastic vessels at 4°C.

EPR studies. Generation of free radicals catalyzed by MPO and in the presence of activated neutrophils was studied with spin traps using an E-4 radiospectrometer (Varian, USA) at room temperature. The spectra were recorded at magnetic field strength 0.33 T, UHF radiation power 10 mW, modulation amplitude 1 mT, and time constant 0.03 sec. Spectral parameters of the spin adducts were analyzed using the hyperfine splitting constants a_β^H and a^N and ΔH_{PP} (the linewidth) [11].

In experiments with MPO, specimens were prepared as follows: in the medium of measurement (50 mM phosphate buffer, pH 7.4) a spin trap (80 mM) and $(CH_3)_3COOH$ (1 mM) were placed, then 20 μM H_2O_2, 63 mM NaCl, and 70 nM MPO were added, the mixture was mixed rapidly, and in a flat quartz cuvette placed into the radiospectrometer resonator. In experiments with activated neutrophils, the specimens were prepared as follows: neutrophils ((15-23)·10^6 cell/ml) in Hanks' solution containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+} (pH 7.4) were supplemented with the spin trap (80 mM), zymosan (6 mg/ml), and $(CH_3)_3COOH$ (1 mM), the mixture was mixed rapidly, and in a flat quartz cuvette placed into the radiospectrometer resonator.

Processing of EPR spectra. Constants of hyperfine splitting of the EPR spectra were calculated and the recorded spin adducts were identified using a program and database of parameters of EPR spectra of spin adducts presented in Internet (http://epr.niehs.nih.gov). The program and its application for modeling EPR spectra are described in detail in [14]. Based on empirically determined parameters of the experimental EPR spectrum, this program allowed us to obtain a simulated spectrum, optimize it by comparing with the experimental spectrum, and thus determine more accurately the constants of hyperfine splitting (as compared to values of empirical constants).

Results were processed statistically. The significance of differences was determined using Student's *t*-test. The differences were considered significant at $p < 0.05$.

RESULTS

Generation of free radical intermediates during decomposition of hydroperoxide in the presence of MPO. Hypochlorite is produced in the organism during the reaction (1) catalyzed by MPO. Using spin traps, we studied the generation of free radical intermediates during the hydroperoxide group decomposition in the presence of MPO and its substrates, Cl^- and H_2O_2.

The experimental EPR spectrum recorded in the system of MPO + H_2O_2 + NaCl in the presence of the spin trap is presented in Fig. 1a. We supposed that this experimental spectrum should be a sum of two signals and succeeded in modeling it. The model spectrum is virtually identical to the experimental one (Fig. 1, a and b). From the model spectrum individual signals of two components were isolated which could belong to two spin adducts (data not presented). The signal of the first adduct was characterized by the following spectral parameters: a_β^H = 0.230 mT, a^N = 1.500 mT, ΔH_{PP} = 0.042 mT. These parameters were close to spectral parameters of the adduct obtained earlier on the interaction of hypochlorite or Ce^{4+} with $(CH_3)_3COOH$ and identified as an adduct of *tert*-butylperoxyl radical $(CH_3)_3COO^\bullet$ [9, 10]. The constants of hyperfine splitting of the second component of the EPR spectrum were as follows: a_β^H = 0.289 mT, a^N = 1.640 mT, ΔH_{PP} = 0.068 mT, i.e., close to parameters of the spin adduct obtained by us earlier on the interaction of Fe^{2+} with $(CH_3)_3COOH$ [9, 10]. Most likely, it was an adduct of the *tert*-butoxyl radical $(CH_3)_3CO^\bullet$. With increase in the MPO concentration in the incubation medium the intensity of the total EPR signal and, respectively, the quantity of produced radicals increased and flattened out (Fig. 2). In the next experiments, we used 70 nM MPO.

In control experiments in the absence of MPO or its substrate H_2O_2, the EPR spectrum of spin adducts was not recorded at all. If the medium of measurement lacked Cl^-, the intensity of the EPR signal was noticeably decreased (Table 1).

To elucidate the mechanism of generation of free radical intermediates in the system of MPO + H_2O_2 +

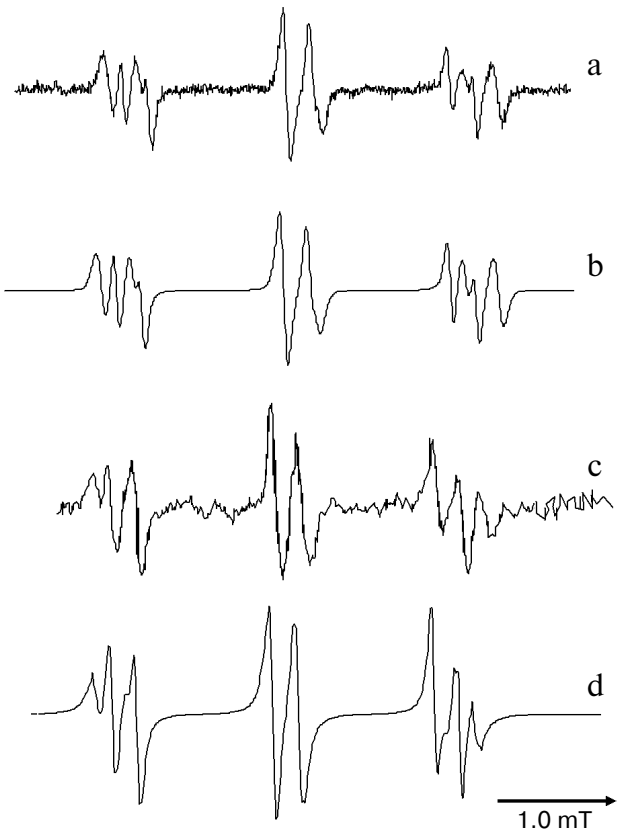


Fig. 1. EPR spectra. a) The EPR spectrum obtained in the system of MPO (70 nM) + H_2O_2 (20 μM) + NaCl (63 mM) + $(CH_3)_3COOH$ (1 mM) in the presence of the spin trap 4-POBN (80 mM). b) Modeling of the spectrum (a). The measurements were performed at room temperature in 50 mM phosphate buffer (pH 7.4). c) The EPR spectrum obtained in the system of neutrophils ($15 \cdot 10^6$ cell/ml) + zymosan (6 mg/ml) + $(CH_3)_3COOH$ (1.8 mM) in the presence of the spin trap 4-POBN (80 mM). d) Simulation of the spectrum (c). The measurements were performed at room temperature in Hanks' solution containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+} (pH 7.4).

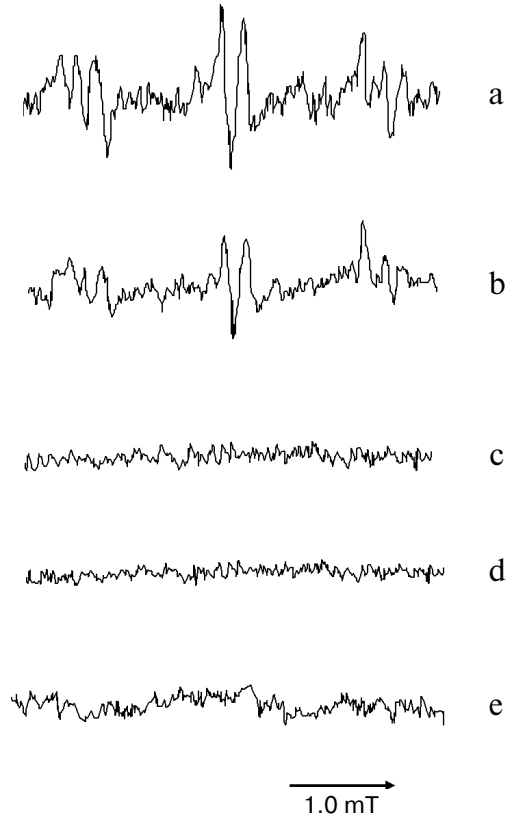


Fig. 3. EPR spectra of spin adducts of the 4-POBN trap recorded in the following systems: a) neutrophils ($18 \cdot 10^6$ cell/ml) + 4-POBN (80 mM) + $(CH_3)_3COOH$ (1.8 mM) + zymosan (6 mg/ml); b) neutrophils ($18 \cdot 10^6$ cell/ml) + 4-POBN (80 mM) + $(CH_3)_3COOH$ (1.8 mM); c) neutrophils ($18 \cdot 10^6$ cell/ml) + 4-POBN (80 mM); d) neutrophils ($18 \cdot 10^6$ cell/ml) + 4-POBN (80 mM) + zymosan (6 mg/ml); e) 4-POBN (80 mM) + $(CH_3)_3COOH$ (1.8 mM) + zymosan (6 mg/ml). The measurements were performed at room temperature in Hanks' solution containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+} (pH 7.4).

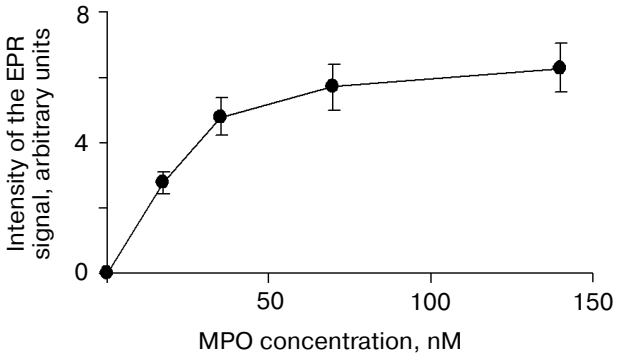


Fig. 2. Dependence of the EPR signal intensity of spin adducts on the MPO concentration in the system of MPO + H_2O_2 (20 μM) + NaCl (63 mM) + $(CH_3)_3COOH$ (1 mM) in the presence of the spin trap 4-POBN (80 mM). The measurements were performed at room temperature in 50 mM phosphate buffer (pH 7.4).

NaCl + $(CH_3)_3COOH$, the incubation medium was presupplemented with free radical or hypochlorite scavengers or MPO inhibitors. The results are presented in Table 1. In the presence of radical scavengers mannitol and BHT, the EPR signal was considerably lower. This finding additionally confirms that free radicals are generated during the hydroperoxide group decomposition in the presence of functioning MPO. The HOCl scavengers taurine and methionine also pronouncedly prevented the generation of spin adducts, and this suggested the involvement of HOCl in the arising of free radical intermediates on addition of H_2O_2 into the system of MPO + NaCl + $(CH_3)_3COOH$. And finally, we failed to record the EPR signal when MPO inhibitors (salicylhydroxamic acid or 4-aminobenzoic acid hydrazide) were introduced into the incubation medium. Thus, we confirm that the generation of all adducts in the system of 4-POBN +

Table 1. Effects of varied agents on the intensity of EPR signal of the spin adducts generated on the incubation of *tert*-butyl hydroperoxide with MPO and its substrates in the presence of the spin trap

System studied	Intensity of EPR signal, % of control
MPO + H_2O_2 + Cl^- (control)	100 ± 6
without MPO	0
without H_2O_2	0
without Cl^-	67 ± 5
+ mannitol (10 mM)	35 ± 4
+ BHT (50 μM)	37 ± 7
+ taurine (1 mM)	45 ± 5
+ methionine (1 mM)	37 ± 5
+ salicylhydroxamic acid (100 μM)	0
+ 4-aminobenzoic acid hydrazide (50 μM)	0

MPO + H_2O_2 + NaCl + $(CH_3)_3COOH$ is caused by functioning of the enzyme.

Generation of free radicals during hydroperoxide decomposition in the presence of neutrophils. It is known that activation of polymorphonuclear leukocytes is accompanied by production of HOCl/OCl^- in reaction (1) catalyzed by MPO [1, 4, 6-8]. We used a model system, which allowed us to study the generation of free radicals during decomposition of hydroperoxide in the presence of activated neutrophils, i.e., under conditions maximally approximating those *in vivo*.

Figure 3a presents the experimental EPR spectrum, which was obtained on the incubation of neutrophils activated with opsonized zymosan in the presence of the spin trap after addition of *tert*-butyl hydroperoxide. The signal indicated the generation of free radicals. In the absence of zymosan, we also recorded an EPR signal (Fig. 3b), but its intensity was lower than in the presence of zymosan (Fig. 3a). In the absence in the incubation medium of hydroperoxide (Fig. 3, c and d) or neutrophils (Fig. 3e) no EPR spectrum was recorded. Zymosan itself added to the spin trap and hydroperoxide gave no signal (Fig. 3e). Thus, it was suggested that neutrophils (better the activated ones) and hydroperoxide should be present for generation of free radicals.

To identify free radical intermediates produced in the system under study, we simulated an experimental spectrum in the belief that it is a sum of two signals. The experimental and model spectra are shown in Figs. 1c and 1d, respectively. Obviously, they are virtually identical. We have also simulated individual spectra of these two components, which seemed to belong to two spin adducts (data not presented). The signal of the first adduct had the following constants of the hyperfine splitting: a_β^H = 0.234 mT, a^N = 1.493 mT, ΔH_{PP} = 0.067 mT. These values were close to values of spectral parameters of the adduct obtained by us on the interaction of hypochlorite or Ce^{4+} with $(CH_3)_3COOH$ and identified earlier as the adduct of *tert*-butylperoxyl radical $(CH_3)_3COO^\bullet$ [9, 10]. It contributed 55% to the total spectrum. The spectral parameters of the second component were as follows: a_β^H = 0.286 mT, a^N = 1.628 mT, ΔH_{PP} = 0.092 mT. These values were close to values of the parameters of the spin adduct obtained on the interaction of Fe^{2+} with $(CH_3)_3COOH$ [9, 10]. Most likely, it was the adduct of *tert*-butoxyl radical $(CH_3)_3CO^\bullet$. Its fraction in the total spectrum was 45%.

Figure 4 presents the kinetic curve of changes in the intensity of the EPR signal recorded in the system of 4-POBM + neutrophils + zymosan + $(CH_3)_3COOH$. The produced adducts were relatively stable. Moreover, their contents in the incubation medium increased with time, and the hyperfine splitting constants did not change. Thus, the generated primary spin adducts did not transform into chemically different adducts.

Table 2 presents data on the comparative effects of free radical and hypochlorite scavengers, MPO inhibitors on production of 4-POBN spin adducts in two systems. System 1 includes human blood neutrophils activated with opsonized zymosan supplemented with *tert*-butyl hydroperoxide. In system 2 *tert*-butyl hydroperoxide was added to hypochlorite solution. Both systems were characterized by a decreased level of the spin adducts generat-

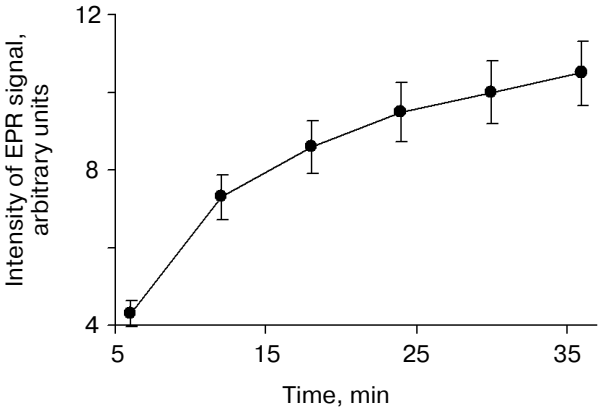


Fig. 4. Kinetic curve of changes in the intensity of the central component of the EPR signal recorded in the system of 4-POBN (80 mM) + neutrophils ($23 \cdot 10^6$ cell/ml) + zymosan (6 mg/ml) + $(CH_3)_3COOH$ (1.8 mM). The measurements were performed at room temperature in the Hanks' solution containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+} (pH 7.4).

Table 2. Intensity of EPR signal (% of control) of spin adducts produced in the systems of 4-POBN (80 mM) + neutrophils ((20 ± 2)·10^6 cell/ml) + $(CH_3)_3COOH$ (2 mM) (system 1) and 4-POBN (40 mM) + $(CH_3)_3COOH$ (2 mM) + HOCl (1 mM) (system 2) in the presence of varied additions*

Addition	System 1	System 2
—	85 ± 9	100 ± 9 (control)
Zymosan (6 mg/ml)	100 ± 9 (control) **	87 ± 8**
Mannitol (10 mM)	50 ± 8**	44 ± 5
BHT (50 μM)	56 ± 9**	44 ± 7
Taurine (10 mM)	68 ± 8**	38 ± 5
Methionine (10 mM)	53 ± 6**	26 ± 3
Salicylhydroxamic acid (50 μM)	44 ± 5**	89 ± 11
4-Aminobenzoic acid hydrazide (50 μM)	41 ± 5**	65 ± 7

* The measurements were performed at room temperature in Hanks' solution containing 1 mM Ca^{2+} and 0.5 mM Mg^{2+} (pH 7.4).

** The measurements were performed in the presence of zymosan (6 mg/ml).

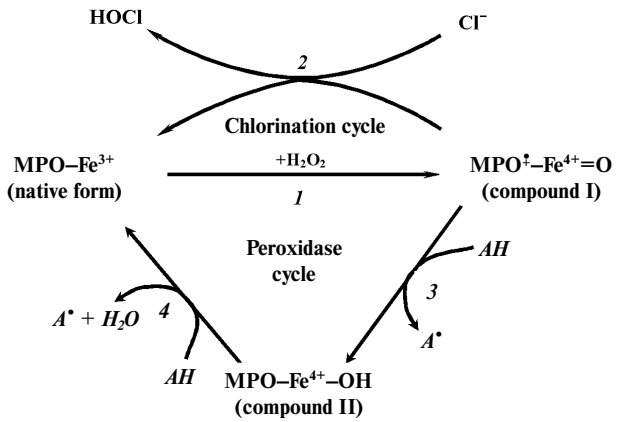
ed on the addition of hypochlorite scavengers (taurine and methionine) and free radical traps (BHT and mannitol). MPO inhibitors (4-aminobenzoic acid hydrazide and salicylhydroxamic acid) considerably prevented the generation of spin adducts in system 1 and slightly affected it in system 2. Obviously, their effect in system 1 was mainly caused by inhibiting MPO of neutrophils. A slight decrease in the level of spin adducts in system 2 in the presence of 4-aminobenzoic acid hydrazide seemed to be due to a direct interaction of hypochlorite with the amino group of this agent. Zymosan somewhat decreased the level of spin adducts in system 2 that most likely was caused by the interaction of hypochlorite with serum components adsorbed on the surface of zymosan particles during its opsonization. However, zymosan also increased the production of spin adducts in system 1. Most likely, it was due to increased functioning of the enzyme systems, including MPO, as a result of the enzyme secretion into the extracellular space during activation of neutrophils.

DISCUSSION

The only pathway of hypochlorite generation in a living organism is a two-electron oxidation of chloride in the MPO-catalyzed reaction (1). A simplified mechanism of the MPO functioning is presented by the scheme. The catalytic cycle results in the successive oxidation and reduction of the heme iron. The native MPO (ferri-enzyme MPO–Fe^{3+}) reacts with H_2O_2, and compound I (MPO‡–Fe^{4+}=O) is produced which contains two oxidizing equivalents as compared to the ferri-form (reaction *1* in the scheme) [15-17]. Compound I is unstable (its half-life is 100 msec), highly reactive, and catalyzes two-electron oxidation of halogenides to hypohaloid acids with production of native ferri-form of the enzyme (reaction *2* in the scheme) [15, 18, 19]. Reactions *1* and *2* (scheme) present the so-called chlorination cycle, which is described by the overall equation (1).

In the presence of one-electron donors of hydrogen (AH – phenols, anilines, β-diketones, nitrite, etc.), compound I can be reduced to compound II (MPO–Fe^{4+}–OH), which is unable to catalyze the production of HOCl (reaction *3* in the scheme) [16, 18]. Compound II, similarly to compound I, catalyzes oxidation of AH and is converted into the native enzyme (reaction *4* in the scheme) [15]. Thus, reactions *1*, *3*, and *4* (the scheme) present a classic peroxidase cycle. The relative concentration of Cl^- and AH determines the mechanism of the enzyme functioning (chlorination or peroxidation).

In the present work the EPR spectrum was recorded upon the addition of *tert*-butyl hydroperoxide to MPO or neutrophils isolated from human blood in the presence of the spin trap 4-POBN, and this indicated the generation of free radical intermediates in these systems. Simulation of the experimental EPR spectra allowed us to determine precise hyperfine splitting constants which characterized the EPR signals belonging to two adducts generated in the two systems studied: 4-POBN + MPO + H_2O_2 + NaCl + $(CH_3)_3COOH$ and 4-POBN + neutrophils + zymosan + $(CH_3)_3COOH$. Values of these spectral parameters are given in Table 3. For comparison, in the same table the hyperfine splitting constants are presented of the EPR



Scheme of MPO transformations

spectra of the spin adducts recorded by us on addition of hypochlorite to $(CH_3)_3COOH$ and also during the reactions

$$(CH_3)_3COOH + Fe^{2+} \rightarrow (CH_3)_3CO^{\cdot} + Fe^{3+} + OH^-, \quad (2)$$

$$(CH_3)_3COOH + Ce^{4+} \rightarrow (CH_3)_3COO^{\cdot} + Ce^{3+} + H^+, \quad (3)$$

where alkoxyl and peroxyl radicals, respectively, are known to be generated [20, 21]. One can see that the spectral parameters of the spin adducts recorded by us in the systems of 4-POBN + MPO + H_2O_2 + NaCl + $(CH_3)_3COOH$ and 4-POBN + neutrophils + zymosan + $(CH_3)_3COOH$ are in coincidence with those of the adducts produced during reactions (2) and (3). Based on this finding, we confirm that *tert*-butyl hydroperoxide is decomposed in the presence of MPO or neutrophils producing two types of O-centered radicals (peroxyl and alkoxyl ones).

The generation of free radicals upon addition of $(CH_3)_3COOH$ to hypochlorite, MPO, or neutrophils was also confirmed by the finding that the intensity of the EPR signal of the recorded spin adducts was considerably decreased in the presence of free radical scavengers (BHT or mannitol) in the reaction medium (Tables 1 and 2).

In the absence of MPO or its substrate (H_2O_2) and also in the presence of MPO inhibitors, the EPR signal was not detected at all (Table 1). Thus, the generation of spin adducts observed in the system of 4-POBN + MPO + H_2O_2 + NaCl + $(CH_3)_3COOH$ was caused only by functioning of MPO.

Is the production of O-centered radicals during decomposition of hydroperoxide in the presence of MPO associated with the chlorination cycle, i.e., with generation of hypochlorite, or does this occur due to peroxidase cycle of the enzyme? The decrease in the EPR signal in the presence of hypochlorite scavengers taurine and methionine unambiguously indicated the involvement of hypochlorite in the generation of free radicals (Table 1). The EPR spectrum of spin adducts was recorded, although its intensity was decreased. Thus, some of the free radicals were produced without involvement of hypochlorite.

In fact (Table 1), spin adducts were nevertheless produced in the absence of chloride in the reaction medium, whereas in the absence of hydrogen peroxide the EPR signal was not recorded at all. This means that free radicals are produced only in the case when the native enzyme can convert into compound I (reaction *1* in the scheme). Then in the presence of chloride, the chlorination cycle results in generation of hypochlorite (reaction *2* in the scheme) which was earlier shown to oxidize $(CH_3)_3COOH$ to $(CH_3)_3COO^{\cdot}$ [9, 10]. But some of $(CH_3)_3COO^{\cdot}$ radicals seem to be generated as a result of one-electron oxidation of $(CH_3)_3COOH$ with compound I. This was shown by authors of work [22] who succeeded in demonstrating the reduction of compound I to compound II in the presence of $(CH_3)_3COOH$.

Activated neutrophils destroy *tert*-butyl hydroperoxide with involvement of MPO, because inhibitors of this enzyme considerably prevent the production of free radical intermediates recorded with spin traps. Moreover, this was at least partially associated with the chlorination cycle because taurine and methionine lowered the yield of free radicals during this reaction (Table 2).

Note, that during the reaction of hypochlorite with $(CH_3)_3COOH$ we recorded only the adduct of *tert*-butylperoxyl radical (Table 3 and [9, 10]). However, in the systems of 4-POBN + MPO + H_2O_2 + NaCl + $(CH_3)_3COOH$ and 4-POBN + neutrophils + zymosan +

Table 3. Spectral parameters of spin adducts of radicals produced in the systems of MPO + H_2O_2 + NaCl + $(CH_3)_3COOH$ and neutrophils + zymosan + $(CH_3)_3COOH$ and parameters of adducts of the radicals recorded earlier in the reaction of $(CH_3)_3COOH$ with Fe^{2+}, Ce^{4+}, or $HOCl/OCl^-$ in the presence of the spin trap 4-POBN

System under study	Resulting radical	Parameters of the EPR spectrum of the spin adduct		
		a^H_β, mT	a^N, mT	ΔH_{PP}, mT
MPO + H_2O_2 + NaCl + $(CH_3)_3COOH$	$(CH_3)_3COO^{\bullet}$ $(CH_3)_3CO^{\bullet}$	0.230 ± 0.009 0.289 ± 0.007	1.500 ± 0.008 1.640 ± 0.005	0.042 ± 0.007 0.068 ± 0.008
Neutrophils + zymosan + $(CH_3)_3COOH$	$(CH_3)_3COO^{\bullet}$ $(CH_3)_3CO^{\bullet}$	0.234 ± 0.007 0.286 ± 0.008	1.493 ± 0.012 1.628 ± 0.006	0.067 ± 0.003 0.092 ± 0.005
Fe^{2+} + $(CH_3)_3COOH$*	$(CH_3)_3CO^{\bullet}$	0.276 ± 0.009	1.633 ± 0.007	0.085 ± 0.008
Ce^{4+} + $(CH_3)_3COOH$*	$(CH_3)_3COO^{\bullet}$	0.233 ± 0.008	1.510 ± 0.009	0.063 ± 0.007
$HOCl/OCl^-$ + $(CH_3)_3COOH$*	$(CH_3)_3COO^{\bullet}$	0.233 ± 0.007	1.484 ± 0.015	0.063 ± 0.009

* Results of previous studies [9, 10].

$(CH_3)_3COOH$ not only *tert*-butylperoxyl but also *tert*-butoxyl radical was detected. Most likely, the $(CH_3)_3COO^{\cdot}$ radical is primarily produced, and it can recombine with production of *tert*-butoxyl radical according to the reaction equation [23]:

$$2(CH_3)_3COO^{\cdot} \rightarrow (CH_3)_3COOOOC(CH_3)_3 \rightarrow$$

$$\rightarrow 2(CH_3)_3CO^{\cdot} + {}^3O_2.$$

Thus, destruction of the hydroperoxide group in the presence of isolated MPO or activated neutrophils accompanied by generation of peroxyl or alkoxyl radicals is directly caused by functioning of MPO. However, only some of the radical intermediates appear during the MPO chlorination cycle, which is the stage of hypochlorite production. The other radical intermediates seem to appear independently of hypochlorite, but with involvement of the peroxidase cycle of MPO. Thus, the activated neutrophils, which are located in inflammatory foci and secrete MPO into the extracellular space can indeed convert hydroperoxide into free radicals and initiate lipid peroxidation and also other free radical reactions thus promoting destruction of protein–lipid complexes. This hypothesis seems more reasonable on taking into account an increased amount of hydroperoxides in inflammatory foci [1, 3, 24].

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