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CELL BIOPHYSICS

The Effects of Antioxidants and Hypohalous Acid Scavengers on Neutrophil Activation by Hypochlorous Acid-Modified Low-Density Lipoproteins

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Abstract—Hypochlorous acid-modified human blood low density lipoprotein (LDL–HOCl) was shown to stimulate neutrophils and to increase the luminol- (lm-CL) or lucigenin-enhanced chemiluminescence (lc-CL) of neutrophils. Antioxidants and HOCl scavengers (glutathione, taurine, cysteine, methionine, ceruloplasmin, and human serum albumin (HSA)) were tested for effects on lm-CL, lc-CL, H_2O_2 production, and degranulation of azurophilic granules of neutrophils. All agents used in increasing concentrations were found to decrease lm-CL produced by neutrophils upon stimulation with LDL–HOCl or subsequent treatment with the activator phorbol 12-myristate 13-acetate (PMA). The agents exerted a far lower, if any, effect on lc-CL and the H_2O_2 production by neutrophils in the same conditions. In the majority of cases, a decline in neutrophil chemiluminescence in the presence of the agents was not related to their effect on neutrophil degranulation, but was most likely due to their direct interactions with reactive halogen (RHS) or oxygen (ROS) species generated upon neutrophil activation or to myeloperoxidase (MPO) inhibition. Antioxidants and HOCl scavengers present in the human body were assumed to decelerate the development of oxidative or halogenative stress and thereby prevent neutrophil activation.

Keywords: halogenative stress, oxidative stress, low density lipoprotein, neutrophil activation, reactive halogen species, chemiluminescence

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INTRODUCTION

The human body contains enzymes of the mammalian heme peroxidase (donor: H_2O_2 oxidoreductase) family (EC 1.11.1.7), which includes myeloperoxidase (MPO) [1]. MPO is secreted into the extracellular space as a result of neutrophil activation and degranulation in an inflammation focus and produces reactive halogen species (RHS), such as HOCl, HOBr, and other species, which act as potent oxidants and halogenating agents [1, 2]. This property allows peroxidases to perform a bactericidal function and to protect the body from pathogens and, on the other hand, determines their involvement in a number of events that are associated with host cell and tissue damage and lead to oxidative or halogenative stress [2, 3]. Several biologically important molecules, such as human serum albumin (HSA) [4], phosphatidylcholine [5], and serum low density lipoprotein (LDL) modified with HOCl (LDL–HOCl) or HOBr, increase neutrophil degranulation and MPO exocyto-

Abbreviations: MPO, myeloperoxidase; RHS, reactive halogen species; HSA, human serum albumin; LDL, low density lipoprotein; ROS, reactive oxygen species; PMA, phorbol 12-myristate 13-acetate; fMLP, N-formyl-Met-Leu-Phe; PBS, phosphate-buffered saline; LDL–HOCl, HOCl-modified LDL; lm-CL, luminol-enhanced chemiluminescence; lc-CL, lucigenin-enhanced chemiluminescence.

sis and thereby aggravate oxidative or halogenative stress.

To aggravate LDL oxidative modification, MPO binds to the LDL surface while catalyzing RHS production and thereby further modifies LDL to facilitate the LDL uptake by endothelial cells, cholesterol accumulation in LDL, and early atherosclerosis [6]. RHS-modified LDL is known to activate neutrophils to stimulate production of reactive oxygen species (ROS), neutrophil adhesion to the endothelium, and degranulation of azurophilic and specific granules [6, 9]. These events increase MPO exocytosis and close the circle of modified LDL generation [6].

At the same time, so-called antihalogenating agents are present in the body. Some of these act as scavengers as a result of the high rate constant of their interaction with RHS. As an example, the rate constants of the reaction with HOCl/HOBr exceed 10^5 $M^{-1}s^{-1}$ for methionine, cysteine, glutathione, taurine, ascorbate, urate, carnosine, histamine, and NADH, which are all found in the human body [10]. Other agents act to inhibit peroxidase activity. As an example, the acute-phase protein ceruloplasmin binds with MPO and eosinophil peroxidase and inhibits their peroxidase and halogenating activities [11–13]. Thiocyanate competes with halogenides for MPO compound I and thereby hinders RHS generation [14, 15]. Tryptophan converts MPO compound I to compound II, which is not involved in the halogenation cycle, and thereby renders HOCl and HOBr production less likely [16]. The balance between pro- and antihalogenation systems of the body determines the likelihood of oxidative or halogenative stress, LDL modification, and atherosclerosis. We have previously shown that HSA modified in halogenative stress conditions stimulates the respiratory burst in neutrophils [17]. The objective of this work was to study how endogenous compounds that possess antioxidant and antihalogenation properties (ceruloplasmin, HSA, glutathione, taurine, cysteine, and methionine) affect neutrophil activation induced by LDL after its modification in conditions that simulate oxidative/halogenative stress.

MATERIALS AND METHODS

Reagents. Salts to prepare buffer solutions, Krebs–Ringer solution (cat. no. K4002), NaOCl, 10-acetyl-3,7-dihydrophenoxazine (Ampliflu Red), Histopaque, *o*-dianisidine, phorbol 12-myristate 13-acetate (PMA), 4-aminobenzoic acid hydrazide, luminol, lucigenin, neomycin trisulfate, cysteine, reduced glutathione, sodium citrate, scopoletin, N-formyl-Met–Leu–Phe (fMLP), phenyl-agarose, Sephacryl S-200 HR, VivaSpin 20 concentrators (molecular weight cut-off 1 MDa), superoxide dismutase, HSA, D-glucose, elastase substrate MeO–Suc–Ala–Ala–Pro–Val–MCA, horseradish peroxidase, and NaN_3 were from Sigma-Aldrich (United States); taurine and methionine were from Fluka (Switzerland); Tween 20, UNO-Sphere Q, and A-1.5m agarose gel were from Bio-Rad (United States); and Lymphoprep was from Nycomed (Norway).

The ceruloplasmin preparation, which was stable during storage, had a more than 95% content of 132-kDa protein, and showed A_{610}/A_{280} greater than or equal to 0.050, was obtained via ion-exchange chromatography of a citrate-stabilized plasma sample on UNO-Sphere Q and affinity chromatography on neomycin-agarose [18].

Myeloperoxidase (A_{430}/A_{280} greater than or equal to 0.83) was isolated from a leukocyte extract by affinity chromatography on heparin-agarose, hydrophobic chromatography on phenyl-agarose, and gel filtration on Sephacryl S-200 HR [13].

The sodium hypochlorite (NaOCl) concentration of a commercial solution was calculated from the hypochlorite anion (OCl^-) absorption measured spectrophotometrically at 290 nm and pH 12, using the molar extinction coefficient $\varepsilon_{290} = 350$ $M^{-1}cm^{-1}$ [19]. Given that HOCl has p$K = 7.5$ [19] and that half of it occurs in a molecular form while the other half occurs in a dissociated form at physiological pH values, HOCl is hereafter understood as a HOCl/OCl^- mixture that is present in a medium.

Neutrophil isolation. Neutrophils were isolated from a donor blood sample, which was stabilized by adding one volume of 3.8% sodium citrate to nine volumes of blood. The sample was centrifuged through a Lymphoprep density gradient at 400 *g* for 35 min according to a published protocol [20]. Alternatively, we used a Histopaque double density gradient (1.119 and 1.078 g/mL). Cells were washed with phosphate-buffered saline (PBS; 10 mM Na-phosphate, 140 mM NaCl, pH 7.4) containing 2.7 mM KCl and 5 mM D-glucose, stored at 4°C, and used in experiments within 4–6 h. The neutrophil content in a cell suspension was 97–98%; the cell viability was no less than 96% according to the Trypan Blue exclusion test.

LDL isolation and modification. Fasting blood samples were collected in vacuum blood tubes with EDTA and centrifuged at 3000 *g* at 4°C for 5 min to obtain the plasma. Trace amounts of chylomicrons and platelets were removed by centrifugation at 13000 *g* at 4°C for 15 min. The plasma sample was combined with 153 mg NaCl and 354 mg KBr per 1 mL plasma, the salts were allowed to dissolve, and the sample was filtered through a 0.22-μm filter. One volume of the plasma was added to a centrifuge tube, two volumes of a 1.063 g/mL solution (33 mg/mL NaCl, 59 mg/mL KBr) were layered; two volumes of a 1.019 g/mL solution (14.5 mg/mL NaCl, 13.6 mg/mL KBr) were then layered. The tubes were placed in a JS-24.38 bucket rotor and ultracentrifuged in an Avanti J-30I centrifuge (Beckman Coulter, United States) at 110500 *g* at 4–8°C for 18 h. The LDL-containing interphase,

which was orange in color, was collected with a syringe and transferred into a VivaSpin 20 device with a 1-MDa molecular weight cut-off. Centrifugation at 3000 *g* at 4°C for 1 h reduced the LDL sample volume to 0.4 mL. The sample was diluted with PBS to 20 mL. The procedure of LDL concentration and dilution with PBS was repeated two more times. The resulting LDL was homogeneous according to 0.8% agarose-gel electrophoresis [8]; the KBr content was lower than 1 μM.

Oxidative/halogenative stress conditions were simulated by treating LDL with NaOCl at a 1 : 160 molar ratio. For this purpose, 3.2 mM NaOCl in PBS was combined with LDL (10 mg/mL protein) and the mixture was incubated at 37°C with periodic agitation. Control experiments showed lack of *o*-dianisidine oxidation with excess NaOCl, indicating that the total NaOCl amount reacted with LDL.

Neutrophil chemiluminescence was continuously recorded with a LKB Wallc 1251 luminometer (Finland) at 37°C with continuous agitation. Neutrophils were added to a 1-mL sample (0.2 mM luminol or lucigenin in Krebs–Ringer solution, pH 7.4) to a final concentration of $0.4 \cdot 10^6$ cells/mL, and spontaneous chemiluminescence was measured. Native LDL or LDL–HOCl was then added to 0.25 mg/mL protein, and chemiluminescence was measured again (chemiluminescence response to LDL). Approximately 3 min later, the cells were stimulated with 0.16 μM PMA, and the chemiluminescence response to the activator was recorded. The chemiluminescence intensity was measured as a peak height (mV). Compounds to be tested were added as PBS solutions immediately before adding LDL.

Neutrophil H_2O_2 production was assayed by a fluorescence method, using a LSF1211A computerized spectrofluorimeter (SOLAR, Minsk, Belarus) and scopoletin as a peroxidase reaction substrate [20]. A neutrophil suspension (10^6 cells/mL in PBS, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.35) along with 1 μM scopoletin, 20 μg/mL horseradish peroxidase, and 1 mM NaN_3, was incubated in a cuvette at 37°C with continuous agitation for 3 min, with or without test compounds: methionine, cysteine, taurine, ceruloplasmin, and HSA. LDL or LDL–HOCl was then added to 0.1 or 0.4 mg/mL protein, respectively, and the kinetics of scopoletin oxidation was recorded by measuring the intensity of fluorescence at 460 nm (excitation at 350 nm). The H_2O_2 production rate of the cells was determined as the slope of a linear region of the kinetic curve, which showed how the scopoletin fluorescence intensity decreased as scopoletin was oxidized by H_2O_2. The linear region included at least ten experimental points. Calculations were performed using Orign 7.0 statistics software.

Supernatants obtained after neutrophil degranulation. A neutrophil suspension (10^6 cells/mL) in Krebs–Ringer solution (pH 7.4) was combined with an antioxidant (methionine, cysteine, taurine, glutathione, ceruloplasmin, or HSA) to 0.25 mg/mL protein. Some of the samples were also combined with 100 ng/mL PMA. The samples were incubated at 37°C for 15 min with periodic agitation; cells were pelleted by centrifugation (400 g, 15 min); the supernatant was collected, frozen immediately, and stored at –40°C.

Myeloperoxidase activity in the supernatant was estimated from the resorufin fluorescence. Resofurin was a product of 10-acetyl-3,7-dihydrophenoxazine oxidation by brominating MPO, which was bound to rat anti-MPO antibodies adsorbed on the surface of plate wells [21]. Antibodies (5 μg/mL in 40 mM Na_2CO_3, 80 mM $NaHCO_3$, pH 9.4) were adsorbed on 96-well polystyrene plates at 4°C overnight. The plates were washed three times with PBS with 0.05% Tween 20; MPO was added to the wells at 0, 0.625, 1.25, 2.5, 5, 10, 20, and 40 ng/mL; the supernatants were diluted 10- to 80-fold with PBS containing 0.05% Tween 20 and added to the wells. The plates were incubated on a shaker (270 rpm) at 37°C for 60 min and washed three times with PBS containing 0.05% Tween 20. Then 1 μM 10-acetyl-3,7-dihydroxyphenoxazine in 24 mM Na-citrate (pH 6.0) containing 0.01 mM H_2O_2, 20 mM NaBr and 200 mM $(NH_4)_2SO_4$ was added to the wells and the plates were incubated on a shaker (270 rpm) at 37°C for 30 min. The resorufin fluorescence was measured at 580–620 nm (excitation at 535–555 nm) using a CLARIOstar monochromator multimode microplate reader (BMG LABTECH, Germany). A calibration plot of the fluorescence intensity I as a function of the MPO concentration ([MPO]) was processed as the binomial function $I = a[\mathrm{MPO}]^2 + b[\mathrm{MPO}] + c$ in Excel 2002 spreadsheets (the coefficient of determination R^2 normalized from 0 to 1 was no less than 0.98). The MPO activity in the supernatant was calculated with regard to its dilution and expressed in ng/mL purified MPO.

The elastase activity in the neutrophil suspensions was assayed by a fluorescence method, using a LSF1211A computerized spectrofluorimeter (SOLAR, Minsk, Belarus) and MeO–Suc–Ala–Ala–Pro–Val–MCA as a specific substrate. A 1-mL aliquot of a neutrophil suspension ($2 \cdot 10^6$ cells/mL in PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$) was combined with 20 μM MeO–Suc–Ala–Ala–Pro–Val–MCA, incubated at 37°C for 3 min, and combined with a stimulating agent (1 μM fMLP). The kinetics of elastase-driven cleavage of the substrate to release the fluorophore aminomethylcoumarin were recorded as the fluorescence intensity at 460 nm (excitation at 380 nm). The elastase activity was obtained as the slope of a linear region of the kinetic curve, which showed the fluorescence intensity increasing with the generation of the fluorescent product [22].

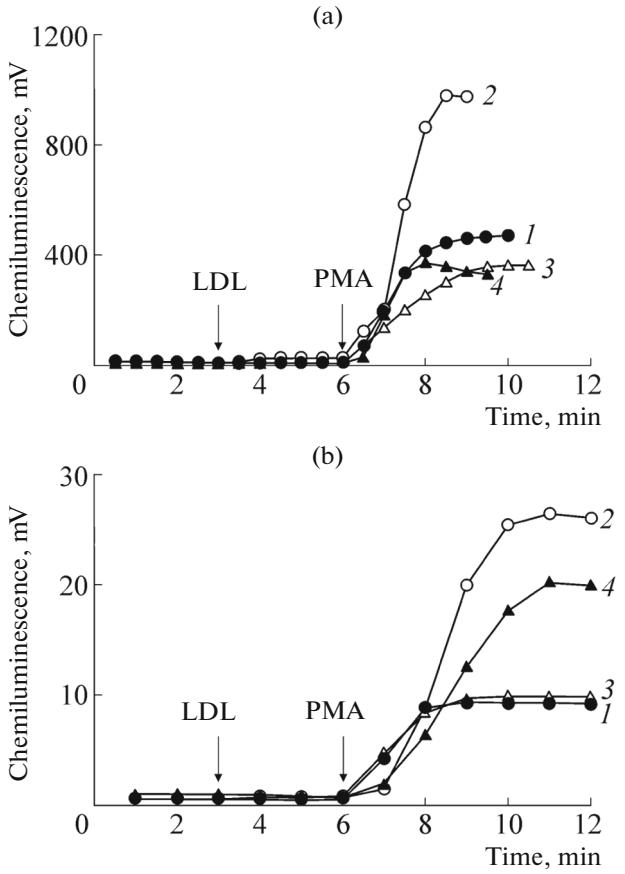

Fig. 1. Typical kinetic curves of (a) lm-CL and (b) lc-CL of neutrophils in response to (*1*) native LDL or (*2*) LDL–HOCl and subsequent addition of PMA. Curve *3*, control experiment where PBS (10 mM Na-phosphate, 140 mM NaCl, pH 7.4) was added in place of LDL or LDL–HOCl. Curve *4*, variant (*2*) in the presence of (a) 4 mM cysteine or (b) 3.8 μM ceruloplasmin. Addition of LDL or PMA is indicated with arrows. Measurements were performed in Krebs–Ringer bicarbonate solution (pH 7.4) with 1.3 mM $CaCl_2$ and 0.2 mM luminol or lucigenin at 37°C. Neutrophils ($4 \cdot 10^5$ cells/mL), LDL (250 μg/mL, and PMA (0.16 μM) were added to the sample.

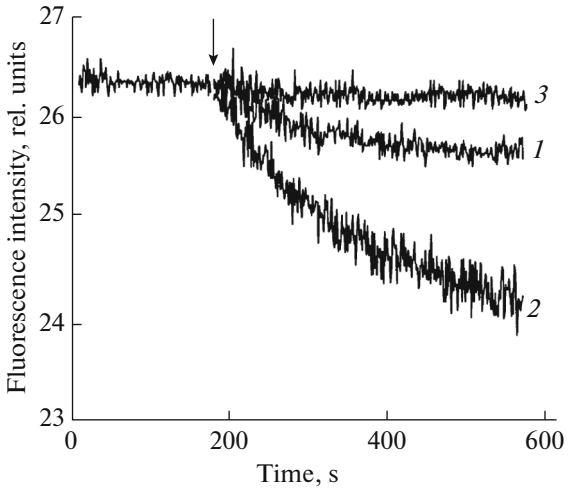

Fig. 2. Typical kinetic curves of scopoletin oxidation in a suspension of neutrophils activated with (*1*) native LDL or (*2*) LDL–HOCl. Curve *3*, effect of HSA (7.5 μM) on scopoletin oxidation in a neutrophil suspension in response to LDL–HOCl. Scopoletin fluorescence was excited at 350 nm and measured at 460 nm. Addition of LDL or LDL–HOCl is indicated with an arrow.

Statistical analysis. The results are presented as mean ± standard deviation. The differences between the mean values were tested for significance using the Student's *t*-test and were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Neutrophil activation with native LDL and LDL–HOCl. Two methods were used to detect activation of neutrophils isolated from the donor blood. One was based on luminol- or lucigenin-enhanced chemiluminescence (lm-CL and lc-CL, respectively); in the other, H_2O_2 production was assayed by the fluorescence method using scopoletin as a peroxidase reaction substrate. Typical curves of neutrophil lm-CL and lc-CL in response to consecutive addition of LDL or LDL–HOCl and then PMA are shown in Figs. 1a and 1b. Typical curves that characterize scopoletin oxidation by neutrophils after adding LDL or LDL–HOCl that reflect the H_2O_2 production rate are shown in Fig. 2. The results of experiments on neutrophil acti-

Table 1. The intensities of lm-CL and lc-CL and the scopoletin oxidation rate of neutrophils in response to LDL or LDL–HOCl

Agent	lm-CL, mV	lc-CL, mV	Scopoletin oxidation rate, pmol/s/10^6 cells	
	LDL, 250 μg/mL	LDL, 250 μg/mL	LDL, 100 μg/mL	LDL, 400 μg/mL
Control	5.0 ± 1.9	0.60 ± 0.10	0.15 ± 0.03	0.21 ± 0.03
LDL	3.8 ± 1.3	0.55 ± 0.05	0.15 ± 0.05	0.25 ± 0.01
LDL–HOCl	13.6 ± 5.0*	1.10 ± 0.40*	0.23 ± 0.03*	0.38 ± 0.04*

* Differences from LDL and the control were significant at $p < 0.05$.

vation in the absence of PMA are summarized in Table 1.

Native LDL did not significantly stimulate lm-CL or lc-CL in the absence of PMA as compared to the control. The scopoletin oxidation rate and, accordingly, the H_2O_2 generation rate of neutrophils also did not significantly increase with native LDL. In contrast, LDL–HOCl activated neutrophils and caused small, but significant increases in the lm-CL (~3.6-fold), lc-CL (~2-fold), and H_2O_2 generation rate (~1.5-fold) of neutrophils as compared with native LDL.

The effects of LDL and LDL–HOCl on the lm-CL and lc-CL responses of neutrophils to PMA are summarized in Table 2. Native LDL did not increase the chemiluminescence response to the activator, while LDL–HOCl determined a significantly greater response to PMA and increased lm-CL and lc-CL by factors of approximately 3.2 and 6.2, respectively. This finding points to a priming effect of LDL–HOCl on the neutrophil oxidative burst.

As is known, lm-CL of neutrophils is due to luminol oxidation by HOCl [23, 24], which is a product of MPO-driven halogenation, while lc-CL is due to lucigenin oxidation by superoxide anion radicals ($^{\bullet}O_2^-$), which is generated by NADPH oxidase [23, 25]. Hence, lm-CL reflects the clorinating activity of MPO [12], and lc-CL reflects the NADPH oxidase activity [26]. The MPO inhibitor 4-aminobenzoic acid hydrazide did, in fact, efficiently reduce the intensity of PMA-stimulated neutrophil lm-CL in both the absence and presence of native LDL or LDL–HOCl (Fig. 3, Table 3). The lc-CL intensity was not affected.

Effects of antioxidants and HOCl scavengers on neutrophil activation. As examples, Figs. 1a and 1b (curves *4*) illustrate how cysteine and ceruloplasmin affected the lm-CL and lc-CL responses of neutrophils to LDL–HOCl and subsequent PMA. Figure 2 (curve *3*) shows the effect of HSA on the kinetics of scopoletin oxidation, which reflects the H_2O_2 production, by neutrophils stimulated with LDL–HOCl. It should be noted that HSA did not appreciably affect the H_2O_2 production rate of neutrophils (data not shown). The effects of testing antioxidants and HOCl scavengers for effects on the above parameters are summarized in Tables 4 and 5. All of the compounds acted in a concentration-dependent manner and decreased the lm-CL responses of neutrophils to LDL–HOCl (Table 4) and subsequent PMA (Table 5). However, a substantially lower, if any, effect was observed on the lc-CL response of neutrophils in similar conditions or the H_2O_2 production of neutrophils. As an example, cysteine used at 3.3 mM caused a 60% decrease in the lm-CL response of neutrophils to LDL–HOCl, but did not affect both the H_2O_2 production and lc-CL response of neutrophils (Table 4). When PMA was added after LDL–HOCl, cysteine similarly caused a 85% decrease in lm-CL without affecting lc-CL of neutrophils (Table 5).

Table 2. The effects of native and modified LDL on lm-CL and lc-CL intensities of PMA-stimulated neutrophils

Agent	lm-CL, mV	lc-CL, mV
Control	340 ± 186	9.6 ± 0.4
LDL	342 ± 111	9.4 ± 1.7
LDL–HOCl	1086 ± 590*	58.1 ± 35.3*

* Differences from LDL and control were significant at $p < 0.05$.

Table 3. The effect of 4-aminobenzoic acid hydrazide (100 μM) on lm-CL and lc-CL relative intensities (% of the control) of PMA-stimulated neutrophils in the presence of native LDL or LDL–HOCl

Agent	lm-CL, %	lc-CL, %
Control (without inhibitor)	100	100
LDL + inhibitor	37.8 ± 1.9*	120 ± 25
LDL–HOCl + inhibitor	20.6 ± 2.0*	126 ± 23

* Differences from the control were significant at $p < 0.01$.

Because the MPO halogenation cycle and HOCl production are mostly responsible for lm-CL of neutrophils [23, 25], the reason that agents that interact with HOCl or inhibit MPO are efficient in decreasing the lm-CL of neutrophils is clear. At the same time, methionine, cysteine, glutathione, and taurine are known to possess antioxidant properties and to interact with ROS, including $^{\bullet}O_2^-$ [27]. This probably explains the reason that these compounds decreased, to a certain extent, neutrophil lc-CL, which is mostly

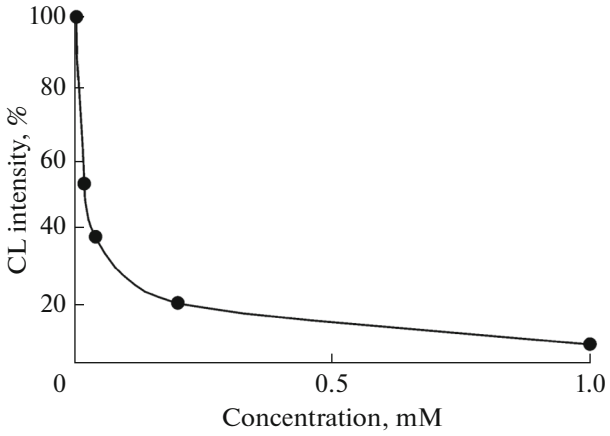

Fig. 3. The lm-CL response of neutrophils to PMA as dependent on the concentration of the MPO inhibitor 4-aminobenzoic acid hydrazide. The lm-CL intensity in the absence of the inhibitor was taken as 100%. Measurements were performed in Krebs–Ringer bicarbonate solution (pH 7.4) containing 1.3 mM $CaCl_2$, 0.2 mM luminol, $4 \cdot 10^5$ cells/mL neutrophils, and 0.156 μM PMA at 37°C.

Table 4. The effects of antioxidants and HOCl scavengers on the lm-CL and lc-CL responses and scopoletin oxidation rate of neutrophils exposed to LDL–HOCl (% of the control)

Agent	lm-CL, %	lc-CL, %	Scopoletin oxidation rate, pmol/s/10^6 cells	
	LDL–HOCl, 250 μg/mL	LDL–HOCl, 250 μg/mL	LDL–HOCl, 100 μg/mL	LDL–HOCl, 400 μg/mL
Control (without any agent)	100	100	100	100
Methionine, mM				
0.1	97	96	–*	63
2.7	76	91	–	–
5.0	56	–	–	–
Cysteine, mM				
0.1	86	114	–	109
2.1	74	–	–	–
3.3	40	118	–	–
Taurine, mM				
1.0	86	118	58	68
3.2	63	77	–	–
Reduced glutathione, mM				
0.3	48	–	–	–
1.3	42	108	–	–
Ceruloplasmin, μM				
2.3	51	81	59	59
3.8	53	90	–	–
HSA, μM				
3	–	90	–	–
6	–	123	–	–
7.5	54	100	71	37
Superoxide dismutase, U/mL				
150	45	62	–	–

* (–), measurements were not performed at the relevant concentration.

due to the function of neutrophil NADPH oxidase [26]. It should be noted that the antioxidant proteins (ceruloplasmin and HSA) exerted an inhibitory effect at 2.3–7.5 μM (Tables 4, 5). Ceruloplasmin is a natural protein that inhibits enzymatic activity and regulates bactericidal activity of MPO [28]. Ceruloplasmin acts as an acute-phase protein and binds with MPO in a complex, thus inhibiting both peroxidase and halogenating activities of the enzyme [11, 12]. In addition, ceruloplasmin possesses superoxide dismutase activity. Ceruloplasmin has been shown to decrease the $^{\bullet}O_2^-$ production of neutrophils activated by opsonized zymosan [29]. In our experiments, ceruloplasmin strongly suppressed the lm-CL response of neutrophils to LDL–HOCl and subsequent PMA and reduced the H_2O_2 production and lc-CL of neutrophils. HSA most likely acts as a nonspecific scavenger of both RHS and ROS to decrease the lm-CL response and H_2O_2 production of neutrophils (Tables 4, 5). An HSA-determined increase in the lc-CL response of neutrophils to PMA (Table 5) is conceivable given that HSA efficiently interacts with HOCl and thereby competes with $^{\bullet}O_2^-$ for the reaction with HOCl [30]. Moreover, NADPH oxidase is activated by chlorinated HSA [17], which results from the interaction of HSA with HOCl. The local $^{\bullet}O_2^-$ concentration is elevated in either case, increasing the lc-CL response of neutrophils. Superoxide dismutase also reduced the lm-CL and lc-CL responses of neutrophils (Tables 4, 5). Superoxide dismutase specifically catalyzes the $^{\bullet}O_2^-$ disproportionation. The enzyme substantially decreased the lc-CL responses of neutrophils to LDL–HOCl and, especially, PMA (Table 5). The minor decrease in lm-CL in the presence of superoxide dismutase may be explained by a nonspecific interaction with RHS; moreover, superoxide dismutase converts $^{\bullet}O_2^-$ to H_2O_2 and thereby increases the

local H_2O_2 concentration, thus possibly inhibiting MPO [31].

Effects of antioxidants and HOCl scavengers on neutrophil degranulation. Considering that neutrophil lm-CL is due to luminol oxidation by RHS that results from the MPO function [23, 24], several mechanisms are possible for the effects that antioxidants and HOCl scavengers exerted on lm-CL in our experiments. One is inhibition of MPO activity, another is the interaction with RHS, and a third one is based on RHS-induced changes in MPO secretion due to neutrophil degranulation. To study this issue, cell supernatants were obtained after incubating neutrophils with LDL or LDL–HOCl in the presence of antioxidants and HOCl scavengers with or without a subsequent addition of PMA. The supernatants were tested for MPO activity by modified ELISA. The results are summarized in Table 6. LDL–HOCl caused neutrophil degranulation and MPO exocytosis in the absence of PMA. A subsequent addition of PMA exerted almost no effect on the process. Of all of the compounds we examined, only glutathione slightly decreased MPO exocytosis. This effect might explain in part the fact that the lm-CL of neutrophils decreased in the presence of glutathione (Tables 4, 5). It is noteworthy that ceruloplasmin and HSA strongly increased the MPO secretion by neutrophils exposed to LDL–HOCl. Ceruloplasmin is known to prime neutrophils by activating NADPH oxidase via its ferroxidase activity [32]. The ceruloplasmin-induced priming is possibly associated with neutrophil degranulation. We have previously shown that HSA modified in the presence of RHS increases degranulation of azurophilic neutrophil granules, which harbor MPO [4]. It can be hypothesized that HSA was modified by RHS or ROS in the presence of activated neutrophils and that modified HSA increased MPO exocytosis. In the presence of cysteine, MPO activity was substantially lower in the supernatants after neutrophil degranulation in response to LDL–HOCl or PMA (Table 6). Control experiments showed that cysteine substantially reduced the numerical MPO activity estimate, although special conditions were created to prevent the effect of protein inhibitors in our ELISA protocol (data not shown). To determine whether cysteine affects neutrophil degranulation, exocytosis in the presence of cysteine was studied for elastase, which is another marker of azurophilic granules. A cell suspension was combined with a substrate that was hydrolyzed to produce a fluorophore in the presence of elastase. The results are shown in Fig. 4. Cytochalasin B substantially increased the fluorescence response to the activator fMLP, suggesting elastase exocytosis. However, cysteine (3.3 mM) did not appreciably affect the fluorescence curve and, therefore, degranulation of azurophilic granules of neutrophils. It can be supposed that the decrease in degranulation was not responsible for the cysteine-induced decrease in the lm-CL and lc-CL responses of neutrophils to LDL–HOCl and PMA. The interaction of cysteine with RHS and ROS is the most probable mechanism.

Table 5. The effects of antioxidants and HOCl scavengers on the lm-CL and lc-CL responses of neutrophils to PMA in the presence of LDL–HOCl (% of the control)

Agent	lm-CL, %	lc-CL, %
Control (without any agent)	100	100
Methionine, mM		
0.1	100	100
2.7	72	103
5.0	58	–*
Cysteine, mM		
0.1	81	114
2.1	49	110
3.3	15	100
Taurine, mM		
1.0	95	100
2.4	88	–
3.2	56	49
Reduced glutathione, mM		
0.13	31	–
0.3	25	74
1.3	28	43
Ceruloplasmin, μM		
2.3	81	100
2.7	70	–
3.8	63	81
HSA, μM		
1.5	58	–
3	59	217
6	64	160
7.5	–	216
Superoxide dismutase, U/mL		
150	33	5

* (–), measurements were not performed at the relevant concentration.

As has been demonstrated, halogenated derivatives of proteins [4], phospholipids [5], and LDL [6], which may form via a MPO-dependent pathway in inflammation sites, activate neutrophils and stimulate their ROS/RHS production and degranulation with a subsequent release of MPO into the extracellular space. These effects further increase the modification of biologically important molecules and facilitate the further development of oxidative/halogenative stress. Our findings indicate that antioxidants and RHS scavengers that occur in the human body are capable of affecting the development of oxidative/halogenative stress and suppressing its progress. To play these roles,

Table 6. The effects of antioxidants and HOCl scavengers on MPO activity in the supernatants obtained after neutrophil degranulation induced by LDL-HOCl in the presence or absence of PMA

Composition	Without PMA		With PMA	
	MPO, ng/mL	MPO, %	MPO, ng/mL	MPO, %
Krebs–Ringer solution	0	0	–	–
Krebs–Ringer solution + LDL–HOCl	0	0	–	–
Neutrophils	22.5 ± 3.2	29	80.0 ± 7.6	91
Neutrophils + LDL	38.3 ± 6.6	50	82.5 ± 1.9	94
Neutrophils + LDL–HOCl	76.8 ± 1.0	100*	88.2 ± 1.6	100*
Neutrophils + LDL–HOCl + methionine (2.7 mM)	105.4 ± 24.5	137	89.6 ± 0.3	102
Neutrophils + LDL–HOCl + cysteine (3.3 mM)	2.4 ± 2.1	3	2.3 ± 2.0	3
Neutrophils + LDL–HOCl + taurine (3.2 mM)	87.5 ± 3.2	114	76.8 ± 1.8	87
Neutrophils + LDL–HOCl + glutathione (1.3 mM)	57.3 ± 7.5	75	56.6 ± 5.4	64
Neutrophils + LDL–HOCl + ceruloplasmin (2.3 μM)	190.6 ± 14.1	248	122.3 ± 5.7	139
Neutrophils + LDL–HOCl + HSA (3 μM)	179.9 ± 33.6	234	86.4 ± 0.8	98

The MPO contents in the supernatants obtained after neutrophil incubation with LDL–HOCl with or without subsequent addition of PMA were taken as 100%.

the substances interact with RHS/ROS and prevent neutrophil activation and degranulation, which are caused by LDL modified in the inflammation site. Activation of the halogenation-prevention system, which includes RHS scavengers, substances that regulate degranulation of azurophilic granules of neutrophils, and inhibitors of MPO and other peroxidases, plays an important role in the regulation of the inflammatory response and in anti-inflammation therapy.

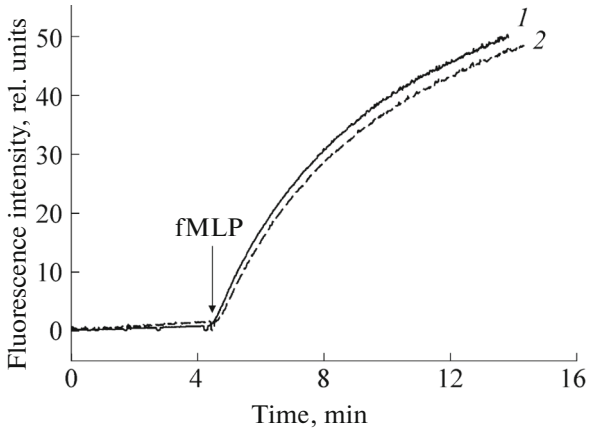

Fig. 4. (*1*) The effect of cysteine on fMLP-induced elastase exocytosis from neutrophils in the presence of cytocholasine B and (*2*) a control curve, which was obtained in the absence of cysteine. Measurements were performed in 10 mM Na_2HPO_4/KH_2PO_4 (pH 7.3) containing 137 mM NaCl, 2.7 mM KCl, and 5 mM D-glucose. Components were used at the following concentrations: $2 \cdot 10^6$ cells/mL neutrophils, 1 μM fMLP, 20 μM elastase substrate MeO–Suc–Ala–Ala–Pro–Val–MCA, 3.3 mM cysteine, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$. The kinetics of elastase-driven cleavage of the specific substrate to release the fluorochrome aminomethylcoumarin was recorded as the intensity of fluorescence excited at 380 nm and detected at 460 nm.

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