Vol. 200, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1994 Pages 408-416

REDUCED CHOLESTEROL EFFLUX TO MILDLY OXIDIZED HIGH DENSITY LIPOPROTEIN

Diane W. Morel

Department of Biochemistry
The Medical College of Pennsylvania
Philadelphia, PA 19129

Received February 28, 1994

SUMMARY: Mild oxidation (<6h) of human high density lipoprotein (HDL) induced by incubation with copper ions increased its thiobarbituric acid reactivity, decreased its phospholipid content, increased its free cholesterol to phospholipid ratio and reduced its ability to mediate cholesterol efflux from cells. While protein modification did occur with further oxidation (>12h), diminished efflux of cell cholesterol was apparant for HDL samples without increased electrophoretic mobility or altered apolipoproteins. Thus, reduced cholesterol efflux to mildly oxidized HDL may reflect changes in its lipids rather than its apoproteins. By limiting cholesterol removal from cells, any mild oxidation of HDL which might occur *in vivo* could contribute to cellular cholesterol accumulation.

High density lipoprotein (HDL) is correlated with decreased risk of atherosclerosis (1,2) and it is believed that this is because of its ability to stimulate removal of cholesterol from peripheral cells and transport this cholesterol back to the liver for excretion (reverse cholesterol transport) (3,4). Cellular cholesterol efflux, the first step in reverse cholesterol transport, is governed in part by the aqueous diffusion of cholesterol from the plasma membrane and association with HDL particles in solution (3,4). In certain systems HDL may associate with a putative receptor, stimulating translocation of cholesterol from intracellular pools to the cell surface and enhancing efflux (5,6). In these systems it is the primary apoprotein of HDL, apo AI, which is thought to mediate the association of HDL with the putative receptor.

HDL has other effects on lipid metabolism as well. For example, HDL inhibits the oxidation of low density lipoprotein (LDL) when present in a co-incubation (7) and ameliorates the cytotoxicity of previously oxidized LDL (8,9) *in*

Abbreviations: high density lipoprotein, HDL; low density lipoprotein, LDL; butylated hydroxytoluene, BHT; denaturing polyacrylamide gel electrophoresis, SDS-PAGE; thiobarbituric acid reacting substances, TBARS; ethylenediaamintetraacetic acid, EDTA; bovine serum albumin, BSA.

0006-291X/94 $5.00

vitro. Since it has been widely speculated that oxidized LDL may contribute to the development of the atherosclerotic plaque (10,11), this is another means by which HDL may protect against atherosclerosis.

In contrast to oxidized LDL, for which there is evidence that oxidation occurs *in vivo* (12,13), there is no evidence as yet that oxidation of HDL occurs *in vivo*. However, in *in vitro* studies, HDL has been reported to be both more and less resistant to oxidation than LDL (14,15). Extensive copper-induced oxidation or chemical modification of apo A1 reduces efflux of cellular cholesterol to that HDL (16,17). In addition, oxidized HDL is reported to inhibit *de novo* cholesterol biosynthesis in fibroblasts (18) and to induce and promote platelet aggregation (19).

The present studies are the first to examine the changes in chemical composition and function of HDL upon mild, copper-stimulated, oxidation. They show that mild oxidation of HDL alters the lipid composition of the HDL and reduces its ability to mediate efflux of plasma membrane cholesterol even without an alteration in its apoproteins.

MATERIALS AND METHODS

Human HDL3 (1.12<d<1.21 g/ml) was isolated by sequential ultracentrifugation from fresh human plasma as described previously (20). To oxidize HDL, ethylene diaminetetraacetic acid (EDTA) used during preparation was removed by overnight dialysis against deaerated phosphate-buffered saline, then copper sulfate was added to HDL3 (adjusted to 1.5-3 mg protein/ml) to a final concentration of 4 uM. The reaction was stopped after the indicated times by the addition of 2 mM EDTA and 50 uM butylated hydroxytoluene (BHT). The relative electrophoretic mobility was measured using 0.5% agarose gels (Beckman Lipo gels). The extent of oxidation was monitored by the thiobarbituric acid assay (in duplicate) as described before (TBARS, 9). HDL lipids were extracted according to Bligh-Dyer (21), then assayed for phospholipids (in triplicate) by the method of Sokoloff and Rothblat (22) and total and free cholesterol (in duplicate) according to Gamble et al. (23), with esterified cholesterol calculated by difference; HDL protein was measured (in duplicate) by the Markwell modification (24) of the Lowry method . Apo AI was measured by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and by a commercial immunoturbidometric assay (Raichem ^{TM}, Reagents Applications, San Diego, CA).

Fu5AH cells, a rat hepatoma cell line, were employed to examine the efflux of plasma membrane cholesterol to HDL3 using procedures established in this department (25). Several days after plating in 6 well plates, cell cholesterol (primarily plasma membrane cholesterol, 26) was radiolabelled by exposure to medium containing 5 % fetal bovine serum and 0.1 μCi/ml [^{14}C]cholesterol (4-[^{14}C]cholesterol, NEN) followed by equilibration overnight in medium containing 0.2% bovine serum albumin (BSA). In some experiments, the free cholesterol content of cells was increased by pretreatment of cells for 24 h with lipoprotein-deficient medium followed by exposure to dispersions of free cholesterol and phosphatidylcholine in a molar ratio of 2:1 (100 μg phospholipid/ml) with 1 μCi/ml [^{14}C]cholesterol as described previously (27). Cells were then washed twice with PBS, and the experimental media containing 0.2% BSA plus the various HDL

samples, adjusted to a final concentration of 300 or 500 ug protein/ml, were added. Esterification of cholesterol was inhibited throughout by the presence of Sandoz compound 58-035 (1 μg/ml). All experiments used triplicate wells per group and included a control group with no HDL. During incubation at 37°C, samples of the medium were removed at various times, centrifuged to remove any cell debris and aliquots were taken for liquid scintillation counting. Cell lipids were extracted by isopropanol at the end of the incubation and aliquots were taken for liquid scintillation counting.

RESULTS

Table 1 shows the time course of HDL oxidation and resulting changes in chemical composition (up to 12h exposure to 4μM copper at room temperature). In initial experiments, the extent of oxidation per particle generated by this oxidation protocol was less for HDL samples than for comparably treated LDL samples (data not shown). With increasing time of oxidation, there was a slight decrease in total cholesterol content (6% in 12 h), with minimal change in the free cholesterol content; phospholipid content decreased by 44% over the same time period, leading to almost a doubling in the free cholesterol or total cholesterol to phospholipid ratio. Only the oxidized HDL exposed to copper for 12 h showed an increase in electrophoretic mobility or polymerization of apo AI by SDS-PAGE

TABLE 1. HDL oxidation and changes in its chemical composition

	Time of oxidation (h)			
	0	3	6	12
TBARS (nmol/mg chol)	0.0	0.3	1.7	3.6
Electrophoretic Mobility	1.0	1.0	1.0	1.2
CONCENTRATIONS (μg/ml)				
Protein	1520	1490	1500	1470
Phospholipid	625	630	420	348
Total Cholesterol	510	516	500	480
Free Cholesterol	151	149	150	146
RATIOS (w:w)				
TC:PROTEIN	0.34	0.35	0.32	0.31
PL:PROTEIN	0.41	0.42	0.28	0.24
FC:PL	0.24	0.24	0.36	0.42
TC:PL	0.82	0.82	1.20	1.40

HDL was oxidized for the times indicated as described in Methods then assayed for TBARS, electrophoretic mobility on agarose relative to non-oxidized HDL, protein, phospholipid and free and total cholesterol contents as described in Methods. Data are representative of three such experiments.

TABLE 2. Phospholipid content in HDL lipid extracts

Lipoprotein	Extract phase	Phospholipid Content (μg phosphorous/ml)
HDL	organic	408±5
	aqueous	5±6
oxidized HDL (12h)	organic	273±7
	aqueous	127±6

HDL was oxidized as described in Methods and the lipids, in triplicate samples before and after oxidation, were extracted. Phosphorous content was determined in both the organic phase (phospholipid) and in the aqueous phase of the extracts. Data are means ± S.D. for the triplicate samples.

(data not shown). Samples oxidized for ≤ 6h, i.e., "mildly" oxidized HDL, exhibited no increase in electrophoretic mobility, apo AI polymerization and unaltered immunoreactive apo AI (data not shown). In other experiments, when BHT (20 μM) was added to HDL prior to exposure to copper, no change in cholesterol or phospholipid content was detected (data not shown).

The most dramatic compositional change detected during the mild oxidation of HDL was the apparent loss of phospholipid and this was enhanced in HDL oxidized for 12 h. Since the phospholipid assay measures inorganic phosphorous content, to account for the apparent loss in phospholipid, the phosphorous content in both the organic and aqueous phases of the lipid extract of oxidized (12 h) and non-oxidized HDL samples was measured. As shown in Table 2, the phosphorous apparently lost in oxidized HDL was recovered in the aqueous phase of the lipid extract, presumably due to conversion of phospholipid to a more polar form.

The rat Fu5AH hepatoma cell line was used to study the effect of mild oxidation of HDL on its ability to mediate cholesterol efflux; these cells were chosen because the characteristics of cholesterol efflux in this cell line have been studied extensively (20,25,28). Figure 1 shows the effect of oxidation time on the ability of HDL_3 samples to stimulate cholesterol efflux from cells to the media. As the time (and degree, see Table 1) of HDL oxidation increased, the rate and extent of [^{14}C]cholesterol release from the cells decreased. In other experiments, when BHT was included during the oxidation protocol , cholesterol efflux to that HDL remained unchanged (data not shown). Even though HDL oxidized for 6 hours exhibited no increase in electrophoretic mobility or degradation of apo AI, it did stimulate less cholesterol efflux than non-oxidized HDL. What did change in oxidized HDL was its phospholipid content (33% decrease, Table 1) and the cholesterol to phospholipid ratio. Reduced cholesterol efflux to mildly oxidized HDL was also observed in L cells, a mouse embryonic fibroblast cell line (data not shown).

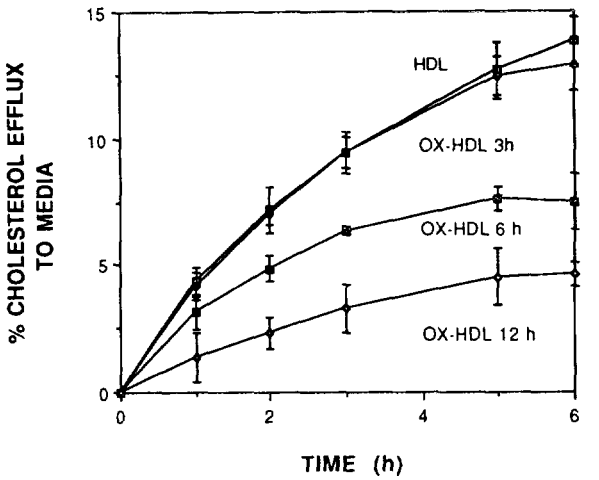

Figure 1. Efflux of cellular [^{14}C]cholesterol from Fu5AH cells to HDL or oxidized HDL (oxidized for 3h, 6h or 12 h). Data are expressed as percent of the [^{14}C]cholesterol in the cells at the start of the efflux period and are shown as the mean ± S.D. for triplicate wells. Data are representative of three similar experiments.

To further examine the effect of mild oxidation of HDL on its ability to mediate cholesterol efflux from cells, Fu5AH cells were enriched with cholesterol as described in methods. Since esterification was inhibited (by 58035), this incubation produced an enrichment in cellular free cholesterol; this cholesterol is presumed to be primarily in the plasma membrane (26). Efflux of cholesterol from cholesterol-rich cells to BSA alone (0.2%) or to BSA plus either HDL or mildly oxidized HDL (oxidized for either 3 or 6 h) was measured. The ratios of free cholesterol to phospholipid in HDL, HDL oxidized for 3h and HDL oxidized for 6h were 0.17, 0.18 and 0.24, respectively. A more severely oxidized HDL (oxidized for 24 h) added at the same concentration (500 μg protein/ml) was cytotoxic (data not shown), similar to the observation of Nagano et al. (16). Figure 2A shows the percent of cellular ^{14}C-cholesterol which accumulated in the medium after nine hours of efflux. Efflux of [^{14}C]cholesterol to BSA alone was negligible whereas 70% of the [^{14}C]cholesterol was removed from the cells by HDL. Efflux of [^{14}C]cholesterol to the mildly oxidized HDL was significantly reduced. Figure 2B shows the cellular free cholesterol content prior to enrichment, at the start of the efflux period (t=0) and after nine hours efflux. Cellular cholesterol was approximately doubled by the enrichment protocol. After incubation with HDL the cellular cholesterol content was reduced to the level observed prior to enrichment. In contrast, cells incubated with mildly oxidized HDL remained enriched with cholesterol, although those incubated with HDL oxidized for 3h did show a significant decrease in cell cholesterol mass ($p<0.02$). Cellular protein increased slightly during the enrichment protocol and remained constant during

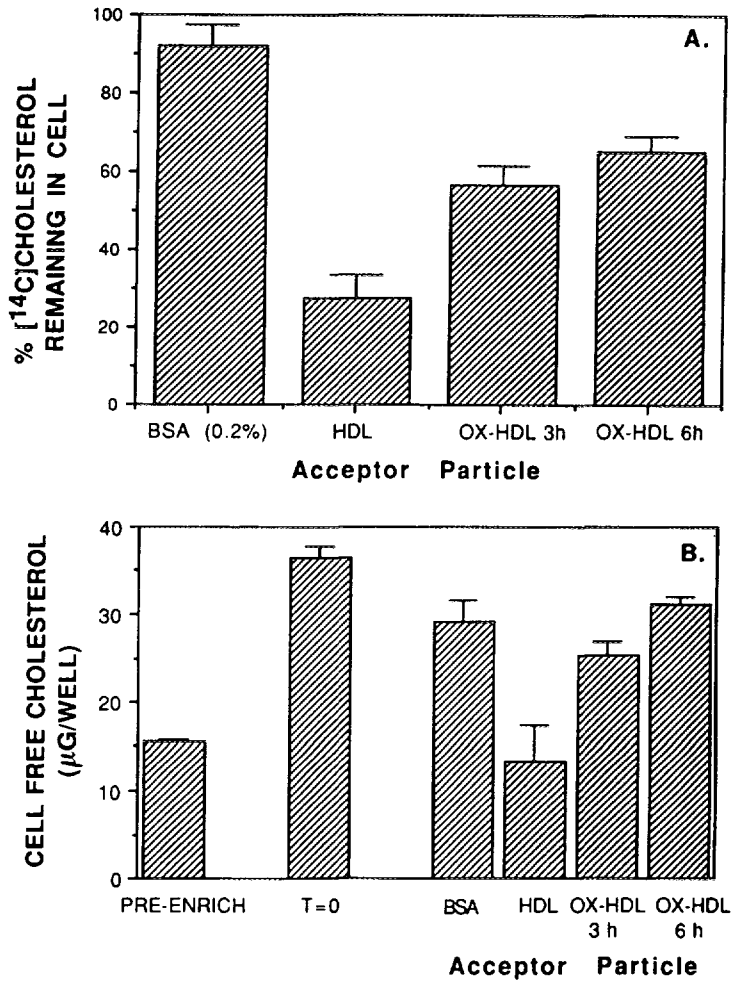

Figure 2. Accumulated (9h) efflux of cholesterol from free cholesterol-enriched Fu5AH cells to various acceptor particles. Data shown are means ± S.D. for triplicate wells and are representative of two similar experiments. **A.** Percent of [^{14}C]cholesterol in cells at start of efflux period appearing in the medium after 9h incubation with 0.2% BSA, 0.2% BSA plus HDL, HDL oxidized for 3h (ox-HDL 3h), or HDL oxidized for 6 h (ox-HDL 6h) at 500 μg protein/ml. Ox-HDL 3h and ox-HDL 6h are significantly different from HDL and from BSA (Students' unpaired t test, $p<0.005$) **B.** Cellular free cholesterol content before enrichment with free cholesterol, after enrichment and after subsequent incubation with acceptors described above. HDL and ox-HDL 3h, but not ox-HDL 6h, are significantly different from BSA control (Students' unpaired t test with $p< 0.002$ and $p<0.02$, respectively).

the efflux period (data not shown). Thus, mild oxidation of HDL also limited its ability to remove cholesterol mass from cholesterol-enriched cells.

DISCUSSION

Since covalent modification of HDL apoproteins reduces cholesterol efflux to that HDL (16,17), the observation that both apoprotein polymerization and

reduced efflux occur with oxidation of HDL (17) has been used to infer cause and effect. As described herein, however, there are other compositional changes in HDL which occur during oxidation that may explain the reduced efflux, namely oxidation of phospholipids with a marked increase in the free cholesterol to phospholipid ratio. This change was the earliest compositional change detected and was associated with the observed reduction in cholesterol efflux. Moreover, the reduced cholesterol efflux occurred in the absence of discernible apoprotein modification.

That the loss of phospholipids due to oxidation and the concomitant increase in the free cholesterol to phospholipid ratio of the particle might produce the observed reduction in efflux to oxidized HDL is consistant with the studies of Johnson et al. (28) in which the net movement of free cholesterol between cells and HDL was shown to occur by mass-action effects. Cholesterol molecules diffuse through the aqueous phase between the cell plasma membrane and the HDL particle; HDL may act as a net acceptor or donor of cellular cholesterol to cells depending on its cholesterol to phospholipid ratio relative to that of the cell plasma membrane. This idea is also consistent with the findings of Oram et al. (29) in which the HDL subclasses with higher densities (i.e., HDL_3 and VHDL) displayed greater ability to remove cholesterol from cells; these fractions also exhibited lower cholesterol to phospholipid ratios than HDL_2. Furthermore, in other studies, HDL depleted of phospholipid by treatment with phospholipase A_2 (with a resulting increase in cholesterol:phospholipid) also exhibited decreased ability to mediate cholesterol efflux (30). The rationale for conversion of HDL to a less effective cholesterol acceptor due to its altered lipid composition is seen more clearly in the experiment using cholesterol-enriched cells (Fig. 2A and 2B). Unlike HDL, oxidized HDL did not stimulate the net removal of cholesterol mass, despite the appearance of radiolabelled cholesterol in the medium. HDL removed from the cells 16 ug cholesterol/well, increasing its ratio of free cholesterol to phospholipid from 0.17 to 0.23, similar to that of HDL oxidized for 6h (0.24). Thus, it is not surprising that HDL oxidized for 6h (with higher free cholesterol to phospholipid ratio) did not stimulate the net removal of cholesterol mass; the medium radiolabel in this case most likely reflects equilibration between oxidized HDL and the cell plasma membrane.

Whereas oxidized LDL may occur *in vivo* (12,13), no such evidence is available yet for the existence of oxidized HDL *in vivo*. The possibility that HDL can occur *in vivo* is suggested by the studies of Nishigaki et al. (31) in which increased levels of lipid peroxides were found in plasma from diabetic patients, with the greatest increase found in the HDL fraction; no functional studies, however, were done by these workers. Since reduced ability to mediate cholesterol efflux is observed prior to apoprotein modification, it may be that detection of oxidized HDL *in vivo* will require identification and detection of the oxidized

lipids. If oxidized HDL does occur *in vivo* it could contribute to the development of atherosclerosis by limiting removal of cholesterol from cells, particularly lipid-laden cells, and exacerbating the lipid accumulation in foam cells.

ACKNOWLEDGMENTS

Thanks to Pamela Bedell for excellent technical assistance and to Drs. George H. Rothblat and Michael C. Phillips for helpful discussions. These studies were funded by a FIRST award from the NIH (HL-45306), a PPG (HL-22633) and the Howard Heinz Endowment.

REFERENCES

1. Gordon, D.J., Probstfield, J.L., Garrison, R.J. et al.(1989) Circulation 79, 8-15.
2. Franceschini,G., Maderna, P. and Sirtori, C.R. (1991) Atherosclerosis 88, 99-107.
3. Rothblat, G.H., Mahlberg, F.M., Johnson, W.J. and Phillips, M.C. (1992) J. Lipid Res. 33, 1091-1096.
4. Phillips, M.C., W.J. Johnson and Rothblat, G.H. (1987) Biochim. Biophys. Acta 906, 223-276.
5. Oram, J.F., Albers, M.C., Cheung, M.C.,and Bierman, E.L.(1981) J. Biol. Chem. 256, 8348-8356.
6. Slotte, J.P. (1987) J. Biol. Chem. 262:12904-12907.
7. Parthasarathy, S., Barnett, J. and Fong, L.G. (1990) Biochim. Biophys. Acta 1044, 275-283.
8. Hessler, J., Morel, D.W., Lewis. L.J, and Chisolm,G.M.(1983) Arteriosclerosis 3, 215-222.
9. Morel, D.W., Hessler, J.R. and Chisolm, G.M. (1983) J. Lipid Res. 24, 1070-1076.
10. Steinbrecher, UP, Zhang, H, and Lougheed, M. (1990) Free Rad. Biol. Med. 9, 155-166.
11. Chisolm, G.M. (1991) Current Opinion in Lipidology 2, 311-316.
12. Azizova, O.A., Panasenko, O.M., Vol'nova, T.V. and Vladimirov, Y.A. (1989) Free Rad. Biol. Med. 7, 251-257.
13. Babiy, A.V., Gebicki, J.M. and Sullivan, D.R. (1990) Atherosclerosis 81, 175-182.
14. Palinski, W., Rosenfeld, M.E., Yla-Herttuala,S, Gurtner,G.C., Socher, S.S., Butler, S.W. and Witztum, J.L. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 1372-1376.
15. Rosenfeld, M.E., Palinski, W., Yla-Herttuala,S., Butler, S.,and Witztum, J.L. (1990) Arteriosclerosis 10, 336-349.
16. Nagano, Y., Arai,H. and Kita, T. (1991) Proc. Natl. Acad.Sci. U.S.A. 88,6457-6461.
17. Salmon, S., Maziere, C., Auclair, M., Theron, L., Santus,R.and Maziere, J-C. (1992) Biochim. Biopys. Acta 1125, 230-235.
18. Ghiselli, G. Giogini, L., Gelati, M. and Musanti,R. (1992) Arteriosclerosis and Thrombosis 12, 929-935.
19. Ardlie, N.G., Selley, M.L. and Simons, L.A. (1989) Atherosclerosis 76, 117-124.
20. Johnson, W.J., Bamberger, M.J., Latta, R.A., Rapp, P.E., Phillips, M.C. and Rothblat, G.H. (1986) J. Biol. Chem. 261,5766-5776.
21. Bligh,E.G. and Dyer, W.J. (1959) Can. J. Biochem. 37:911-917.

22. Sokoloff, L. and Rothblat, G.H. (1972) Biochim. Biophys.Acta 280, 172-181.
23. Gamble, W., Vaughan,M., Kruth, H.S. and Avigan,J. (1978) J.Lipid Res. 19, 1068-1070.
24. Markwell, M.A.K., Haas, S.M., Bieber, L.L. and Tolbert, N.E.(1978) Anal. Biochem. 87, 206-210.
25. Rothblat, G.H., Bamberger, M. and Phillips, M.C. (1986) Methods Enzymol. 129, 628-644.
26. Lange, Y. and Ramos, B.V. (1983) J. Biol. Chem. 258, 15130-15134.
27. Arbogast, L.Y., Rothblat, G.H., Leslie, M.H. and Cooper, R.A. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3680-3684.
28. Johnson, W.J., Mahlberg, F.M., Chacko, G.K., Phillips, M.C. and Rothblat, G.H. (1988) J. Biol Chem. 263, 14099-14106.
29. Oram, J.F., Albers, J.J., Cheung, M.C. and Bierman, E.L. (1981) J. Biol. Chem. 256, 8348-8356.
30. Slotte, J.P., Oram, J.F. and Bierman, E.L. (1987) J. Biol. Chem. 262, 12904-12907.
31. Nishigaki, I., Hagihara, M., Tsunekawa, H., Maseki, M. and Yagi, K. (1981) Biochem. Med. 25, 373-378.