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Identification and properties of complexes formed by myeloperoxidase with lipoproteins and ceruloplasmin

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ABSTRACT

The first evidence of multi-component complexes formed by myeloperoxidase (MPO), ceruloplasmin (CP), and very low/low density lipoproteins (VLDL/LDL) obtained by electrophoresis, gel filtration, and photon-correlation spectroscopy (PCS) is presented in this paper. Complexes were observed when isolated MPO, CP, and VLDL/LDL were mixed and/or when MPO was added to the blood plasma. Complex LDL–MPO–CP was detected in 44 of 100 plasma samples taken from patients with atherosclerosis, and 33 of 44 samples also contained the VLDL–MPO–CP complex. MPO concentration in these patients' plasma exceeded 800 ng/ml. Interaction of MPO with high density lipoproteins (HDL) was not revealed, as well as binding of CP to lipoproteins in the absence of MPO. Adding antibodies against apoB-100 to VLDL–MPO–CP and LDL–MPO–CP complexes results in release of lipoproteins. Using PCS the diameters of complexes under study were evaluated. By comparing concentrations of the components in complexes formed by MPO, CP, and lipoproteins their stoichiometry was assessed as 2VLDL:1MPO:2CP and 1LDL:1MPO:2CP. Lipoproteins affected the inhibition of MPO peroxidase activity by CP. The affinity of lipoproteins to MPO–CP complex was assessed using apparent dissociation constants determined as ~0.3 nM for VLDL and ~0.14 nM for LDL.

1. Introduction

Myeloperoxidase (MPO, E.C. 1.11.1.7), a heme-containing dimeric peroxidase, is stored within azurophilic granules of leukocytes (Klebanoff, 2005). Like other peroxidases MPO generates a number of potent oxidant compounds. These products play an important role in killing invading parasites and pathogens, but are also capable of promoting oxidative modifications in host tissues. Due to its specific activity, MPO catalyzes oxidation of halogenides and pseudohalogenide (SCN^-) in a reaction with hydrogen peroxide, which results in generation of highly reactive hypohalogenites. MPO performs its important role as a component of innate immunity mainly by the *in vivo* production of hypochlorite deleterious for microbial cells. However, hypohalogenites can damage various molecules of the host organism, and thereby can cause a cytotoxic effect and oxidative ("chlorinative") stress development (Klebanoff, 2005; Yap et al., 2007).

Oxidative modifications of host tissues may provoke a number of serious diseases (Klebanoff, 2005). For instance, numerous studies demonstrate that oxidative damage of blood lipoproteins, mainly of low density lipoproteins (LDL), by the products of MPO catalysis should be regarded as one of the pathological mechanisms of atherosclerosis (Nicholls and Hazen, 2009). The list of pro-oxidants, generated with participation of MPO and capable of damaging LDL, includes hypochlorite, chloroamines, tyrosylic radical and nitrogen dioxide (Carr et al., 2000a). Atherosclerotic plaques contain active MPO, since hypohalogenite-damaged LDLs were obtained from the plaques (Daugherty et al., 1994). The content of 3-chlorotyrosine, a specific marker of MPO-catalyzed oxidation, is notably elevated in LDLs isolated from human atherosclerotic intima (Hazen and Heinecke, 1997). Lipid peroxidation and protein nitration are facilitated when LDLs are exposed to activated monocytes through MPO-generated reactive nitrogen species (Podrez et al., 1999). This entails conversion of LDL into a high uptake form readily engulfed by macrophages via CD36 scavenger receptor (Podrez et al., 2000). MPO is also able to catalyze carbamylation of LDL, converting the

Abbreviations: ABTS, sodium 2,2′-azino-bis(3-ethylbenzotriazoline-6-sulphonate); CP, ceruloplasmin; CTAB, cetyltrimethylammonium bromide; HDL, high density lipoproteins; K_i^*, apparent dissociation constants; LDL, low density lipoproteins; MPO, myeloperoxidase; PCS, photon-correlation spectroscopy; TMB, tetramethylbenzidine; VLDL, very low density lipoproteins.

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latter into a ligand for the scavenger receptor SRA-1 (Wang et al., 2007). High systemic levels of MPO were found to be associated with the adverse cardiovascular outcomes in patients investigated in the setting of acute ischemic syndromes, and independently predict cardiovascular risk, regardless of evidence of myocardial necrosis (Baldus et al., 2003). Taken together, all these data demonstrate the capability of MPO to generate *in vivo* modified forms of LDL.

It was shown that MPO can modify high density lipoproteins (HDL) along with LDL (Zheng et al., 2004, 2005). The role of MPO in the generation of dysfunctional HDL particles seems to be important in view that heart ischemia and its consequences can occur even in the presence of high levels of HDL cholesterol (Zheng et al., 2004, 2005). MPO-induced modifications of apoA-I have a pronounced effect on the reverse cholesterol transport pathway, in particular, they interfere with binding of nascent HDL to LCAT, thus reducing LCAT functional activity (Wu et al., 2007). This finding is in line with the observed correlation between the degree of apoA-I modification and impairment of HDL promoting ABCA-1-dependent cholesterol efflux from macrophages, which is regarded as an increased risk of atherosclerotic plaque development and cardiovascular diseases (Zheng et al., 2004; Bergt et al., 2004; Pennathur et al., 2004). A likely mechanism of MPO impact on mobilization of cholesterol from peripheral tissues to the liver was suggested recently (Nicholls and Hazen, 2009). The authors demonstrated that HDL exposed to the [MPO + H_2O_2 + Cl^-] system becomes a competitor of the native HDL as a ligand for the scavenger receptor BI (SRBI).

Thus, MPO causes oxidative modifications, which increase atherogenicity of LDL and interfere with the proper functionality of HDL, and fine regulation of its activity might underlie a therapeutic approach to control status of lipoproteins in bloodstream.

MPO is a polycationic protein (p*I* > 10), and since lipoprotein particles have negative net charge (Panasenko et al., 1985), their interaction with the enzyme seems highly probable. Indeed, electrophoretic mobility of MPO was changed when it was added to LDL, which demonstrates their affinity to each other (Carr et al., 2000b). The authors also observed a co-precipitation of MPO and apoB-100-containing lipoproteins. It was shown that MPO binds to apoA-I at the HDL surface (Zheng et al., 2004). ApoA-I isolated from plasma of patients with coronary heart disease contained greater amounts of nitrotyrosine and chlorotyrosine than the protein of healthy donors (Zheng et al., 2005). Once HDLs are modified by hypochlorite, their affinity towards MPO increases (Marsche et al., 2008). Thus, MPO-dependent oxidative modification of HDLs favors their contact with MPO, which results in their conversion to pro-atherogenic forms (Zheng et al., 2004).

A copper-containing protein and acute phase reactant ceruloplasmin (CP) seems to be the physiological inhibitor of MPO whose peroxidase activity is suppressed upon interaction with CP (Segelmark et al., 1997; Griffin et al., 1999). Both peroxidase and chlorinating activities of MPO are essentially suppressed by non-proteolyzed CP (Panasenko et al., 2008; Sokolov et al., 2008). Inhibition seems to depend on a steric barrier that prevents or strongly limits substrate binding in the active center of MPO. Indeed, proteolysis of the peptide loop in CP that contacts with the entrance to the heme pocket in MPO cancels the inhibitory effect of CP (Sokolov et al., 2008). CP in a complex with MPO can be found in blood plasma and exudates of patients with inflammation, when MPO concentration increases (Sokolov et al., 2007).

Thus, on the one hand, there are numerous data on an "unfavorable" outcome of MPO binding to lipoprotein particles, but on the other hand, we know that MPO interacts with CP, its physiological inhibitor. We attempted to study the behavior of MPO in the presence of both CP and lipoproteins belonging to different classes (VLDL, LDL, and HDL). This study shows the possibility of formation of multi-component complexes composed of CP, MPO, and very low density lipoproteins (VLDL)/LDL, and some characteristics of such complexes are presented.

2. Experimental

2.1. Chemicals

Goat antibodies against rabbit IgG conjugated with horseradish peroxidase, skimmed dry milk, and Tween 20 (BioRad, USA), 4-chloro-1-naphtol (Fluka, Switzerland), hydrogen peroxide (Merck, Germany), chromatographic resins (Pharmacia, Sweden), Coomassie G-250 and R-250, tetramethylbenzidine (TMB) (Serva, Germany), cetyltrimethylammonium bromide (CTAB), Freund's adjuvant, *o*-dianisidine dihydrochloride, *o*-phenylendiamine, sodium 2,2′-azino-bis(3-ethylbenzotriazoline-6-sulphonate) (ABTS) (Sigma Aldrich, USA), reagents for electrophoresis (Laboratory Medigen, Russia), and heparin (SPOFA, Poland) were used. All solutions were prepared using deionized water and treated, when necessary, with Chelex-100 (BioRad, USA) to eliminate traces of heavy metals.

2.2. Samples

Samples of blood plasma were obtained upon informed consent of patients with atherosclerosis taking a course of treatment at the Clinical Department of the Institute of Experimental Medicine by courtesy of Prof. A.D. Denisenko. Blood plasma and buffy coat for protein isolation were obtained from healthy donors with their informed consent thanks to assistance of Prof. V.N. Kokryakov (Institute of Experimental Medicine).

2.3. (Lipo)proteins preparation

Monomeric CP, stable at storage and containing more than 95% of non-fragmented 132 kDa-protein (Laemmli, 1970) with ratio $A_{610}/A_{280} > 0.045$, was purified from human blood plasma by affinity chromatography on protamine–Sepharose (Sokolov et al., 2005). Human leukocytic MPO with $Rz = A_{430}/A_{280} = 0.85$, typical of the homogeneous protein, was purified from buffy coat by affinity chromatography on heparin–Sepharose, hydrophobic chromatography on phenyl-Sepharose and gel filtration on Sephacryl S-200 HR (Sokolov et al., 2007). A separate portion of MPO was additionally extracted from buffy coat with 0.5% CTAB (a cationic detergent usually applied for extraction of proteins from leukocytes) according to Olsen and Little (1983). This MPO with $Rz = A_{430}/A_{280} = 0.83$ is henceforth named MPO_{CTAB}.

VLDL, LDL, and HDL were isolated from plasma of healthy donors by preparative ultracentrifugation in NaBr solutions of varying density (Lindgren, 1975). All preparations of lipoproteins were dialyzed for 15 h at 4 °C against PBS (150 mM NaCl, 10 mM Na-phosphate buffer, pH 7.4). Mouse monoclonal antibodies to human apoB-100 (Yanushevskaya et al., 1999) were generously provided by E.V. Yanushevskaya and T.N. Vlasik (Russian Cardiologic Scientific-Industrial Complex, Moscow).

2.4. Electrophoresis

Lipoproteins and their complexes with CP and MPO were subjected to PAGE in four-layered gel (Wada et al., 1973) stained for proteins (Coomassie R-250) or for enzymatic activities of CP and MPO. CP activity was revealed in gel after electrophoresis due to enzymatic oxidation of specific chromogenic substrate *o*-dianisidine (Owen and Smith, 1961). MPO activity was detected by oxidation of 4-chloro-1-naphtol in the presence of H_2O_2 (Sokolov et al., 2007).

Table 1
Estimation of diameter (d) of proteins and of their complexes (formation of complex marked by *) by PCS.

Protein/mixture (number of measurements)	Final concentration, μM	d, nm ($M \pm$ SD, measured by PCS)	d, nm (data from literature)
CP (12)	0.45	7.4 ± 0.8	7.2 (Sokolov et al., 2009)
MPO (12)	0.22	7.8 ± 1.2	7.6 (Sokolov et al., 2009)
CP-MPO (12)*	0.22–0.45	9.8 ± 1.4	–
LDL (15)	0.23	23.0 ± 1.4	22.5 ± 2.5 (Patsch et al., 1976)
LDL + CP (10)	0.23–0.45	15.8 ± 5.8	–
LDL + MPO (15)*	0.23–0.22	28.0 ± 1.9	–
LDL + MPO + CP (15)*	0.23–0.22–0.45	29.0 ± 1.7	–
HDL (15)	0.56	10.7 ± 2.0	10.6 ± 0.8 (Patsch et al., 1976)
HDL + CP (10)	0.56–0.45	8.8 ± 4.8	–
HDL + MPO (10)	0.56–0.22	9.0 ± 3.9	–
HDL + MPO + CP (10)	0.56–0.22–0.45	8.9 ± 4.7	–
VLDL (12)	0.44	46.5 ± 7.6	55 ± 15 (Sata et al., 1972)
VLDL + CP (10)	0.44–0.45	7.5 ± 0.9 and 46.2 ± 8.3	–
VLDL + MPO (12)*	0.44–0.22	85.9 ± 8.9	–
VLDL + MPO + CP (12)*	0.44–0.22–0.45	129.0 ± 9.7	–

Electrophoresis of lipoproteins and their complexes with MPO was carried out in 1% agarose gel (w/v) prepared on Tris–glycine–barbital buffer with ionic strength 0.08 and pH 8.8 (Laurell, 1967).

2.5. Gel filtration

Analytical gel filtration was performed on a column (20 cm × 0.5 cm) packed with Sephacryl S-300 *Superfine* in PBS, elution speed kept at 0.6 ml/min. Samples (0.2 ml) contained 0.5 μg MPO, 10 μg CP, as well as VLDL, LDL or HDL (protein content 20 μg) in 10% (w/v) sucrose. The column was sequentially loaded with MPO, CP, the mixture of CP and MPO, and then with analogous samples to which lipoproteins had been added. CP and MPO contents were assayed in chromatographic fractions (0.2 ml) by ELISA. Concentrations of lipoproteins were evaluated by a total protein microassay using Coomassie G-250, with the sensitivity limit of 0.5 μg/ml (Bradford, 1976).

Gel filtration of healthy donors' plasma (1 ml), to which MPO had been added (5 μg), was performed on a column (50 cm × 1 cm) packed with Superose 6 in PBS, elution speed kept at 0.2 ml/min. Chromatographic fractions (0.2 ml) were analyzed by electrophoresis, and their CP and MPO contents were measured by ELISA. Then CP- and MPO-containing fractions were subjected to PAGE in four-layered gel.

2.6. ELISA

Protocols for obtaining antibodies against CP and MPO and for performing ELISA are described in details by Gorudko et al. (2009). Briefly, we immunized rats (with MPO) and rabbits (with MPO and CP) and isolated affinity antibodies against these proteins using MPO- or CP-Sepharose. All solutions for ELISA were prepared on 3% defatted milk (w/v) with PBS to which 0.05% Tween was added (BLOTTO-T). To provide immunoglobulin adsorption on polystyrene plates, 100 μl of antibodies' solution in 0.1 M sodium bicarbonate buffer, pH 9.4 were added into each well, followed by 12-h incubation at +4 °C. The chromogenic mixture for horseradish peroxidase detection consists of 1% *o*-phenylendiamine solution in ethanol (1 ml), 0.1 M sodium-citrate buffer, pH 4.0 (11 ml) and 10 M H_2O_2 (6 μl). 100 μl of the mixture were added into each well, and after 4–5 min the reaction was stopped by 50 μl of 6 M H_2SO_4. Then optical density (A_{492}) of the solution was registered using a 2100 Microplate Reader (Awareness Technology, USA).

CP concentration in fractions was measured by competitive ELISA. Rabbit antibodies against human CP (10 μg/ml) were adsorbed on the plates. The plate was washed with PBS, blocked with BLOTTO-T, and incubated with a mixture of a CP-containing sample with fixed amount of CP conjugated to horseradish peroxidase in BLOTTO-T. Peroxidase activity in wells was revealed, and then CP concentration in samples was evaluated by a calibration curve plotted using standard CP dilutions ranging from 10 to 150 ng/ml.

Our ELISA modification applied for MPO measurements involved using antibodies against human MPO raised both in rabbits and in rats. Rat antibodies against MPO (5 μg/ml) were adsorbed on a plate. Then the plate was washed with 0.05% Tween 20 (v/v) in PBS and blocked with BLOTTO-T. Three consecutive 1-h incubations, each followed by a washing step, were performed: wells were loaded by MPO-containing samples, by rabbit antibodies against human MPO (10 μg/ml), and by horseradish-conjugated goat antibodies against rabbit IgG (1:5000; BioRad), all solved in BLOTTO-T. Peroxidase activity was assayed, and then MPO concentration was determined with a calibration curve plotted using standard MPO dilutions ranging from 3 to 250 ng/ml. The results of three measurements per sample are presented as mean values with standard deviation.

2.7. Photon-correlation spectroscopy

Photon-correlation spectroscopy (PCS) is widely used to evaluate the diameter of particles in solution and to study the formation of protein complexes (Berland et al., 1996). In this method Stokes–Einstein equation is used, which is represented as $D = k_B T/3\pi\eta d$, where D is diffusion coefficient of particles in a fluid, k_B is Boltzmann constant, T is temperature (K), η is solvent viscosity (poise), and d is a particle's diameter (cm). Scattered-light intensity was registered and an autocorrelation function was computed to estimate D. Measurements were carried out by a submicron analyzer of particle diameter N5 Beckman Coulter equipped with the program pack "Contin". Light was emitted by a 632.8 nm laser; the range of measurements was 3–3000 nm. Protein and lipoprotein solutions in 10 mM Tris–HCl, pH 7.4, with 150 mM NaCl were filtered through 0.22 μm pores prior to measurements. Total protein concentration in solutions was empirically varied each time (from 1 to 10 mg/ml) to keep the intensity of scattered light within the limits proposed for the device. Proportions of proteins for formation of complexes were also chosen empirically: one component was added to another till the deviation of the measured d value became less than 20%. In such cases the polydispersity index obtained did not exceed 0.3, which implied monodispersity of the system. Final amounts of components are given in Table 1. The diameters of complexes are presented as mean values with standard deviations; the number of measurements for each sample is given in Table 1.

2.8. Assaying MPO activity

To study the effect of CP and lipoproteins on MPO-catalyzed oxidation of chromogenic substrates such as ABTS and TMB, the reaction rate at various concentrations of substrates (Sokolov et al., 2008) was measured. Reaction mixture in case of ABTS contained 5 nM MPO, 0.4–1.4 mM ABTS in 0.1 M sodium-phosphate buffer, pH 6.0, and 100 μM H_2O_2. In case of TMB the mixture was composed of 10 nM MPO, 0.1–0.5 mM TMB in 0.1 M sodium-phosphate buffer, pH 7.4, and 100 μM H_2O_2. H_2O_2 was added to initiate reaction. Reaction rate was registered at 20 °C as ΔA_{414}/min (ABTS) and ΔA_{650}/min (TMB) on SF 2000-02 spectrophotometer. Enzymatic activity was assayed both in the absence of proteins interacting with MPO and in the presence of CP (0.5 μM) and lipoproteins (protein concentration 0.5 μg/ml). This amount of lipoproteins corresponds to the particle concentration of 0.65 nM for VLDL, 0.68 nM for LDL and 3.4 nM for HDL. Experiments were repeated three times for each point. The respective K_M and V_{max} were determined from the graphs in Hanes–Woolf coordinates using least squares fitting (Microsoft Excel). Coefficients of determination for linear regression were all above 0.98. The type of inhibition and an adequate formula for calculating the inhibition constants (K_i) were chosen taking into consideration the review (Krupyanko, 2007).

3. Results

3.1. Interaction of lipoproteins with CP and MPO

To study features of interaction of MPO with CP and lipoproteins belonging to different classes gel filtration on Sephacryl S-300 was used. Separation of proteins without adding lipoproteins is illustrated by Fig. 1A. Pure CP and MPO were eluted within the same volume, their maximum yield was observed in fraction 9. Loading together CP and MPO on the column caused a reliable displacement of MPO- and CP-containing elution peaks, indicating the formation of CP-MPO complex. The elution peak of the complex overlapped with the peak of excess CP because the resin used does not allow complete separation of the complex from non-complexed proteins. MPO, loaded on the same column together with VLDL or LDL (Fig. 1B and C), was distributed among two elution peaks, of which the first one included mostly lipoproteins (fractions 2–5) while the second one contained free MPO (fractions 7–11). In turn, MPO applied together with HDL was eluted as a single peak corresponding to free MPO, so we did not reveal MPO affinity towards HDL (Fig. 1D). When a mixture of MPO with CP and lipoproteins was subjected to gel filtration, the elution profiles demonstrating CP and MPO content in fractions were composed of two peaks in case of VLDL and LDL, but not of HDL (Fig. 1B–D). It means that MPO and CP partially entered into the complex VLDL–MPO–CP (LDL–MPO–CP), and the rest of the portion ran as MPO–CP complex and non-complexed proteins. CP did not interact with lipoproteins in the absence of MPO, as it was eluted alone in fractions 8–10 (data not shown).

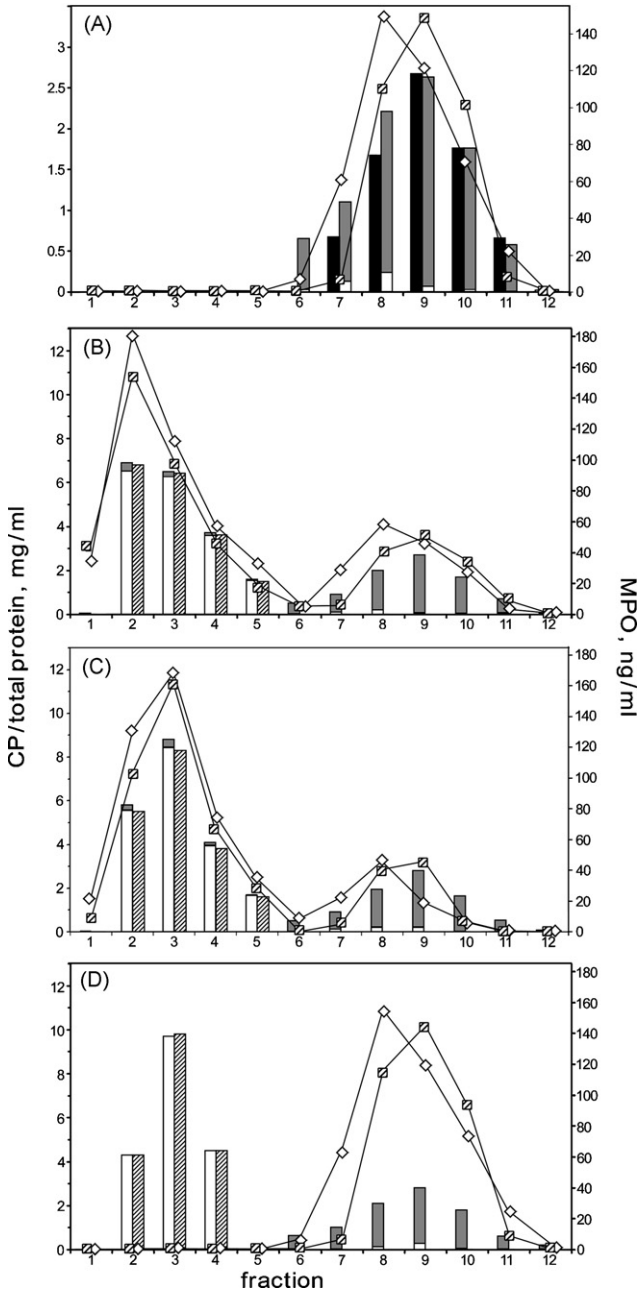

Fig. 1. Interaction of MPO with CP and lipoproteins examined by gel filtration on Sephacryl S-300. (A) Control runs of MPO, CP, and MPO with CP; (B–D) runs of MPO without and with CP in the presence of VLDL, LDL and HDL, respectively. Loaded samples (200 μl) contained 0.5 μg MPO, 10 μg CP and 20 μg lipoproteins. (Legend: ■ CP in control run; ▨ MPO and ▨ total protein in runs without CP; –◇– MPO, ■ CP and □ total protein minus CP in runs with CP).

Similar results were obtained by means of PAGE in four-layered gel. Such gel provides a resolution with retention of VLDL, LDL and HDL, respectively, at the entrance to the second and third layers and below the entrance to the fourth layer (Fig. 2A). CP had the highest mobility and was revealed in the fourth layer (Fig. 2B: 1). Meanwhile, cationic MPO did not enter the gel, therefore its activity was not found (Fig. 2C: 1). Upon mixing CP with MPO the proteins formed a complex with an intermediate electrophoretic mobility (Fig. 2B: 4, 2C: 2). Part of CP moved at its original pace. VLDL and LDL also changed the mobility of MPO and its activity was revealed in bands corresponding to the respective lipoproteins, which indicates binding of MPO to them (Fig. 2C: 4, 5). No interaction of HDL with MPO was observed (Fig. 2C: 3). CP added to lipoproteins in the absence of MPO had a mobility intrinsic for non-complexed protein (Fig. 2B: 2, 3). When excessive amounts of CP were added to MPO and LDL (or VLDL), we observed LDL–MPO–CP and VLDL–MPO–CP complexes along with free CP and CP–MPO complex (Fig. 2B and C: 6, 7). Addition of antibodies against apoB-100 provided less intensively stained bands containing complexes of LDL and VLDL with CP and MPO, which is explained by their dissociation (Fig. 2B and C: 8, 9). This was accompanied by a noticeable enhancement of the band corresponding to CP–MPO complex. These experiments evidenced that (i) LDL and VLDL are likely to interact with MPO via apoB-100; and (ii) upon mixing VLDL/LDL, MPO, and CP multi-component complexes are formed, in which MPO is the crucial link.

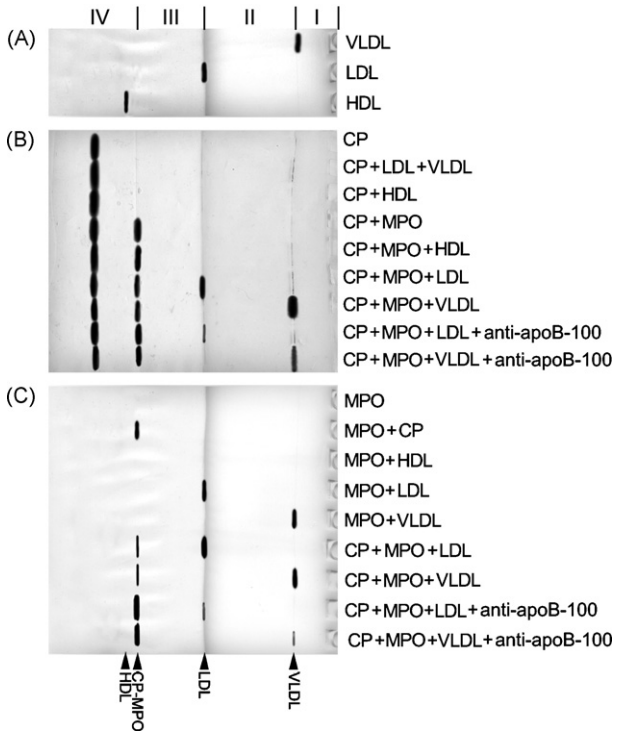

Fig. 2. Resolution pattern in four-layered PAGE of the mixture of CP (1 μg), MPO (0.5 μg), lipoproteins (4 μg of protein), and antibodies against apoB-100 (5 μg). Panel A, Coomassie R-250 staining: 1 – VLDL, 2 – LDL, 3 – HDL. Panel B, *o*-dianisidine staining: 1 – CP, 2 – CP + LDL + VLDL, 3 – CP + HDL, 4 – CP + MPO, 5 – CP + MPO + HDL, 6 – CP + MPO + LDL, 7 – CP + MPO + VLDL, 8 – CP + MPO + LDL + anti-apoB-100, 9 – CP + MPO + VLDL + anti-apoB-100. Panel C, 4-chloro-1-naphtol and H_2O_2 staining: 1 – MPO, 2 – MPO + CP, 3 – MPO + HDL, 4 – MPO + LDL, 5 – MPO + VLDL, 6 – CP + MPO + LDL, 7 – CP + MPO + VLDL, 8 – CP + MPO + LDL + anti-apoB-100, 9 – CP + MPO + VLDL + anti-apoB-100.

Although some publications described MPO binding to HDL particles (Zheng et al., 2004; Marsche et al., 2008), we did not succeed in revealing such interaction using either gel filtration or PAGE. This discrepancy may result from purification peculiarities of commercial MPO preparations. MPO used in the majority of studies is likely to contain admixtures of CTAB that may enforce the cationic charge of MPO and promote its interaction with lipoproteins. To verify

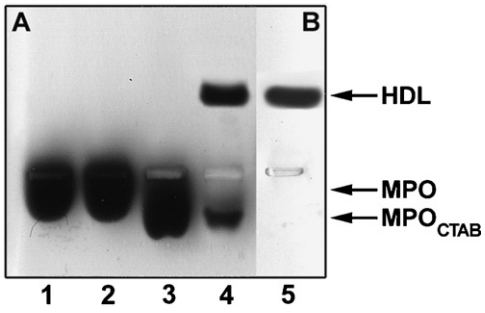

Fig. 3. Electrophoresis of the mixture MPO/MPO$_{CTAB}$ (0.5 μg) with HLD (4 μg of protein), Panel A, 4-chloro-1-naphtol and H_2O_2 staining, Panel B, Coomassie R-250 staining: 1 – MPO, 2 – MPO + HLD, 3 – MPO$_{CTAB}$, 4 – MPO$_{CTAB}$ + HDL, 5 – HDL.

this suggestion, we isolated some portion of MPO, adding CTAB to solutions at all stages but the last one. Preparations obtained without CTAB (MPO) and in its presence (MPO$_{CTAB}$) were compared using electrophoresis in agarose gel. MPO band was slightly shifted towards cathode, and this shift was more pronounced in MPO$_{CTAB}$ (Fig. 3, lanes 1 and 3). MPO mobility was not changed in the presence of HDL (lane 2). However, when HDL was added to MPO$_{CTAB}$, the enzymatic activity of MPO was revealed in the band that moved towards anode (lane 4) with the mobility of pure HDL (lane 5), which most likely results from complex formation between this protein and HDL. It can be suggested that a direct interaction between MPO and HDL in blood plasma or in arterial wall in the absence of CTAB either does not exist or is very weak.

Tests were made to learn whether components of blood plasma prevent the interaction of MPO with apoB-100-containing lipoproteins. In fractions obtained by gel filtration of a healthy donor's plasma on Superose 6 we did not reveal MPO even by highly sensitive ELISA. Therefore, 5 μg of MPO was added to 1 ml of blood plasma and gel filtration was repeated. In this case MPO were added virtually in even moieties between the high-molecular fraction 2 and fractions 9–12 that also contained CP (Fig. 4). The result of PAGE with gel staining for CP and MPO activities showed that the first peak included VLDL–MPO–CP and LDL–MPO–CP complexes, while the second one contained CP–MPO and non-bound CP (Fig. 4, insert).

Plasma samples from 65 healthy donors were screened using PAGE, and no MPO-containing complexes were detected. MPO con-

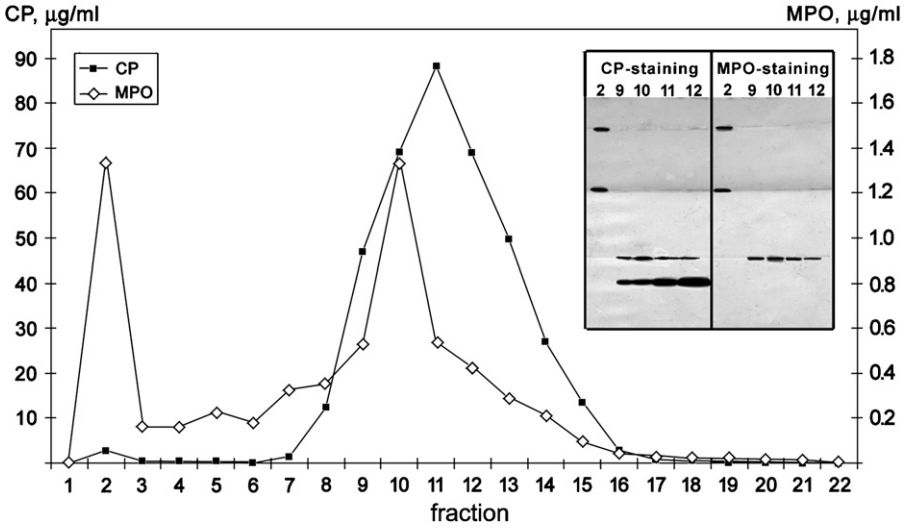

Fig. 4. CP and MPO content in fractions obtained by gel filtration on Superose 6 of blood plasma (1 ml) to which 5 μg of MPO were added. Inserts: electrophoretic resolution of 200 μl fractions containing MPO complexes. *o*-Dianisidine staining for CP activity; 4-chloro-1-naphtol and H_2O_2 staining for MPO activity.

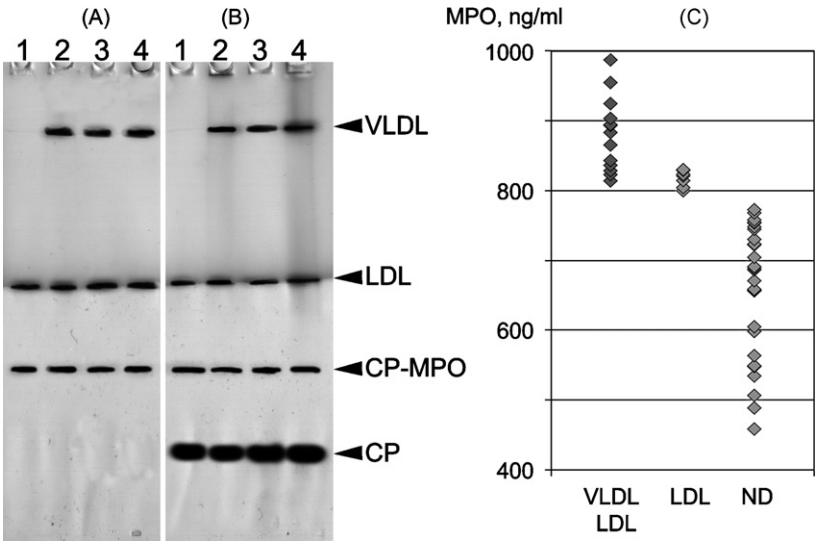

Fig. 5. Detection of VLDL/LDL–MPO–CP complexes in plasma (10 μl samples) of patients with atherosclerosis by four-layered PAGE. Panel A, 4-chloro-1-naphtol and H_2O_2 staining; panel B, *o*-dianisidine staining; panel C, level of MPO in group of patients: VLDL/LDL – complexes VLDL/LDL–MPO–CP (lines 2–4), LDL – complexes LDL–MPO–CP (line 1), ND–no complexes.

centration in samples assessed by ELISA was below 200 ng/ml. It is known that MPO concentration is increased in the blood of patients with atherosclerosis (Baldus et al., 2003). We checked whether plasma of such patients (100 samples) contains complexes of lipoproteins with MPO and CP (Fig. 5), assaying MPO concentration each time. No complexes of lipoproteins with MPO and CP were found in samples where MPO level was below 780 ng/ml (56 samples). When the content of MPO in patients' blood was increased, either LDL–MPO–CP complex (11 samples, 800–831 ng/ml MPO), or complexes VLDL–MPO–CP and LDL–MPO–CP (33 samples, 815–1993 ng/ml MPO) were revealed. The CP–MPO complex and a noticeable amount of non-complexed CP in all samples containing lipoprotein complexes were also found. These results showed that MPO in plasma can interact with CP and apoB-100-containing lipoproteins at the same time.

3.2. Determination of diameters and stoichiometry of the complexes by PCS

Diameters of separate proteins (CP, MPO) and lipoproteins (VLDL, LDL, HDL) as well as of two- and three-component complexes, which they are able to form, were estimated by PCS. The data obtained are summarized in Table 1. Formation of a complex is evidenced by a larger diameter registered upon mixing the components under study. For example, CP has a diameter of 7.4 ± 0.8 nm and that of MPO is 7.8 ± 1.2 nm, while after mixing these proteins the diameter increased to 9.8 ± 1.4 nm. If the components did not interact, the diameter value given by PCS was between the sizes of separate proteins, and the polydispersity index was high. For instance, mixing MPO (7.8 ± 1.2 nm) with HDL (10.7 ± 2.0 nm) produced a set of particles with the average diameter 9.0 ± 3.9 nm. When non-interacting particles' diameters were strongly different they could be registered as separate values (see CP and VLDL). By comparing concentrations of the components in formed complexes of MPO, CP, and lipoproteins (Table 1), their stoichiometry was assessed as 2VLDL:1MPO:2CP and 1LDL:1MPO:2CP.

Thus, we confirmed the interaction of MPO with CP and apoB-100-containing lipoproteins in solution, and for the first time determined the size of VLDL–MPO, VLDL–MPO–CP, LDL–MPO, and LDL–MPO–CP complexes.

3.3. Effect of CP and lipoproteins on peroxidase activity of MPO

MPO is able to provoke oxidative modifications of lipoproteins, and their affinity to each other may facilitate the deleterious effect. Since it is known that CP inhibits peroxidase activity of MPO, the tests were made to learn whether it retains the capability to suppress the MPO-catalyzed oxidation of ABTS (Fig. 6) and TMB in multi-component complex with lipoproteins. The values K_M and V_{max}, deduced from Hanes–Woolf plots, as well as calculated K_i, are summarized in Tables 2 and 3.

Kinetic parameters of ABTS and TMB oxidation in the presence of sole MPO (line 1) were close to those measured upon addition of lipoproteins to the reaction mixture (line 2) (Fig. 6). It means that lipoproteins at concentrations used in our experiment (0.65 nM VLDL, 0.68 nM LDL, 3.4 nM HDL) had no effect on peroxidase activity of MPO. Comparing parallel lines 1 and 3 (Fig. 6), one can ascertain that CP is a competitive inhibitor of MPO activity, as we have found previously (Sokolov et al., 2008). Addition of CP caused an increase of K_M, while V_{max} remained the same (Tables 2 and 3). However, lipoproteins alter the inhibitory effect of CP (Fig. 6A and B): line 4, which demonstrates oxidation of the substrates in the presence of CP and VLDL/LDL, is not only shifted with regard to line 2, but also turned. Both V_{max} and K_M are decreased in this case (see Tables 2 and 3), which implies non-associative inhibition of MPO activity. Addition of HDL did not affect the inhibition of MPO peroxidase activity by CP (Fig. 6C).

Besides K_i, that characterizes the CP influence on MPO-catalyzed reactions in the presence and in the absence of apoB-100-containing lipoproteins, we estimated the "inhibition constants" (K_i^*, apparent dissociation constants) for the alleviating effect of LDL/VLDL on the suppression of MPO activity by CP. These values were calculated using the formula for non-associative inhibition (Krupyanko, 2007)

$$K_i^* = \frac{[I]}{\sqrt{(K'_M/K_M - 1)^2 + (V_{max}/V'_{max} - 1)^2}},$$

where $[I]$ is the concentration of VLDL or LDL, K_M and V_{max} are the values of kinetic parameters of the substrates oxidation measured when MPO was inhibited by CP only, and K'_M and V'_{max} are the same parameters obtained in the presence VLDL or LDL. In case of both ABTS and TMB we found K_i^* equal to 0.3 nM for VLDL, and

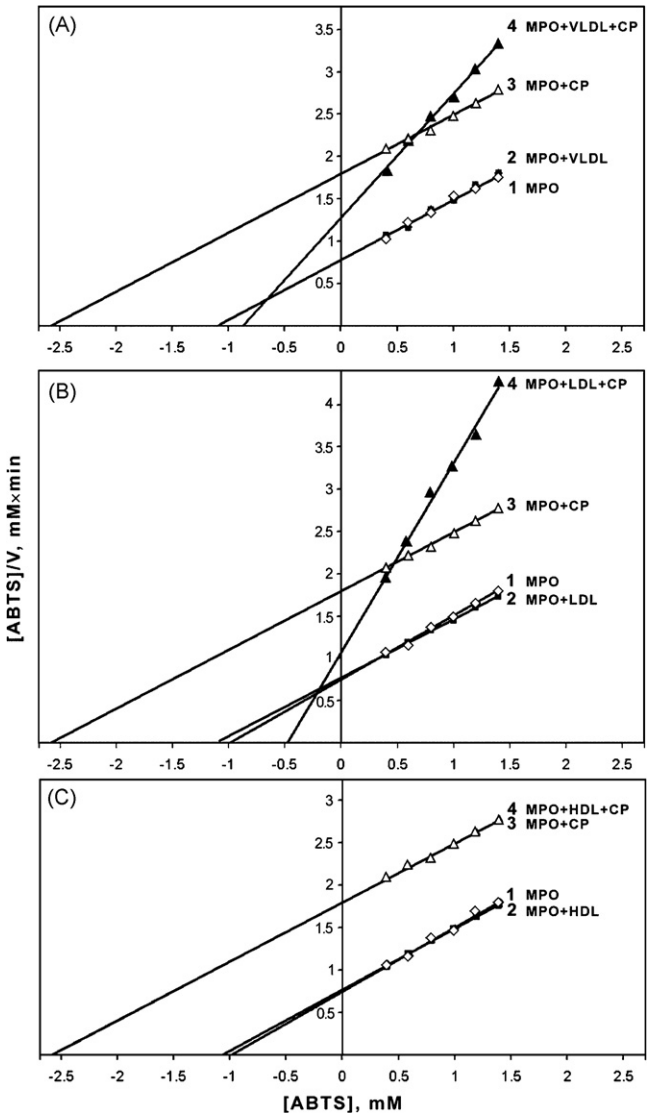

Fig. 6. Hanes–Woolf plots illustrating the effect of CP on kinetics of MPO-catalyzed ABTS oxidation. The mixture contained 5 nM MPO, 100 μM H_2O_2, ABTS (0.4–1.4 mM) in 0.1 M sodium-phosphate buffer, pH 6.0; CP and/or lipoproteins were added to achieve concentrations, respectively, of 0.5 μM and 0.5 μg/ml.

roughly 0.14 nM for LDL (Tables 2 and 3). These values allow us to assess the affinity of lipoproteins towards MPO.

4. Discussion

Using three independent methods, i.e. electrophoresis, gel filtration, and PCS, formation of multi-component complexes containing VLDL/LDL, MPO, and CP was demonstrated. Complexes were formed both upon adding isolated MPO to purified CP and lipoproteins, and to the blood plasma. Taking into account previous data on interaction of MPO with lipoproteins and with CP, it seems likely that the key link in formation of these complexes is the MPO molecule. In fact, our results show that CP alone is not able to bind to lipoproteins.

The attempts to reveal an affinity of MPO to HDL by any of the used methods failed, perhaps due to their insufficient sensitivity. It seems likely that interaction of MPO with HDL particles, if it only exists, is much weaker than its binding to VLDL or LDL. It was observed that the affinity of MPO to HDL becomes higher as the extent of lipoproteins' oxidative modifications increases (Marsche et al., 2008). Moreover, some data indicate that MPO interacts with a certain site on the surface of apoA-I and thus causes oxidative alteration of tyrosines (Zheng et al., 2004). It means that something like a vicious circle arises: the higher the extent of MPO-mediated damages in HDL, the stronger the affinity of HDL to MPO. We dealt mostly with the lipoproteins purified from healthy donors' plasma, and therefore, pronounced damages of the proteins on the surface of HDL were not likely. Nevertheless, in blood samples from patients with atherosclerosis we also did not reveal a band that would contain MPO and HDL (Fig. 5). MPO binding to HDL was observed only when the protein preparation contained CTAB admixtures. This detergent routinely used in the course of MPO purification may enhance its affinity towards lipoproteins, thus distorting the final results. When HDL immobilized in plate wells is used to detect MPO interaction with HDL (Marsche et al., 2008), correctness of the results also can be affected, since adsorption of HDL might alter the native state of lipoproteins, enhancing their binding to MPO. Consequently, direct interaction between MPO and native HDL in blood plasma or in arterial wall is unlikely. Yet, in spite of lesser affinity of MPO to HDL as compared to VLDL and LDL, involvement of MPO in formation of dysfunctional HDL particles via modifications produced by MPO-derived oxidative species cannot be excluded. Particularly, apoA-I isolated from plasma of patients with coronary heart disease contains greater amounts of specific markers of MPO-catalyzed oxidation, nitrotyrosine and chlorotyrosine, than apoA-I from healthy controls (Zheng et al., 2004; Bergt et al., 2004; Pennathur et al., 2004).

Authors who described the interaction of MPO with apoB-100-containing lipoproteins (Carr et al., 2000b), mentioned that no unequivocal data were obtained to tell whether MPO contacts the lipid, protein, or even carbohydrate moiety of lipoproteins. In our study dissociation of complexes VLDL/LDL–MPO–CP into CP–MPO upon adding antibodies against apoB-100 was observed. *In vitro* studies of oxidative modifications in apoB-100 of LDL, caused by either hypochlorite or the [MPO + H_2O_2 + Cl^-] system, indirectly evidenced in favor of apoB-100 interaction with MPO (Yang et al., 1999). Our data confirm the notion that MPO indeed contacts apoB-100, since antibodies against this protein displaced MPO from complexes with lipoproteins. Further study is required to localize the interaction site(s) for MPO on apoB-100 surface. Obtaining synthetic peptides or other agents, able to prevent binding of MPO to lipoproteins, might lay the ground for a novel regulation of MPO activity deleterious for lipoproteins.

The dimensions of complexes formed by CP, MPO, and lipoproteins were measured using PCS. Compared to chromatography, this method is advantageous as it allows to eliminate the possibility of interaction between components of a complex and a resin. Such an interaction may noticeably alter the results obtained in a gel filtration study of complexes formed by cationic and anionic proteins. Some part of the net charge of the proteins becomes shielded upon electrostatic coupling, which can increase the elution volume. It is noteworthy that dimensions of separate proteins and lipoproteins that we determined by PCS (Table 1) are in good agreement with previously published data (Sata et al., 1972; Patsch et al., 1976; Sokolov et al., 2009). To our knowledge this is the first measurement of the sizes of VLDL–MPO, VLDL–MPO–CP, LDL–MPO, and LDL–MPO–CP complexes. No interaction of MPO with HDL was registered by PCS. In a previous work using a calibration plot we determined by PCS the molecular mass of CP–MPO complex and estimated its stoichiometry as 1MPO:2CP (Sokolov et al., 2009). In the current study we cannot make suggestions about the molecular mass of the complexes observed due to the absence of proper calibration. However, it should be mentioned that upon interaction of VLDL with MPO and CP a substantial increase of the particles' diameter was noticed (Table 1), which is likely to indicate the inclusion of several VLDL molecules into the complex.

Table 2
Changes of kinetic parameters of MPO-catalyzed oxidation of ABTS in the presence of CP and lipoproteins. (K_i^* – apparent dissociation constants for the alleviating effect of LDL/VLDL on the suppression of MPO activity by CP).

Mixture	K_M, mM	V_{max}, ΔA_{414}/min	K_i CP, nM	K_i^*, nM
MPO	0.99 ± 0.06	1.32 ± 0.08	–	–
MPO + VLDL	1.09 ± 0.05	1.41 ± 0.07	–	–
MPO + LDL	1.12 ± 0.05	1.44 ± 0.07	–	–
MPO + HDL	1.06 ± 0.06	1.39 ± 0.08	–	–
MPO + CP	2.58 ± 0.06	1.44 ± 0.07	310 ± 30	–
MPO + CP + VLDL	0.88 ± 0.05	0.68 ± 0.07	530 ± 40	0.33 ± 0.03
MPO + CP + LDL	0.48 ± 0.05	0.45 ± 0.06	390 ± 30	0.15 ± 0.02
MPO + CP + HDL	2.58 ± 0.06	1.44 ± 0.07	–	–

We attempted to evaluate the stoichiometry of complexes formed by MPO, CP, and lipoproteins on the basis of amounts of mixed components. No assumption could be made on the basis of the concentrations in the PAGE study (Fig. 2). The components were mixed roughly with molar ratio 1MPO:2CP (in the presence of excessive amount of lipoproteins), and some part of CP retained the mobility typical for non-bound protein. Probably, MPO and lipoproteins also did not fully participate in the complex formation, although we could not detect it because non-bound MPO does not enter the gel. The amounts of lipoprotein particles interacting with CP and MPO in the course of gel filtration also cannot be estimated, since non-bound lipoproteins do not separate from VLDL/LDL–MPO and VLDL/LDL–MPO–CP complexes (Fig. 1, B and D). Gel filtration allowed us only to determine the ratio between CP and MPO within multi-component complexes. Both in experiments with purified preparations (Fig. 1) and in case of fractionation of blood plasma to which MPO was added (Fig. 4) the molar ratio of CP to MPO in fractions containing VLDL/LDL–MPO–CP complexes was close to 2. For example, fraction 2 in Fig. 4 contains 135 ng/ml MPO (0.9 nM) and 280 ng/ml CP (2.1 nM). These data are in agreement with our previous observation (Sokolov et al., 2009) and mean that each subunit of dimeric MPO contacts with one molecule of CP. Since lipoproteins do not alter this ratio, they seem unlikely to compete with CP for the binding sites on the surface of MPO. Comparing the final molar concentrations of the components that formed the complexes under PCS study (Table 1), one can see that one MPO molecule contacts either with one LDL particle, or with two VLDL particles. Thus, we assessed the stoichiometry of interactions between MPO, CP, and lipoproteins as 2VLDL:1MPO:2CP and 1LDL:1MPO:2CP.

On account of normal (lipo)proteins content in plasma of healthy humans (i.e. 3 μM CP, 1.5 nM MPO, 150 nM VLDL and 750 nM LDL) one can propose that MPO is a factor that limits the plasma concentrations of VLDL/LDL–MPO–CP and MPO–CP complexes. Even upon addition of "excessive" amounts of MPO to a healthy donor's plasma it was equally divided between VLDL/LDL–MPO–CP and MPO–CP complexes in the course of gel filtration. Considerable part of CP in such a case was not included in the complexes as judged by the elution profile in Fig. 4. Unsuccessful attempts to detect by electrophoresis these complexes in healthy donors' plasma may be explained by insufficient sensitivity of the method used to reveal MPO peroxidase activity in protein bands. Among patients with atherosclerosis VLDL/LDL–MPO–CP and MPO–CP complexes were detected only in plasma samples with MPO concentrations above 800 ng/ml. Perhaps this is the threshold concentration of MPO (about 5.5 nM) above which the complexes with lipoproteins can be detected by PAGE. Anyhow, it seems likely that the entire pool of plasma MPO is included into MPO–CP and VLDL/LDL–MPO–CP complexes. This notion underlies the necessity to study effects of CP and lipoproteins on oxidative properties of MPO.

The inhibitory effect of CP on MPO peroxidase activity described earlier (Sokolov et al., 2008) becomes weaker, but is not totally canceled, when these two proteins form a complex with lipoproteins. Considering obtained values for kinetic parameters (Tables 2 and 3), one can conclude that apoB-100-containing lipoproteins mainly decrease the affinity of CP towards the active centre of MPO. Indeed, when ABTS was oxidized in a solution containing MPO and CP, addition of VLDL to the reaction mixture resulted in a 70% drop of K_M (from 2.58 to 0.88 nM), while adding LDL made this value five times lower (0.48 nM). Simultaneously, V_{max} value for VLDL and LDL was two and three times lower, respectively (Table 2). Values of K_M and V_{max} measured when TMB was oxidized also decreased (Table 3). K_i values calculated for oxidation reactions catalyzed by MPO in the presence of lipoproteins and CP are different for two substrates used (Tables 2 and 3). This discrepancy probably comes from different pH values at which ABTS and TMB are oxidized (6.0 and 7.4, respectively), while the affinity of CP towards MPO decreases as pH goes down (Sokolov et al., 2007). The pH-dependent pattern of oxidation of the two substrates explains the data obtained when MPO is a part of complexes MPO–CP and LDL–MPO–CP. However, in the presence of VLDL the K_i value for TMB oxidation is higher than that for ABTS (770 and 530 nM, respectively).

The significant result of the kinetic tests is an estimation of K_i^* for VLDL and LDL, which quantitatively characterizes how lipoproteins alleviate inhibition of MPO activity by CP. The values define the affinity of an effector's interaction with an 'enzyme–inhibitor' complex, which in our case is binding of lipoproteins to MPO–CP. K_i^* values calculated for VLDL (~0.3 nM) or LDL (~0.14 nM) from the data on oxidation of two substrates were close, indicating that the difference in reaction conditions practically does not influence the affinity of lipoproteins to MPO. The physical meaning of K_i^* is that when lipoproteins are added to the CP- and MPO-containing reaction mixture in concentrations equal to K_i^*, they must bind to a half of MPO-CP complexes and show half of the possible effect.

Table 3
Changes of kinetic parameters of MPO-catalyzed oxidation of TMB in the presence of CP and lipoproteins. (K_i^* – apparent dissociation constants for the alleviating effect of LDL/VLDL on the suppression of MPO activity by CP).

Mixture	K_M, mM	V_{max}, ΔA_{650}/min	K_i CP, nM	K_i^*, nM
MPO	0.22 ± 0.02	0.636 ± 0.021	–	–
MPO + VLDL	0.23 ± 0.02	0.659 ± 0.031	–	–
MPO + LDL	0.20 ± 0.02	0.614 ± 0.039	–	–
MPO + HDL	0.24 ± 0.02	0.665 ± 0.043	–	–
MPO + CP	0.52 ± 0.02	0.636 ± 0.035	210 ± 20	–
MPO + CP + VLDL	0.16 ± 0.02	0.299 ± 0.029	770 ± 30	0.28 ± 0.03
MPO + CP + LDL	0.08 ± 0.02	0.232 ± 0.046	280 ± 20	0.12 ± 0.02
MPO + CP + HDL	0.49 ± 0.02	0.615 ± 0.028	–	–

In our study it was found out that one MPO molecule interacts either with one LDL particle or with two VLDL particles and consequently a double amount of VLDL is necessary for the same effect. The calculated values point at similar, rather high affinities of the MPO–CP interaction with one LDL or two VLDL particles. These stoichiometries seem to be paradoxical, because lipoprotein particles of both types contain a single apoB-100 molecule. Antibodies against apoB-100 completely disrupted the complexes of MPO with either VLDL or LDL, which means that MPO contacts with lipoproteins only through apoB-100. However, this unexpected result is in line with our notion of the absence of mixed complexes, formed by MPO and apoB-100-containing lipoprotein particles of two types. A likely explanation of their absence is a different spatial arrangement of apoB-100 within VLDL and LDL that precludes binding of the second LDL or VLDL particle to the MPO-LDL complex.

Since elevated MPO plasma concentrations is an independent prediction factor of a myocardial infarction risk (Baldus et al., 2003), it can be suggested that formation of complexes by lipoproteins and MPO may be a factor largely determining the development of atherosclerosis and the resulting complications. However, more studies and evidence are required to demonstrate an unequivocal linkage between such complexes and pathogenesis of atherosclerosis.

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