RESEARCH COMMUNICATION

Rapid reduction and removal of HDL- but not LDL-associated cholesteryl ester hydroperoxides by rat liver perfused *in situ*

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To test whether high-density lipoproteins (HDL) could aid in the removal *in vivo* of potentially atherogenic oxidized lipids, we perfused rat liver *in situ* with buffer supplemented with isolated human HDL containing small amounts of cholesteryl linoleate hydro(pero)xides [Ch18:2-O(O)H]. Perfusion resulted in the rapid removal of Ch18:2-O(O)H from HDL with a half-life ($t_{1/2}$) of 11.4 min, faster than that of unoxidized cholesteryl linoleate, and dependent on the presence of the liver. In addition, the liver enhanced the reduction of Ch18:2-OOH associated with HDL

remaining in the perfusate buffer. Perfusion resulted in the release of a hepatic activity that enhanced the reduction of HDL-associated Ch18:2-OOH and was resistant to heat treatment. In contrast with the situation with HDL, low-density lipoprotein (LDL)-associated Ch18:2-O(O)H were neither removed nor reduced by perfused rat liver within the time course studied, in support of a possible role for HDL in the detoxification of circulating lipid hydroperoxides *in vivo*.

INTRODUCTION

High-density lipoproteins (HDL) are generally thought to be anti-atherogenic because they can remove 'excess' cholesterol from peripheral cells and transport it to the liver for elimination into bile. This process, referred to as 'reverse cholesterol transport', involves the initial conversion of cellular cholesterol into HDL-associated cholesteryl esters (CE), which are subsequently removed from the circulation via various pathways. The CE of HDL may be transferred to very-low- and low-density lipoprotein (LDL) [1] and removed temporarily by hepatic clearance of these more buoyant lipoproteins, whereby cholesterol is largely recycled in newly secreted plasma lipoproteins [2]. Removal of whole HDL particles is a small contributor to the overall CE clearance in man [3]. HDL may also acquire apolipoprotein E, which results in increased hepatic uptake of the particle; however, this pathway also appears to be of minor importance *in vivo* [1]. Alternatively, the HDL CE may be taken up 'selectively', i.e. without lipoprotein-particle uptake [1,4,5], a pathway of relevance in the clearance of HDL CE in humans [1]. Importantly, 'selective uptake' of HDL CE by liver parenchymal cells is, at least in the rat, directly coupled to secretion of cholesterol into bile [6].

In contrast with HDL, elevated levels of LDL may accelerate atherogenesis, and there is evidence that 'oxidized' forms of LDL contribute to this process [7]. In the light of this observation and the protective activity of HDL, we were surprised to find that, in fasted plasma of healthy donors, most of the detectable hydroperoxides, primarily those of cholesteryl linoleate (Ch18:2-OOH), are associated with HDL rather than LDL [8]. Also, during oxidation of human plasma by peroxy radicals, Ch18:2-OOH are formed initially in HDL rather than LDL [8]. Furthermore, HDL can acquire pre-existing Ch18:2-OOH from

oxidized LDL in the presence of CE transfer protein [9]. In any case, Ch18:2-OOH associated with HDL are reduced to the corresponding hydroxides (Ch18:2-OH) due to an intrinsic activity [10] enhanced by GSH [11] and ebselen [11,12]. In addition, HDL Ch18:2-OOH and Ch18:2-OH [Ch18:2-O(O)H] are taken up selectively and detoxified by human hepatoma Hep G2 cells [13]. Together, these results are consistent with a role for HDL in the hepatic detoxification of potentially atherogenic oxidized CE, an activity which could complement the already known anti-atherogenic nature of HDL.

To obtain further support for such a protective role of HDL, we examined the fate of HDL-associated Ch18:2-O(O)H during perfusion of rat liver *in situ*. Herein we demonstrate an HDL-specific, hepatic pathway for the rapid removal and reduction of Ch18:2-OOH.

MATERIALS AND METHODS

Materials

2,2'-Azobis-(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-azobis-(2,4-dimethylvaleronitrile) (AMVN) were purchased from Polysciences (Warrington, PA, U.S.A.), Chelex-100 from Bio-Rad Laboratories (Richmond, CA, U.S.A.), PD-10 Sephadex G-25M columns from Pharmacia LKB Biotechnology (Uppsala, Sweden), BSA and egg-yolk lecithin (phosphatidylcholine) from Sigma (St. Louis, MO, U.S.A.), and cholesteryl (9-hydroxy)linoleate from Cayman Chemicals (Ann Arbor, MI, U.S.A.). Ch18:2-OOH standard was prepared from purified cholesteryl linoleate (Sigma) [14]. Centricon-30 concentrators were purchased from Amicon (Beverly, MA, U.S.A.) and [*cholesteryl*-1,2,6,7-³H]cholesteryl linoleate (³H]Ch18:2; sp. radioactivity 74 Ci/mmol) from du Pont-NEN (Boston, MA, U.S.A.). All other chemicals were from Merck (Darmstadt,

Abbreviations used: AAPH, 2,2'-azobis-(2-amidinopropane) dihydrochloride; AMVN, 2,2'-azobis-(2,4-dimethylvaleronitrile); CE, cholesteryl esters; Ch18:2, cholesteryl linoleate; Ch18:2-OH, cholesteryl linoleate hydroxides; Ch18:2-OOH, cholesteryl linoleate hydroperoxides; HDL, high-density lipoproteins; HDL_{ox}, mildly oxidized HDL (HDL_{ox}) treated with ebselen to reduce all Ch18:2-OOH to Ch18:2-OH; LDL, low-density lipoproteins; LDL_{ox}, mildly oxidized LDL.

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Germany). Ethanol (AR grade) was obtained from BDH (Kilsyth, Vic., Australia), while all other organic solvents were from Malinckrodt (Clayton, Vic., Australia) and of HPLC quality. Before use, all aqueous solutions used in the preparation of lipoproteins, were stored over Chelex-100 to remove trace-metal contamination.

Isolation and labelling of lipoproteins

HDL and LDL were isolated from EDTA-plasma obtained from venous blood of healthy male or female donors (aged 22–40 years) as described previously [14]. Aspirated lipoproteins were pooled, concentrated (using Centricon-30 concentrators at 2800 g for 1 h for HDL and 2 h for LDL), gel-filtered on PD-10 columns using PBS and the protein content estimated [15]. Labelling was achieved by incubating lipoproteins overnight at 37 °C and under argon with an equal volume of lipoprotein-deficient plasma and 0.5–1 ml of liposomes, prepared by sonication of egg-yolk lecithin (160 µg/ml in 50 mM phosphate buffer, pH 7.4) containing 50–100 µCi of [³H]Ch18:2 [9]. The labelled lipoproteins were then re-isolated as described.

Oxidation of lipoproteins

Lipoproteins were oxidized in EDTA-free PBS by AAPH (50 mM) at 37 °C for 1.5 h (LDL) or 2 h (HDL), the oxidation terminated by gel-filtration (three successive PD-10 columns) of the reaction mixture, the lipoproteins concentrated and their protein content determined. The resulting oxidized HDL, HDLox, contained 6.7 ± 4.9 µM Ch18:2-OOH plus Ch18:2-OH [Ch18:2-O(O)H], corresponding to approximately seven molecules of Ch18:2-O(O)H per HDL particle, with $48.9 \pm 9.5\%$ being present as the hydroxide. This degree of oxidation corresponds to $\approx 4\%$ of the lipoprotein's CE being oxidized. Similarly, oxidized LDL, LDLox, contained 17.5 ± 6.2 µM Ch18:2-O(O)H or approx. 176 molecules of Ch18:2-O(O)H per LDL particle, with $14.0 \pm 4.4\%$ being present as the hydroxide, corresponding to approx. 7% of the CE being oxidized. Lipoproteins were stored under argon at 4 °C and used within 72 h for unoxidized preparations and within 24 h for oxidized preparations. To obtain HDLox, HDLox, prepared as described above, was incubated at 37 °C for 30 min in the presence of ebselen (68 µM) and GSH (408 µM) to convert all the Ch18:2-OOH into Ch18:2-OH [11]. Ebselen and GSH were removed by gel filtration (two PD-10 columns [16]), and the resulting HDLox was stored under argon at 4 °C and used within 72 h.

Perfusion of rat livers *in situ*

For all perfusion experiments, anaesthetized 250–350 g male Wistar-strain albino rats were used. The rats were anaesthetized by intraperitoneal injection of sodium pentobarbitone (50 mg/kg body weight), and the livers perfused with Krebs–Henseleit bicarbonate buffer equilibrated with O₂/CO₂ (19:1), with a final O₂ concentration of ≈ 1 mM. Two integrated tubing circuits were available, one incorporating a large-volume non-recirculating reservoir, and one from a small-volume recirculating reservoir, with a two-way flow valve to select the mode. The perfusate was delivered at a constant volume of 35 ml/min by means of an LKB2115 peristaltic pump. For each experiment, the liver was equilibrated for 15 min in the non-recirculating mode, and then switched to the small recirculating reservoir, containing HDL(ox) or LDL(ox) at a particle concentration corresponding to 1 or 0.1 µM respectively. These absolute concentrations of lipoproteins are ≈ 10 -fold lower than those in human plasma, which contains 10-times more HDL than LDL

particles. At the time points indicated, a small sample was removed and 1 ml extracted in methanol/hexane (1:5, v/v) and stored at –20 °C for HPLC analysis.

Reversed-phase HPLC analysis of Ch18:2, [³H]Ch18:2-OOH and [³H]Ch18:2-OH

Separation and analysis of Ch18:2, [³H]Ch18:2-OOH and [³H]Ch18:2-OH was as described in [9,17]. Results were quantified by area comparison with appropriate standards.

RESULTS

HDLox and LDLox were prepared by exposure of the native lipoproteins to controlled mild fluxes of aqueous peroxy radicals for short periods of time. During such oxidation, approximately equal amounts and three times more hydroperoxides of CE than phospholipids are formed in HDL and LDL respectively [8,18], and there is insignificant modification of apolipoproteins [19]. HDLox and LDLox obtained in this way and used in the present study contained ≈ 5 and 170 molecules of oxidized cholesterol esters per particle respectively. Oxidation did not change the apparent densities of the lipoproteins, as judged by the position of the lipoprotein bands in density gradients after centrifugation (results not shown). The extent of this oxidation is very mild compared with that used in previous studies addressing the metabolism of oxidized lipoproteins [20–23], though it is some four orders of magnitude larger than that detectable in these lipoproteins when isolated rapidly from the blood of healthy human subjects [8].

Perfusion of rat liver *in situ* with HDLox resulted in a time-dependent disappearance of Ch18:2-O(O)H from the recirculating perfusate, whereas replacement of the organ with a piece of tubing completely eliminated loss of Ch18:2-O(O)H (Figure 1A). In the presence of the liver, Ch18:2-OOH and Ch18:2-OH

Figure 1 Loss of HDL- but not LDL-associated Ch18:2-O(O)H during short-term perfusion of rat liver *in situ*

LDL and HDL were isolated and used unoxidized (for loss of HDL's Ch18:2) or after oxidation (see the Materials and methods section) in the perfusion apparatus in the presence (+) or absence (–) of liver. Results are expressed as a percentage of the Ch18:2-O(O)H or Ch18:2 present at the start of each perfusion and represent the means $\pm$ S.E.M. for six, seven or four separate experiments with unoxidized HDL (A), HDLox (A) and LDLox (B) respectively. The starting levels of Ch18:2-O(O)H in HDLox and LDLox were 6.7 ± 4.9 and 17.5 ± 6.2 µM respectively, with 48.9 ± 9.5 and $14.0 \pm 4.4\%$ of the oxidized Ch18:2 initially present as Ch18:2-OH. The concentration of Ch18:2 in native HDL was 160 ± 77 µM. Student's *t* tests were used to determine statistical significance at each time point, with the hash symbol (#) (liver versus no liver) and the asterisk (*) (HDLox versus unoxidized HDL) showing significance at $P < 0.01$ and $P < 0.025$ respectively.

Figure 2 HDL-specific reduction of Ch18:2-OOH to Ch18:2-OH during perfusion of rat liver *in situ*

HDLox (A) and LDLox (B), prepared as described in the legend to Figure 1, were used in the perfusion apparatus described, in the presence (●) or absence (■) of rat liver. The starting levels of total oxidized Ch18:2 and Ch18:2-OH in HDLox and LDLox were as described in the legend to Figure 1. Results are expressed as the increase in percentage of Ch18:2-OH present at the each time point relative to the total Ch18:2-O(O)H, and represent the means $\pm$ S.E.M. for seven and four separate experiments with HDLox and LDLox respectively. Student's *t* tests were used to determine the statistical significance at each time point, with the hash symbol (#) and asterisk (*) showing significance at $P < 0.01$ and $P < 0.05$ respectively.

of HDLox disappeared at indistinguishable relative rates (results not shown). With HDLox-supplemented buffer, the loss of Ch18:2-OH was comparable with the sum of the losses of Ch18:2-OOH and Ch18:2-OH from HDLox (results not shown). A striking feature of the observed liver-dependent disappearance of HDL-oxidized CE was its rapidity, with a mean half-life of 11.4 min, calculated from the initial rate of Ch18:2-O(O)H loss in Figure 1A. The relative loss of Ch18:2-O(O)H from HDLox was somewhat faster than that of unoxidized Ch18:2 from native HDL (36.8 ± 6.8 as against $57.1 \pm 4.9\%$ remaining after 20 min; $P < 0.025$) (Figure 1A). In absolute terms, however, more Ch18:2 was lost from native HDL than Ch18:2-O(O)H from HDLox (see the legend to Figure 1A). This is probably due to the presence of much higher amounts of Ch18:2 than Ch18:2-O(O)H in both native and oxidized HDL. Consistent with this, the relative and absolute rates of loss of Ch18:2 from native and oxidized HDL were not distinguishable.

Perfusion of rat liver *in situ* with [3 H]Ch18:2-labelled HDL or [3 H]Ch18:2-O(O)H-labelled HDLox resulted in a time-dependent loss of [3 H]Ch18:2 or [3 H]Ch18:2-OOH and [3 H]Ch18:2-OH respectively from the perfusion buffer. The extent of removal of [3 H]Ch18:2-O(O)H from HDLox was not distinguishable from that of Ch18:2-O(O)H from unlabelled HDLox, as verified in two separate experiments using HDLox with different initial content of [3 H]Ch18:2-O(O)H (results not shown). All of the radiolabel lost from the perfusate was recovered in the liver as [3 H]cholesterol ($\approx 92\%$) and [3 H]Ch18:2 ($\approx 7\%$), with traces of [3 H]Ch18:2-OH, but no [3 H]Ch18:2-OOH, detected. In contrast, there was no hydrolysis of [3 H]Ch18:2-O(O)H in the perfusate, as indicated by a lack of formation of [3 H]cholesterol (results not shown).

In addition to the increased net removal of Ch18:2-O(O)H, perfusion of liver *in situ* with HDLox resulted in a time-dependent conversion of perfusate Ch18:2-OOH into Ch18:2-OH (Figure 2A). This reduction was also seen with perfusate supplemented

Figure 3 Enhancement of HDL-mediated reduction of Ch18:2-OOH to Ch18:2-OH by hepatic factor(s) released during perfusion of rat liver *in situ*

Buffer with (squares) or without heparin (400 units/kg rat weight) (circles) was recirculated for 15 min in the absence (open symbols) or presence (closed symbols) of liver and then collected. HDLox, prepared as described in the Materials and methods section, was then added to the collected buffer (1:1, v/v) and the conversion of Ch18:2-OOH into Ch18:2-OH monitored at 37 °C. The starting level of Ch18:2-O(O)H in HDLox was $4.0 \pm 2.2 \mu\text{M}$, with $50.6 \pm 8.9\%$ of the oxidized Ch18:2 initially present as Ch18:2-OH. Results are expressed as the increase in percentage of Ch18:2-OH relative to the total Ch18:2-O(O)H measured at each time point, and represent the means $\pm$ S.E.M. for four or five separate experiments. Student's *t* tests were used to determine statistical significance ($P < 0.05$) at each time point. The asterisk (*) and hash symbol (#) indicate significant differences between heparin-containing buffer in liver perfusate versus control perfusate and liver perfusate in the presence versus absence of heparin respectively.

with [3 H]Ch18:2-O(O)H (results not shown). The observed reducing activity was largely liver-dependent, as in its absence there was an approx. 3-fold lower extent of reduction. The level of reduction seen in the absence of liver was consistent with the reported intrinsic reducing activity associated with HDL that converts 50% of its Ch18:2-OOH into Ch18:2-OH within 4 ± 0.8 h at 37 °C [10].

We next tested whether this enhanced reduction was due to the release of a presently unknown hepatic activity. Heparin-containing perfusion buffer was recirculated, in the absence of lipoprotein, for 15 min in the presence (liver perfusate) or absence (control perfusate) of the liver. HDLox was then added to each of the collected perfusates and the conversion of Ch18:2-OOH into Ch18:2-OH monitored over a period of 2 h. Compared with control perfusate, liver perfusate showed a significantly enhanced ability to reduce HDLox's Ch18:2-OOH (Figure 3), though the extent of reduction was much less than that seen during the perfusion of liver *in situ* with HDLox (Figure 2A). Boiling the liver perfusate did not attenuate the observed reducing activity (results not shown). Omission of heparin from the buffer resulted in the abolition of the liver effect, and heparin was without effect when added to buffer recirculated in the absence of liver (Figure 3).

In sharp contrast with HDLox, inclusion of LDLox in the perfusion buffer resulted in neither significant disappearance of Ch18:2-O(O)H (Figure 1B) nor its reduction to Ch18:2-OH (Figure 2B), independently of whether the recirculating buffer passed through the liver.

DISCUSSION

The results presented here demonstrate that perfusion of rat liver *in situ* with HDLox results in both hepatic removal of Ch18:2-O(O)H and concomitant reduction of Ch18:2-OOH to Ch18:2-OH, which remain in the perfusion buffer. Hepatic removal of CE showed a partial preference for the oxidized over the unoxidized forms and was specific for HDLox, with no significant hepatic removal of Ch18:2-O(O)H associated with LDLox observed. As Ch18:2-O(O)H associated with HDLox are taken up 'selectively' and are subsequently reduced by human hepatoma cells *in vitro* [13], our results support a role for the liver in the removal and detoxification *in vivo* of oxidized CE via an HDL-specific pathway.

In total, two to five molecules of unoxidized plus oxidized CE were removed from each HDL particle during each passage through the liver, as calculated from the flow rate, perfusate volume, and the observed loss of esterified lipids. A comparison of oxidized with unoxidized CE showed that, after 20 min of perfusion with HDLox *in situ*, $\approx$ 2.5-fold more Ch18:2-O(O)H than Ch18:2 were removed, based on the molar ratios of the two types of lipids before and after perfusion. This is less striking than the 9-fold greater 'selective uptake' of HDL's Ch18:2-O(O)H than Ch18:2 observed with Hep G2 cells *in vitro* [8,13], but still results in a significantly greater relative removal of Ch18:2-O(O)H over time. Should our findings with rat liver perfused *in situ* hold true for human liver, the observed removal from HDL of oxidized CE molecules per lipoprotein particle and passage represents a substantial detoxification capacity. As on average every HDL particle passes through the liver once a minute, the half-life of circulating HDL-associated Ch18:2-O(O)H in healthy humans is expected to be short, consistent with the fact that, in human plasma, Ch18:2-OOH is detected at low-nanomolar concentrations [8].

Our second novel finding in support of a detoxification role of liver and HDL for circulating oxidized lipids is the hepatic enhancement of the reduction of HDL's (but not LDL's) Ch18:2-OOH. A reduction of hydroperoxides of CE and possibly other (lipoprotein) lipids to their corresponding stable hydroxides represents an important antioxidant step, as it not only eliminates potentially (cyto)toxic hydroperoxides, but also the precursors for reactive secondary oxidation products, including aldehydes, that have been implicated in, for example, the generation of high-uptake, atherogenic LDL *in vivo*. The mechanism of this enhancement is not known at present. The observed small increase in reducing activity in liver perfusate by heparin together with a lack of effect of boiling on the reducing activity, argue against the involvement of a protein. Apart from cellular phospholipid hydroperoxide glutathione peroxidase [11,24], no known mammalian enzyme is able to reduce lipoprotein-associated Ch18:2-OOH. It is therefore possible that the released hepatic factor supports the intrinsic reducing activity associated with HDL [10]. One possible candidate is GSH [10,25], which is able to enhance HDL- but not LDL-associated reduction of Ch18:2-OOH [11]. If GSH were responsible for the observed enhanced reduction, GSH-releasing white blood cells [26] could also potentially support HDL's reducing activity in circulation.

A striking feature of our results was that both enhanced Ch18:2-OOH-reducing activity and hepatic removal of oxidized CE showed specificity for HDL over LDL. This could be significant in the context of the known anti-atherogenic properties of HDL and the suggested pro-atherogenic properties of oxidized LDL (reviewed in [7]). Our results suggest that HDL could facilitate the reduction of atherogenic oxidized CE, as well as functioning as a vehicle for their rapid hepatic clearance, in each case decreasing the likelihood of accumulation of these potentially atherogenic oxidized lipids in LDL, for example via transfer mediated by cholesteryl ester transfer protein [9]. Thus our previous [8–11,13] and present findings support a protective role of HDL based on the detoxification and elimination of circulating oxidized lipids.

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