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On the Ability of High Density Lipoproteins to Remove Phospholipid Peroxidation Products from Erythrocyte Membranes

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Abstract—To study the transfer of oxidized phospholipids from cell membranes to high-density lipoproteins (HDL), human Cu^{2+}-oxidized erythrocyte membranes were incubated with HDL_3 subfraction for 17 h at 37°C followed by isolation of the supernatant, precipitation from it of HDL_3, and determination of lipid peroxide products (LPP) in them. The incubation increased the content of lipid hydroperoxides in HDL_3 significantly (by 32 and 40% calculated per ml of sample or mg of protein) and of malondialdehyde (by 27 and 34%, respectively) compared to control (incubation of HDL_3 alone). The content of conjugated dienes did not change significantly. Fluorescence analyses of isolated HDL_3 particles showed that the content of fluorescent products (λ_{ex} = 365 nm, λ_{em} = 430 nm) in them was 2.5 times higher than in control, and the number of binding sites for the 1-anilinonaphthalene-8-sulfonic acid probe decreased by 22%. This also confirms accumulation of LPP in the lipoprotein subfraction. It seems likely that an increase in LPP (at least hydroperoxides) in HDL_3 after their incubation with oxidized membranes occurs via transport of phospholipids containing LPP from erythrocyte membranes to lipoproteins. The data on the ability of HDL_3 to accept LPP from erythrocyte membranes *in vitro* suggest that HDL_3 may have a protective action on cell membranes undergoing oxidation *in vivo* as well.

Key words: lipid peroxidation, lipid hydroperoxides, conjugated dienes, malondialdehyde, high-density lipoproteins, phospholipids

In earlier experiments, we showed that high-density lipoproteins (HDL) inhibit the peroxidation of low-density lipoproteins (LDL), both spontaneous and induced by iron ions or xanthine–xanthine oxidase *in vitro* [1]. The results of these experiments were confirmed and expanded in some other laboratories (see review [2]).

The antioxidant effect is most pronounced in the HDL_3 subfraction and is accounted for by the action of lecithin:cholesterol acyltransferase (LCAT) tightly bound to this lipoprotein subfraction [3]. The addition of partially or highly purified LCAT protected LDL from oxidation [3, 4] and also from forming aggregates *in vitro* [4].

The protective antioxidant effect of HDL also occurs *in vivo*. This was evident in experiments on animals where after an intravenous injection of large doses of HDL_3 the content of conjugated dienes and trienes in the plasma was decreased [5, 6] and in the observation of people which showed a negative correlation between the level of HDL cholesterol and the content of conjugated dienes (CD) and lipid hydroperoxides (LHP) [5, 7]. Investigations using transgenic mice expressing human apo A-I showed that an increase in HDL in plasma was followed by a significant decrease in the content of lipid peroxidation products (LPP) [8].

There are grounds to suggest that during the formation of peroxidatively modified LDL part of the oxidized phospholipids undergo further catabolism inside the lipoprotein particles [9] and part is transferred to HDL_3 [10]. The functions of HDL_3 include transportation of cholesteryl esters to the liver to be converted to bile acids, the transfer of oxidized phospholipids from LDL to HDL_3, and participation in the formation of cholesteryl esters. These functions contribute to the elimination of toxic LPP from the body.

Thus, HDL_3 can have a double protective effect: to inhibit oxidation of LDL and to accept oxidized phospholipids from this lipoprotein fraction in case the latter have somehow undergone oxidation. The question therefore arises as to whether HDL can accept oxidized phospholipids from cell membranes. The question seems quite

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reasonable since the cholesterol-accepting function of HDL_3 from cell membranes is well known [11, 12].

The aim of the present work was to determine whether during their interaction with erythrocyte membranes HDL_3 can accept and thus eliminate LPP. Further interest in this problem is prompted by the fact that in the case of ischemic heart disease, LPP accumulate in erythrocyte membranes [13] and modify erythrocyte proteins—hemoglobin and band-3 protein [14].

MATERIALS AND METHODS

Isolation of erythrocyte membranes. Erythrocyte membranes were isolated by the method of Dodge et al. [15]. Erythrocytes were obtained from donor blood (storage did not exceed 24 h) prepared at a donor station by a convenient method with Glugicir (citrate–glucose solution) used as an anticoagulant. The blood was centrifuged (1000*g*, 20 min), and the plasma and buffy coat removed. The erythrocytes were washed three times in 0.9% NaCl and placed in test tubes. Each test tube containing 4 ml of "packed" erythrocytes was subject to osmotic hemolysis by suspending in cooled 10 mM sodium phosphate buffer (pH 8.0) at ratio 1 : 10 (v/v). After hemolysis, the erythrocyte mixture was left to precipitate at 4°C for 30 min and then centrifuged at 4000*g* for an hour at 4°C. The supernatant was removed and another portion of buffer was added. The membranes were washed three times (until the liquid over the precipitate was no longer pink), stored at −20°C, and used within 1-2 days. Erythrocyte membranes isolated in this way contained on average 0.47 mg of phospholipids per mg protein.

Isolation of lipoproteins. HDL_3 were isolated from another portion of donor blood by sequential centrifugation [16] in a 70 Ti rotor (in a Beckman L8-55 centrifuge, USA) in a NaBr density gradient (1.125-1.21 g/ml) with addition of EDTA as antioxidant (1 mg/ml). The isolated HDL_3 subfraction was dialyzed against solution containing 0.9% NaCl and 0.1% EDTA for 24 h at 4°C to remove NaBr.

Induction of peroxidation in membranes. To oxidize the membrane lipids, the suspension of erythrocyte membranes in 0.9% NaCl was incubated in the presence of 100 μM $CuSO_4$ at 37°C for 20 h. The membranes were then precipitated by centrifugation at 4000*g* and resuspended in 0.9% NaCl.

Experimental protocol. HDL_3 were incubated with oxidized membranes for 17 h at 37°C in a water bath with stirring. The incubation mixture contained (per ml): oxidized membranes in 0.9% NaCl (about 0.6 mg protein); HDL_3 in 0.9% NaCl (2.5-3 mg protein), 0.05 M Tris-HCl buffer, pH 7.4 (0.35 ml), and antioxidants—EDTA and β-ionol (butylated hydroxytoluene)—1 mg and 4.4 μg, respectively. The total assay volume was 3 ml. The control samples contained all the above-mentioned components except erythrocyte membranes. After incubation, the membranes and supernatant were separated by centrifugation at 4000*g* for 60 min, and the supernatant was used for precipitation of HDL_3 with sodium tungstate and $MgCl_2$ according to Burstein et al. [17]. The precipitated lipoprotein fraction was resuspended in 0.9% NaCl and 10% Na_2CO_3 was added drop by drop until the precipitate was fully dissolved. The HDL_3 solution was used for determination of protein and LPP. The control experiments showed that according to polyacrylamide gel electrophoresis [18], HDL_3 isolated from the incubation medium were homogenous and did not contain any other lipid-containing admixtures.

In a special series of experiments, erythrocyte membranes with Cu^{2+}-oxidized phospholipids were incubated with HDL_3 for a shorter period, 3 h only. Then the supernatant was taken to determine the concentration of LPP.

Determination of protein. For determination of total protein, the erythrocyte membranes were preliminary treated with 2% SDS with mixing for 30 sec on a Vortex mixer. The protein was determined according to Lowry et al. [19].

Determination of phospholipids and LPP. Phospholipids were determined using the enzymatic method of Boehringer Mannheim (MPR2). Lipids were extracted according to Folch [20] with chloroform–methanol mixture (2 : 1 v/v). To measure the LPP, aliquots of the chloroform layer were evaporated under nitrogen. The quantity of CD was measured after the addition of methanol–heptane (4 : 1 v/v) using an SF-26 spectrophotometer (Russia) at 233 nm [21], and the data were recalculated using a molar extinction coefficient of $2.1 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$. LHP were determined by the iodometric method [22], using the molar extinction coefficient $2.46 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ and malondialdehyde (MDA) from the reaction with 2-thiobarbituric acid [23] using the molar extinction coefficient $1.56 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$.

Fluorescence measurement. The relative intensity of fluorescence as an additional criterion of peroxidation of lipoprotein particles was estimated using a Perkin-Elmer 500 spectrofluorimeter (USA) and taking readings at 365 and 430 nm (excitation and emission, respectively) according to the method of Shimasaki et al. [24]. The HDL_3 protein concentration was 50-100 μg/ml. In addition, quenching of the fluorescence of the probe 1-anilinonaphthalene-8-sulfonic acid (ANS) by LPP was assayed [25]. The final concentration of the ANS probe was 40 μg/ml.

RESULTS AND DISCUSSION

Under physiological conditions many LPP continue to undergo oxidation and interact with each other or with proteins, loosing their capacity to be measured by convenient methods. Thus, in our investigation, to estimate

the degree of peroxidation, three LPP were measured—two primary (CD and LHP) and one secondary (MDA). Since the MDA content was estimated from the reaction with thiobarbituric acid (TBA), the abbreviation "MDA" is used to cover peroxidation products capable of interacting with TBA.

In isolated HDL_3, all these LPP were present (Table 1) but in different quantities: LHP and CD were the major LPP (43.08 and 42.29 nmole/ml of sample, respectively), while MDA was present at a concentration about 6.5 times lower.

From Table 1 it also follows that isolated erythrocyte membranes contained CD and LHP, and the proportion of these products was very similar. Determination of MDA in membranes showed an extremely low concentration of this LPP (on the limit of sensitivity of the method). Table 1 shows that incubation of membranes in the presence of Cu^{2+} led to approximately twofold increase in the content of all LPP studied.

Oxidation of membrane phospholipids was achieved by prolonged incubation with a high concentration of $CuSO_4$ (100 μM) for 20 h. It would appear quite likely that the initial erythrocyte membranes used in this investigation had been partially oxidized in the course of isolation and washing.

First, we tried to detect the possible transfer of LPP from erythrocyte membranes to the incubation medium containing HDL_3. In the first series of investigations, when oxidized erythrocyte membranes were incubated with HDL_3 for 3 h, there was an increase in LPP in the supernatant compared to incubation of HDL_3 alone: CD by 19%, LHP by 11%, and MDA by 24%. This increase, however, was found to be statistically insignificant.

In our investigation, HDL_3 were used as a diluted solution of this lipoprotein subfraction in 0.9% NaCl. Therefore, we cannot exclude the possibility that during incubation some LPP, water-soluble ones in particular, were transferred from the membranes to the aqueous medium and, thus, could be wrongly interpreted as being transferred into HDL_3. To avoid such a possibility, HDL_3 from the incubation medium were precipitated and used for analysis. To achieve a more pronounced effect, incu-

Table 1. Proportion of LPP in isolated HDL_3 and erythrocyte membranes in nmole/ml sample (average data of 6 experiments, M ± SD)

Investigated material	CD	LHP	MDA	Total content
Solution of HDL_3 in 0.9% NaCl	42.29 ± 12.77	43.08 ± 10.36	6.57 ± 0.68	91.94 ± 23.11
Suspension of erythrocyte membranes in 0.9% NaCl				
initial	19.73 ± 1.37	19.85 ± 0.73	0.33 ± 0.08	39.91 ± 1.69
after incubation at 37°C for 20 h with Cu^{2+}	36.06 ± 7.01	40.57 ± 4.11	0.74 ± 0.17	77.38 ± 8.11

Table 2. Content of LPP in HDL_3 subfraction precipitated from the supernatant after incubation of HDL_3 with Cu^{2+}-oxidized erythrocyte membranes for 17 h at 37°C (average data of 6 experiments, M ± SD)

Incubated material	LPP					
	CD		LHP		MDA	
	nmole/ml sample	nmole/mg protein	nmole/ml sample	nmole/mg protein	nmole/ml sample	nmole/mg protein
HDL_3 (control)	44.25 ± 9.76	7.83 ± 1.58	47.22 ± 10.49	8.36 ± 1.34	12.85 ± 1.74	2.30 ± 0.44
HDL_3 + Cu^{2+}-oxidized membranes	40.59 ± 6.09 (n.s.)	7.66 ± 1.38 (n.s.)	62.12 ± 4.84*	11.69 ± 1.19**	16.31 ± 1.77***	3.09 ± 0.54*

Note: Incubation medium contained sodium salt of benzylpenicillin (0.1 IU/ml) and streptomycin sulfate (1 μg/ml); n.s., non-significant differences.

* $p < 0.02$.

** $p < 0.002$.

*** $p < 0.01$.

bation was carried out for 17 h. The data obtained in this series of experiments are given in Table 2.

As can be seen from Table 2, incubating HDL_3 with oxidized erythrocyte membranes increased the content of LPP, especially LHP, in the HDL_3 (by 32 and 40% calculated per ml of HDL solution and per mg of protein, respectively). The content of MDA increased by 27 and 34%. The content of CD was not changed significantly. Thus, the incubation of HDL_3 with erythrocyte membranes resulted in a portion of the oxidized products being transferred from the membranes to HDL_3.

It may well be that such transfer (at least in the case of LHP) occurs through membrane phospholipids via physicochemical exchange between oxidized phospholipids of membranes and non-oxidized phospholipids of HDL_3. It should be mentioned at this point that a transfer of phosphatidylcholine to HDL from monolayer phosphatidylcholine–cholesterol liposomes [26] and from erythrocytes [27] was described earlier. However, the mechanism by which LPP are transferred from oxidized membranes to HDL_3 requires further investigation.

It has been previously reported that a major portion of total lipoprotein MDA is contained in HDL [28, 29]. However, it remains unclear how much of it is formed within lipoprotein particles and how much comes from the outside. It should be noted that in HDL_3 the portion of MDA in the total content of LPP is about 7%, while in erythrocyte membranes (initial and oxidized) it is very small, about 1% (Table 1). The increase in concentration of MDA in HDL_3 from 12.85 to 16.31 nmoles/ml of sample (Table 2) after incubation of oxidized membranes with HDL_3 suggests that MDA is formed from the primary LPP within lipoprotein particles, but not transported from erythrocyte membranes, where as already mentioned its concentration compared to other LPP was very small. Physiologically, the accumulation of LPP, and especially MDA, is most dangerous for the body; it would seem more likely that they are transported within lipoproteins to the liver for further metabolism and detoxication.

In our investigation, in addition to the direct determination of LPP in HDL_3 particles, a fluorescence method for the detection of the oxidized products was also used. The results we obtained are given in Table 3.

As can be seen, in HDL_3 incubated with oxidized membranes the fluorescence intensity ($F_{365/430}$) increased. This indicates the presence in HDL_3 of oxidized products or their fluorescent derivatives. On the other hand, it is well known that with an increase in the LPP content in lipoprotein particles the fluorescence of the ANS probe is quenched [25, 30]. In our experiments, there was a decrease in the number of binding sites for the probe in HDL_3 particles incubated with membranes. Thus, the results of the fluorescence analysis can be regarded as confirming the transfer of LPP from oxidized erythrocyte membranes to HDL_3 particles.

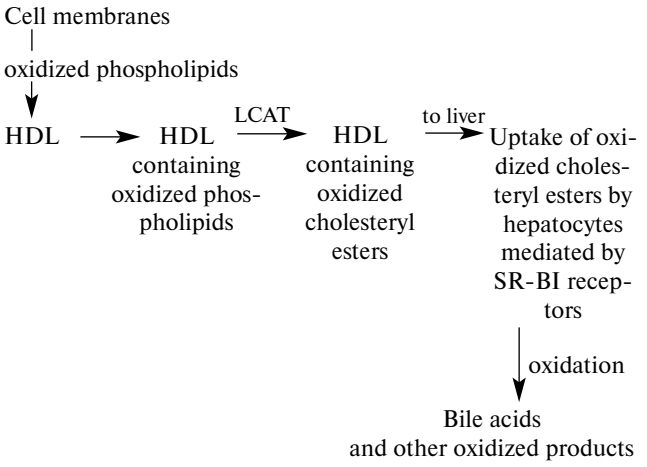
Table 3. LPP determined in HDL_3 using fluorescence analysis (average data of 3 experiments, M ± SD)

Parameter	HDL_3	
	initial	after incubation with Cu^{2+}-oxidized membranes
LPP determined by fluorescence intensity at $\lambda_{ex} = 365$ nm, $\lambda_{em} = 430$ nm (relative units)	1.00	2.56 ± 0.34*
Number of ANS binding sites (relative units)	1.00	0.78 ± 0.10*

* $p < 0.05$.

The turnover of phospholipid phosphorus in erythrocyte membranes is slow (3.3-4.6% in 24 h) [31], but the rate of its turnover is higher than the rate of total turnover of erythrocytes (about 120 days). It seems quite probable that the turnover of oxidized, as compared to non-oxidized, phospholipids of erythrocytes is faster. And we suggest that HDL_3 are involved in this process. The addition of phosphatidylcholine hydroperoxides to human plasma or to a solution of HDL (but not LDL) results in the formation of lysophosphatidylcholine. This reaction is inhibited by 5,5′-dithio-bis(2-nitrobenzoic acid) [32]. Thus, HDL-bound LCAT and phospholipid hydroperoxides take part in the formation of cholesteryl esters containing oxidized fatty acids. It was also shown that oxidized long-chain phosphatidylcholines are substrates for plasma LCAT [33]. Data are available indicating that HDL containing oxidized lipids (oxidized cholesteryl esters, in particular, formed as a result of the LCAT reaction) are eliminated from blood during perfusion of rat liver [34] and are taken up by Hep G2 cell culture [35] faster than HDL containing non-oxidized esters. This furnishes evidence that the body tends to get rid of oxidized lipids and seemingly of oxidized cell membrane phospholipids. HDL_3 and HDL-bound LCAT are both involved in this process.

It is well known that HDL particles passing through the liver interact with SR-BI receptors of hepatocytes followed by uptake of cholesteryl esters by the cells [36]. In the liver, cholesteryl esters undergo oxidation to form bile acids. Taking this into account, the following scheme for transport of oxidized phospholipids from the cell membranes to the liver where they will be oxidized can be suggested:



Thus, HDL_3 and LCAT are involved in protection not only of LDL from oxidation, as mentioned above, but also in protection of cell membranes from accumulating oxidized phospholipids.

It may well be that the ability of HDL_3 revealed in our *in vitro* experiments to accept oxidized phospholipids from erythrocyte membranes also occurs *in vivo* and may occur with membranes of other cells. It may prove to have an important role in the reparation of cell membranes.

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