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Adsorption of Acylhydroperoxy-Derivatives of Phospholipids from Biomembranes by Blood Plasma Lipoproteins

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Abstract—It has been established that acylhydroperoxy derivatives of phospholipids from oxidized rat liver mitochondria are captured predominantly by LDL particles but not by HDL during co-incubation with blood plasma lipoproteins, which refutes the previously suggested hypothesis about the involvement of HDL in the reverse transport of oxidized phospholipids and confirms the possibility of different mechanisms of lipohydroperoxide accumulation in LDL during oxidative stress.

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INTRODUCTION

Lipid transport *in vivo* is executed by blood plasma lipoproteins present as two major classes referred to as low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Particles (nanoscale size) of such lipoprotein classes profoundly differ in terms of related metabolic functions as well as particle size and chemical composition [1-4]. HDL particles bear apoproteins A_1, C_2, and E [1-4], whereas larger-sized LDL particles contain only apoprotein B-100 [1-4]. LDL generated in the liver transport lipids to peripheral tissues, whereas HDL are involved in lipid reverse transport back to the liver [5-8]. It has been demonstrated that accumulation of oxidized LDL is the main event causing development and progression of atherosclerosis [9-11]. In contrast, high level of HDL protects from atherosclerosis development [12, 13].

The only protein in the LDL particles (apoprotein B-100) could be subjected to chemical modification with participation of low molecular weight dicarbonyl agents formed during free radical peroxidation of polyene lipids (such as malonic dialdehyde – MDA) as well as enzymatic oxidation or autooxidation of glucose and other 6-atom carbohydrates (e.g., glyoxal and methylglyoxal) [14-16]. The dicarbonyl-modified LDL particles are recognized by scavenger receptors and are captured by vascular wall cells, which are subsequently converted into the lipid-rich "foam cells" [11, 14-17]. The foam cells create primary pre-atherogenic lipoid lesions in the vascular wall in atherosclerosis and diabetes [11, 14-17]. The oxidized LDL particles can also bind to endotheliocyte LOX-1 scavenger receptor inducing NADPH oxidase expression and generation of superoxide anion radicals [15, 16, 18]. Overproduction of reactive oxygen species (ROS) ultimately promotes apoptosis and leads to endothelial dysfunction [15, 16, 18]. Thus, oxidative transformations of lipoproteins could play a crucial role in the molecular mechanisms of vascular wall damage during atherogenesis and diabetogenesis [14, 15]. Based on our own data and available publications, we hypothesized that vascular wall damage in atherosclerosis and diabetes mellitus occur via the same mechanism in which accumulation of the dicarbonyl-modified LDL plays an essential role [15, 19]. LDL particles are highly susceptible to initiation of free radical peroxidation in the outer phospholipid monolayer, whereas HDL particles remain quite resistant to lipid peroxidation [20]. Nevertheless, it has been shown that HDL could not only inhibit oxidation of LDL upon *in vitro* co-incubation [21], but also

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; LOOH, lipid hydroperoxides.

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exert an antioxidant effect (suppress LDL oxidation) in the bloodstream *in vivo* [22, 23]. Moreover, it was hypothesized that HDL particles are capable of capturing oxidized phospholipids from the supramolecular lipid–protein complexes (lipoproteins and biomembranes) to transport them to the liver, where enzymatic detoxification of lipid peroxides occurs [24]. Unfortunately, this hypothesis was not rigorously validated experimentally by using proper approaches and methods allowing to obtain irrefutable and unambiguous results [25, 26]. Based on the aforementioned, here we assessed ability of the LDL and HDL particles to absorb hydroperoxy-derivatives of phospholipids from the surface of oxidized biomembranes.

MATERIALS AND METHODS

Isolation of various lipoprotein classes (LDL, HDL) and subfractions (HDL_2, HDL_3) with preparative ultracentrifugation. Preparative isolation of blood plasma LDL, total HDL fraction, as well as HDL_2 and HDL_3 subfractions from apparently healthy donors was carried out by using NaBr density gradient centrifugation with an Optima XPN-80 ultracentrifuge (Beckman, USA) as described earlier [20, 27]. Lipoproteins of various classes were isolated from three blood plasma samples obtained from three individual healthy donors. Blood samples were collected into test tubes containing EDTA (1 mM) as an anticoagulant and antioxidant. Protein level in the isolated lipoprotein samples purified further by dialysis [20] was assessed by Lowry method.

Isolation and free radical oxidation of rat liver mitochondria. To isolate mitochondria, rat liver (WKY strain, males, 250-300 g) was perfused with a cooled isotonic KCl solution followed by homogenization (1 : 10, w/v) in a Potter homogenizer with Teflon pestle in a medium containing 0.25 M sucrose solution, 10 mM MOPS and 1 mM EDTA [28]. Nuclei and debris were precipitated by centrifugation at 700*g* for 10 min in an Eppendorf 5804 R refrigerated centrifuge (Eppendorf, Germany) followed by repeated centrifugation of the final supernatant at 8000*g* for 10 min to precipitate heavy mitochondria [28]. The pellet was resuspended in an EDTA-free isolation medium, and mitochondria were precipitated twice followed by resuspension (1 mg protein/ml) in an isotonic K/Na-phosphate buffer (pH 7.4). Free radical peroxidation of polyene phospholipids of mitochondrial membranes was induced by adding 0.5 mM ascorbate to reduce endogenous iron ions. Kinetics of mitochondrial free radical peroxidation under aerobic conditions was measured by assessing accumulation of conjugated double bond-bearing lipid hydroperoxides (LOOH) based on absorption at 233 nm wavelength using a UV-2600 spectrophotometer (Shimadzu, Japan) with an ISR-2600 integrating sphere attachment for measurements in a turbid medium. After a 4-hour incubation (reaching a plateau on kinetic curves), mitochondrial oxidation was inhibited by adding EDTA to a final concentration of 1 mM.

Assessing transfer of acylhydroperoxy phospholipid derivatives from oxidized mitochondrial membranes into lipoprotein particles under conditions of co-incubation. A pellet of oxidized mitochondria was resuspended in a medium containing isotonic K/Na-phosphate buffer (pH 7.4) and 1 mM EDTA, precipitated/resuspended twice at 8000*g* for 10 min (1 mg protein/ml) in an isotonic K/Na-phosphate buffer (pH 7.4) containing 1 mM EDTA. Next, LDL, HDL, or HDL_2 and HDL_3 subfractions (200 μg protein/ml) were added to the medium with oxidized mitochondria and incubated for 6 h. At specific time points, aliquots of the incubation medium were collected, centrifuged at 10,000*g* for 15 min to precipitate mitochondria and fragments of their membranes followed by evaluating LOOH level in the supernatant containing only lipoprotein particles based on absorption of the conjugated dienes at 233 nm. The experiments were repeated three times by using lipoprotein samples obtained from individual donors in each experiment. The methodology we developed allowed quick separation of the oxidized membranes from lipoprotein particles, which facilitated monitoring kinetics of LOOH accumulation in LDL and HDL. The amount of LOOH per LDL and HDL particle was calculated based on the determined levels of apoprotein B-100 and apoprotein A-1, respectively, by using molar extinction coefficient of 22,000 M^{-1} cm^{-1}. Concentration of LOOH per one LDL and HDL particles was calculated based on the level of apoprotein B-100 and apoprotein A_1 in LDL and HDL particles, respectively (each of these apoproteins is found at the level of 1 molecule per LDL and HDL particle, respectively). The level of apoprotein B-100 and apoprotein A_1 was measured using an Architect C8000 chemical analyzer (Abbott, USA) and relevant test kits (Abbott) [20].

Statistical analysis of the data was carried out using the software packages STATISTICA 10 (Statsoft, USA), MedCalc version 12.7.0.0 (MedCalc Software, Belgium), and Microsoft Excel 2010, version 14.0.7263.5000. The data were presented as a mean ± standard error of the mean. Considering that in all cases the analyzed parameters did not exhibit normal distribution, nonparametric statistical methods were applied. Analysis of intergroup differences for quantitative parameters was performed by using the nonparametric Mann–Whitney U-test. Differences were considered significant at $p < 0.05$.

RESULTS

Free radical oxidation of outer membranes of rat liver mitochondria. Kinetics of free radical oxidation occurring in rat mitochondria outer membranes is shown in Fig. 1.

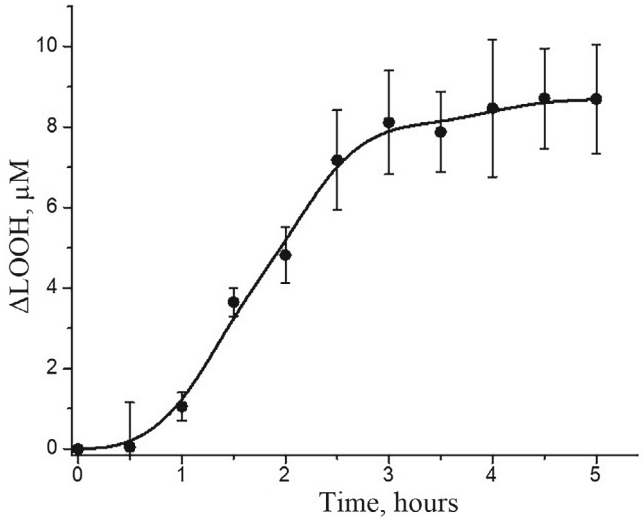

Fig. 1. Kinetics curve of free radical oxidation in the rat liver mitochondria outer membranes. For details of experimental settings see "Materials and Methods" section.

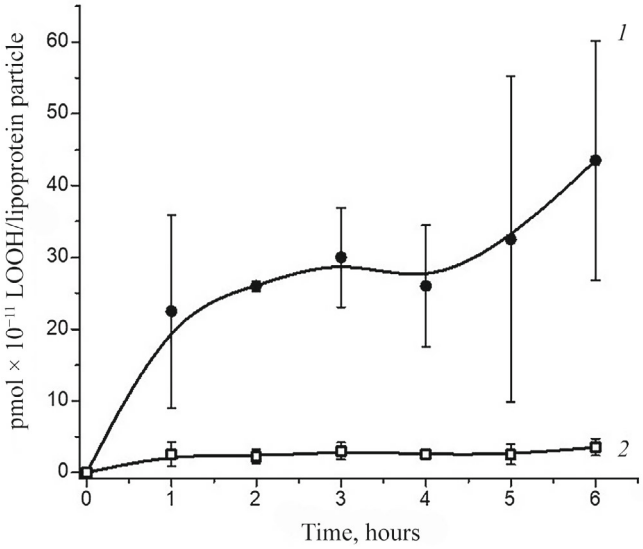

Fig. 2. Kinetic curve depicting incorporation of acylhydroperoxy phospholipid derivatives from the rat hepatic mitochondrial membranes subjected to free radical peroxidation into LDL (curve *1*) and HDL (curve *2*) particles. For details of experimental settings see "Materials and Methods" section.

As can be seen from the kinetic curve no further oxidation of polyene phospholipids within the mitochondrial membranes occurs after 3-hour incubation (Fig. 1).

Based on this fact, 3 h after the onset of incubation, main portion of the medium containing mitochondria was collected followed by suppression of further mitochondrial oxidation by adding EDTA to a final concentration of 1 mM for binding variable valency metal ions that catalyze oxidation. After three precipitation–resuspension cycles to remove membrane fragments and soluble low molecular weight oxidation products, the final preparation of oxidized heavy mitochondria was used to assess absorption of acylhydroperoxy-containing phospholipids of mitochondrial membranes by the isolated unoxidized LDL and HDL particles.

Assessing absorption kinetics of acylhydroperoxy-containing phospholipids of mitochondrial membranes by LDL and HDL particles. The results of investigation of the possibility for transfer of acylhydroperoxy phospholipid derivatives from oxidized biomembranes to LDL and HDL particles are shown in Fig. 2.

The obtained data indicate that the native LDL particles co-incubated with oxidized mitochondria predominantly capture acylhydroperoxy phospholipid derivatives (Fig. 2, curve *1*), whereas the rate of oxidized phospholipid transfer from the biomembranes to HDL particles is insignificant (Fig. 2, curve *2*). Kinetics of oxidized phospholipid absorption by the HDL subfractions – HDL_2 and HDL_3 – is presented in Fig. 3.

Hence, the obtained data allow concluding that the difference in the levels of acylhydroperoxy phospholipid derivatives absorbed from oxidized mitochondrial membranes by HDL_2 and HDL_3 particles were insignificant (Fig. 3, curves *2* and *3*), whereas they were significantly lower than the level of oxidized phospholipids absorbed by the total HDL fraction (Fig. 3, curve *1*) at all time points examined.

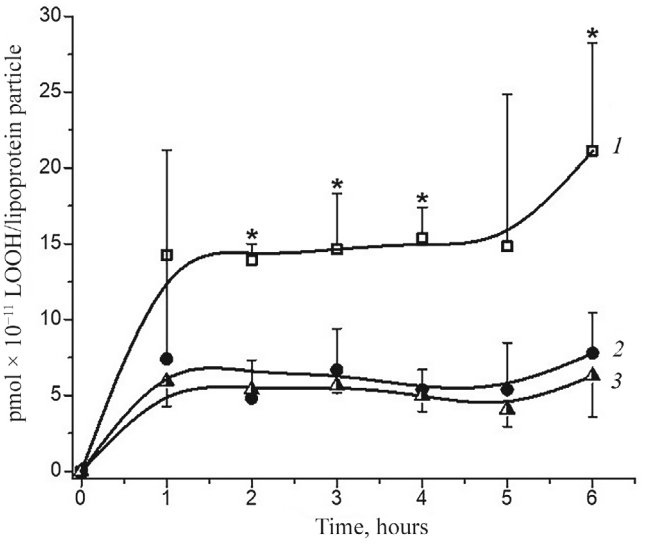

Fig. 3. Kinetics curve of incorporation of acylhydroperoxy phospholipid derivatives from the rat hepatic mitochondrial membranes subjected to free radical oxidation into total HDL fraction (curve *1*), HDL_2 (curve *2*), and HDL_3 (curve *3*) particles. Differences in the levels of incorporated LOOH into HDL_2 and HDL_3 particles found insignificant at all time points; * significant differences in the level of incorporated LOOH for total HDL fraction as well as HDL_2 or HDL_3 particles ($p < 0.05$). For details of experimental settings see "Materials and Methods" section.

DISCUSSION

The data obtained in our study (Fig. 2) contradict the previously proposed hypothesis suggesting the possibility of the HDL-mediated reverse transport of oxidized lipids to the liver [24]. It should be noted that the possibility of the transfer of phospholipid acylhydroperoxy derivatives from the oxidized erythrocyte membranes (shadows of erythrocytes) to co-incubated LDL has been demonstrated earlier [28] and is not considered as unusual. It was shown in other similar studies [29] that the multi-hour oxidation of erythrocyte shadows with the free radical oxidation initiators resulted in generation of a large number of secondary products of aldehyde nature [16] presence of which could be a source of artifacts [16]. It is worth mentioning that the erythrocyte membranes are not particularly suitable for such experiments, because, unlike liver microsomes and mitochondria, erythrocytes are highly resistant even to enzymatic lipid peroxidation [30]. In accordance with our data, LDL particles, unlike HDL, can not only be easily oxidized via the free radical mechanism [20], but also can capture oxidized phospholipids from other lipid–protein supramolecular complexes when they interact (Fig. 2). Destruction of the hydroperoxy derivatives of phospholipids accumulated in the LDL should undoubtedly elicit atherogenic modifications of LDL particles by the secondary products of oxidation such as low molecular weight dicarbonyls, e.g., 4-hydroxy-2-nonenal and MDA [14, 16, 17] contributing to atherogenesis [14-17]. Nevertheless, there are data available showing that the oxidized lipids can be actively transferred from the outer phospholipid layer of LDL particles to HDL particles during their co-incubation [31]. Moreover, oxidized lipids of the hydrophobic core of LDL are not transferred to HDL and oxidized HDL lose their capability to adsorb lipoperoxides from oxidized LDL [31]. Indeed, the acylhydroperoxy derivatives of phospholipids of the outer layer of LDL particles are significantly more polar than the non-oxidized phospholipids [32]. As a result, the phospholipid hydroperoxy acyls should enter the water phase [32] and, probably, could be more efficiently exchanged with the other lipid–protein supramolecular complexes upon contact. Despite that it is HDL subfractions HDL_2 and HDL_3, which are credited with a cardioprotective effect [33-35], our data (Fig. 3) indicate that if such effect exists, it probably has nothing to do with their presumed ability to participate in detoxification of lipid hydroperoxides [24]. It is evident that the overall LOOH absorption by HDL_2 and HDL_3 subfractions is lower (Fig. 3, curves *2* and *3*) than that by the total HDL fraction (Fig. 3, curve *1*). This apparent contradiction may be due to the fact that the number of total HDL subfractions is not limited solely to HDL_2 and HDL_3 [34]. Moreover, preparative isolation of HDL_2 and HDL_3 may be associated with the loss of relevant oxidized species present in the total HDL fraction [35].

CONCLUSION

The data obtained in our study imply existence of diverse mechanisms causing emergence of atherogenic LDL enriched in free radical peroxidation products, which further highlights the threat posed by the pathogenetically-important oxidative transformation of LDL *in vivo* [14-16]. Antioxidant action of HDL particles [36] may be related to the presence of paraoxonase-1, which is capable of reducing lipid peroxides within LDL particles upon co-incubation [29, 37]. Obviously, convincing data are necessary to validate this putative mechanism for blood plasma lipid peroxides utilization *in vivo* involving an effective exchange between the LDL-derived oxidized phospholipids and HDL particles. Unfortunately, no such data are currently available. What is clear that enrichment of the LDL particles with the oxidized lipids, as shown by our data, may occur both during intense lipid free radical peroxidation as well as by the capture of oxidized lipids from the membranes of blood cell (erythrocytes, etc.). Both these processes should contribute to the increased atherogenicity of the oxidatively modified LDL, i.e., facilitate their involvement in the vascular wall damage [14-16].

Contributions. V.Z.L. – supervised the study, discussed the data; A.K.T. – wrote and edited the manuscript; V.Y.K., G.G.K. – performed experiments, prepared the manuscript.

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Ethics declarations. The authors declare no conflict of interests in financial or any other sphere. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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