The kidney composition of high-density lipoprotein affects its re-absorption in the kidney by proximal tubule epithelial cells

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INTRODUCTION

HDL (high-density lipoprotein) metabolic research has shown that the kidney and the liver are the two major sites of HDL catabolism [1]. The kidney cortex appears to be an important site of apoA-I (apolipoprotein A-I) catabolism. This uptake has been thought to be the result of glomerular filtration, tubular reabsorption and intracellular degradation of lipid-poor apoA-I [1,2]. However, there is also evidence for whole HDL particle uptake by the kidney. Intact apolipoproteins have been isolated from urine of normolipidemic subjects, at a density of 1.24 g/ml, suggesting that small amounts of HDL-like particles are excreted in the urine [3,4]. Peterson and colleagues have demonstrated glomerular filtration, luminal uptake and lysosomal proteolysis of HDL, particles in the microperfused rabbit proximal nephron [5].

HDL particle uptake has been reported to occur in a wide number of tissues [6,7] and to be facilitated by well-defined HDL receptors, such as SR-BI [6]. A unique HDL receptor in the kidney, cubulin, has been reported to facilitate non-selective endocytosis of HDL and apoA-I [8,9]. Cubulin is expressed in the apical membranes of various absorptive epithelia, including those of the yolk sac, placenta and kidney [10]. The receptor has also been implicated in the endocytosis of intrinsic factor–vitamin B12 complex in the intestine [11,12]. In addition to cubulin, a number of other proteins capable of interacting with apoA-I or HDL have also been found to be expressed in the kidneys [13–17]. Their roles in apolipoprotein or HDL metabolism have not yet been elucidated.

This laboratory has shown that HDL charge can affect the clearance of HDL from the bloodstream and that more negatively charged HDL particles are cleared from the plasma of a rabbit at a slower rate than the positively charged ones [18]. Since the filtration barrier of the glomerulus has both a size and charge selectivity [19], HDL metabolism in the kidney may be affected by the structure and electrostatic properties of this lipoprotein. The effect of lipid composition of reconstituted HDL on the binding and uptake of the lipoprotein has been previously studied in isolated human enterocytes. The study showed that an apoA-I/dimyristoyl-L-α-phosphatidylcholine recombinant particle competed with HDL for HDL receptor-binding sites on enterocytes with equal efficiency, while apoA-I was less efficient in competing with HDL, [20]. The authors suggested that while apoA-I may be a ligand for HDL receptors, the lipid composition of the particle may affect the conformation/structure of the ligand and affect its ability to bind to unique HDL receptors [20]. In the present study we have examined the effect of the lipid and apoprotein composition of HDL particles on the association and uptake of HDL by a human proximal tubule epithelial cell line. We show that the lipid composition of the HDL particle has a strong influence on its cell association and may therefore affect the re-absorptive salvage of HDL in the kidney.

EXPERIMENTAL

Materials

FluoBioLink™ Cy3™ Monofunctional Dye was purchased from Amersham Biosciences (Baie d’Urfé, PQ, Canada). Brain

Abbreviations used: apoA-I, apolipoprotein A-I; CE, cholesteryl olate; DG, diolein; FC, free cholesterol; HDL, high-density lipoprotein; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; rHDL, reconstituted HDL; TG, triolein.

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Table 1  LpA-I complex composition and structure

<table>
<thead>
<tr>
<th>LpA-I</th>
<th>PC/CE/apoA-I (mol)</th>
<th>Hydrodynamic diameter (nm)</th>
<th>Surface potential (− mV)</th>
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</tr>
<tr>
<td>PC</td>
<td>120:0:2</td>
<td>7.7</td>
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sphingomyelin, FC [free (unesterified) cholesterol] and POPC (1-palmitoyl-2-oleyl phosphatidylcholine) were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). CE (cholesterol linoleate) and TG (triolein) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.), while DG (diolein) was obtained from Nuchek Prep (Elysian, MN, U.S.A.). Iodobeads were from Pierce (Rockford, IL, U.S.A.). All other reagents were of analytical grade. The HKC-8 cell line was obtained from the laboratory of Dr Lorraine Racusen (Johns Hopkins School of Medicine, Baltimore, MD, U.S.A.).

Purification of apoA-I

Human HDL (ρ = 1.063–1.210 g/ml) was isolated from fresh plasma by sequential-density-gradient ultracentrifugation according to the procedure of Havel et al. [21]. HDL was delipidated in chloroform/methanol as described [22]. Purified apoA-I was isolated by size-exclusion chromatography on a Sephacryl S-200 HR column [23]. ApoA-I was stored in lyophilized form at −80 °C. Prior to use, it was resolubilized in 6 M guanidine hydrochloride and 10 mM Tris, pH 7.2, and dialysed extensively against PBS.

Iodination of apoA-I

Purified apoA-I was iodinated with Na125I using the IODO-BEAD iodination reagent (Pierce) and manufacturer-recommended protocols. The efficiency of labelling was 52%, and the resultant specific radioactivity of apoA-I was 1 μCi/μg of protein.

Preparation of reconstituted spherical HDL particles by co-sonication

Purified 125I-apoA-I and 125I-apo HDL obtained from delipidated HDL were prepared by IODO-BEAD NaI labelling (Pierce). Reconstituted HDL particles were prepared by co-sonication of a fixed ratio of lipid/apoA-I. Briefly, specific amounts of synthetic or HDL-extracted lipids in chloroform (see Table 1 for molar ratios) were dried under nitrogen in a 12 mm × 75 mm glass tube, and 800 μl of PBS was added. The lipid/buffer mixture was successively sonicated with a Branson Sonifier 450 (Branson Ultrasonics, Danbury, CT, U.S.A.) under nitrogen for 1 min at constant output, incubated at 37 °C for 30 min and sonicated again for 5 min at 95% duty cycle under nitrogen. Then 0.5 mg of 125I-labelled apoA-I and unlabelled apoA-I (diluted to 1.4 mg/ml solution in PBS) were added to the lipid mixture and sonicated for 4 × 1 min at 90% duty cycle under nitrogen, with 1 min cooling periods between sonications. The particles were then filter-sterilized. The size and homogeneity of the particles were estimated by non-denaturing gradient-gel electrophoresis (Phast System; Pharmacia). Particle charge was calculated from electrophoretic mobility in 0.5% agarose gels containing BSA (Paragon Lipo kit; Beckman).

Ligand-binding and degradation assays

HKC-8 cells were seeded into 24-well Falcon plates (Fisher Scientific, Nepean, ON, Canada) and grown to confluency in a CO2 incubator at 37 °C. Cells were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (Life Technologies, Burlington, ON, Canada) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were washed twice with medium and 1 ml of 125I-labelled ligand (100 nM) in medium (with 6 mg/ml BSA/0.1% CaCl2) was added either alone (total binding) or in the presence of an excess (1 mg/ml) of unlabelled HDL (non-specific binding). HDL-specific binding was calculated by subtracting the non-specific from the total binding. Plates were incubated at 37 °C for various times to allow cell association. At the end of the incubation period, the cells were put on ice, washed twice with cold PBS (with 6 mg/ml BSA/0.1% CaCl2) and twice again with cold PBS alone. The 1 ml of 0.2 M NaOH was added to each well and incubated overnight at 23 °C with gentle rocking. Lysates were removed and radioactivity was measured with a Cobra II Auto-Gamma counter (Canberra Packard Canada, Mississauga, ON, Canada). Cell protein was determined using the BCA (bicinchoninic acid) protein assay (Pierce). For degradation analysis, an aliquot of medium post-incubation was added to an equal volume of 25% trichloroacetic acid for precipitation. The radioactivity of the soluble products was measured and expressed as the percentage of total radioactivity.

Fluorescent microscopy

The FluoroLink™ Cy3™ Monofunctional Dye was conjugated with HDL by adding 1 mg of dye to 68 μl of HDL and 932 μl of 0.1 M sodium carbonate buffer at pH 9.3 for a total volume of 1 ml. The 1 mg/ml solution was mixed and incubated at room temperature for 30 min with additional mixing (Amersham Biosciences). The unconjugated dye was removed from the HDL preparation by gel-permeation chromatography. HKC-8 cells grown to confluence on microscopy wells were washed with medium containing Hepes buffer and 2 mg/ml BSA. For cell-surface labelling, the cells were incubated for 1 h on ice with the labelled HDL in the medium at 10 μg/ml. For cell uptake, the cells were incubated at 37 °C up to 60 min after the labelled ligand was added. In both cases, cells were washed twice with cold PBS, fixed in 500 μl of cold paraformaldehyde and washed again twice with cold PBS. The cells were viewed with an Olympus IX50 inverted fluorescent microscope and photographed with a Micromax Camera equipped with Win View software (Princeton Instruments, Whitley, ON, Canada).

RESULTS

Confluent HKC-8 cells were incubated with fluorescently labelled HDL to monitor the binding and uptake of the HDL particles. Cell-surface binding was estimated after removal of the cell media by incubation of labelled HDL with the live cells on ice. Figure 1(A) shows that the Cy3-HDL readily binds to the cell surface of
High-density lipoprotein composition and renal reabsorption

Figure 1 Binding and internalization of Cy3-HDL by HKC-8 cells

(A) HKC-8 cells were incubated with Cy3-labelled HDL particles for 1 h at 4 °C, washed, fixed and viewed by light microscopy. Cy3-HDL appears to be localized to the cell surface. (B) HKC-8 cells were treated as in (A), except at 37 °C. Cy3-HDL appears to be localized in endocytic vesicles in the perinuclear region. Magnification, ×60.

HKC-8 cells, as evidenced by the intense peripheral labelling highlighting the cell membrane. To determine if this cell-surface-bound HDL can be taken up by the cells, HKC-8 cells were incubated with medium containing Cy3-HDL at 37 °C for up to 60 min. Microscopic evaluation of the HKC-8 cells showed a progressive internalization of the labelled lipoprotein over time. After 60 min, most of the surface-bound HDL particles were endocytosed, as evidenced by the appearance of label in endocytic vesicles in the perinuclear region within the HKC-8 cells (Figure 1B).

To differentiate the HDL-specific binding from non-specific interactions with human proximal tubule cells, we performed time-course experiments in HKC-8 cells using 125I-labelled HDL. Cells were incubated with 100 nM of radiolabelled lipoproteins for up to 2 h, either alone or in the presence of an excess of unlabelled HDL. The cells were washed in buffer on ice and lysed in 0.2 M NaOH. The cell lysates were then evaluated for radioactivity by γ counting. Figure 2 shows the time course of 125I-labelled HDL binding to human proximal tubule cells. The amount of cell-associated particles reached a plateau at 2 h, with a rapid increase up to 30 min and a gradual levelling-off thereafter.

Figure 2 shows the time course of 125I-labelled HDL binding to human proximal tubule epithelial cells. Total (HDLₜ) and HDL-specific (HDLₛ) binding of 125I-HDL to HKC-8 cells are presented as cell-associated radioactivity. HKC-8 cells were incubated with 100 nM 125I-HDL, either alone or in the presence of an excess of unlabelled HDL, at 37 °C for various times. HDL-specific binding was calculated by subtracting the non-specific from the total binding. Data are presented as means ± S.D. from triplicate determinations.

Figure 3 Lp2A-I (an HDL containing two molecules of apoA-I) subjected to electrophoresis in 8–25% non-denaturing gradient gels

Gel profiles are shown for high-molecular-mass standards (lane 1), and four different reconstituted Lp2A-I (compositions shown in Table 1) at 1 mg/ml. Lane 2, rHDL prepared from HDL-lipids and pure apoA-I. Lane 3, rHDL prepared from HDL-apoproteins and PC. Lane 4, rHDL prepared from PC and apoA-I. Lane 5, rHDL prepared from PC, CE and apoA-I. Stokes’ diameters for Lp2A-I were determined by comparison with high-molecular-mass standards and are shown in Table 1.

The HDL-specific binding component represented about 58% of the total binding (Figure 2).

To identify the role of specific HDL constituents on the association with HKC-8 cells, a reconstituted HDL system was utilized. Lipid-free apoA-I was obtained from delipidated normolipidaemic HDL and rHDL (reconstituted HDL) was prepared by co-sonication of synthetic or native lipids and apoA-I. To evaluate the size and homogeneity of the rHDL particles, non-denaturing gradient gel electrophoresis was performed. Figure 3 shows the rHDL to be homogeneous preparations with sizes ranging from 7.7 to 7.9 nm (Table 1). Previous work in this laboratory has shown these complexes to be stable for several weeks at 4 °C. While the particle diameter of the various rHDLs

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Figure 4 Comparative binding of 125I-apoA-I and reconstituted 125I-HDL to cultured human proximal tubule epithelial cells

Total (upper panel) and HDL-specific (lower panel) binding are presented as cell-associated radioactivity. HKC-8 cells were incubated with 100 nM 125I-apoA-I or 125I-rHDL particles, either alone or in the presence of an excess of unlabelled HDL, at 37 °C for various times. HDL-specific binding was calculated by subtracting the non-specific from the total binding. The rHDL particles were prepared by co-sonication. See Table 1 for particle composition. Data are presented as means ± S.D. from triplicate determinations.

appeared to be fairly constant, the surface charge potential of the rHDL was more variable and was sensitive to the lipid composition (Table 1).

Figure 4 illustrates an experiment characterizing the interaction of different lipoprotein particles with HKC-8 cells over time. A saturable cell association can be seen for all three particles evaluated, with 125I-rHDL (POPC/apoA-I, 120 mol:2 mol) and 125I-apoA-I reaching plateau more rapidly than 125I-HDL. Some 78 and 65% of total binding was represented by the HDL-specific binding component for 125I-apoA-I and 125I-rHDL respectively. In addition, 125I-apoA-I HDL-specific binding was 2-fold higher than that of the 125I-rHDL. Both particles showed noticeably lower levels of association than native 125I-labeled HDL. It is also evident that the 125I-HDL, 125I-apoA-I and 125I-rHDL undergo minimal degradation over the incubation period. Less than 2% trichloroacetic acid-soluble radioactivity over the 2 h incubation period was evident for all particles (Figure 5). This was not significantly different than control/background values, which showed that negligible protein degradation had occurred.

To evaluate the importance of the lipid and apoprotein constituents of native HDL, these components were purified through a chloroform/methanol organic extraction of HDL. The obtained HDL-lipids and -apoproteins were then used to reconstitute rHDL particles by sonication with apoA-I and POPC. The cell association of the two reconstituted particles was assessed following their incubation with HKC-8 cells at 37 °C for up to 2 h and was compared with that of native 125I-labeled HDL. As seen in Figure 6, a 5-fold decrease in HDL-specific cell association was observed with the 125I-rHDL reconstituted with HDL-apoproteins relative to that for native 125I-labelled HDL. In contrast, HDL-specific cell association of the 125I-rHDL reconstituted with native HDL-lipids was comparable and slightly greater than that observed for native 125I-labelled HDL.

To elucidate the effect of specific HDL-lipids on rHDL particle association with HKC-8 cells, recombinant particles were prepared by co-sonication of pure lipids and apoA-I. rHDL reconstituted with sphingomyelin, DG, TG or FC and apoA-I showed HDL-specific binding equivalent to that of 125I-rHDL containing POPC and pure apoA-I (results not shown). 125I-rHDL reconstituted with POPC and CE showed a 2-fold increase in HDL-specific cell association when compared with that of 125I-rHDL containing no CE, and was about half as effective as native 125I-labeled HDL in binding to HKC-8 cells (Figure 7). rHDL reconstituted with both CE and FC showed comparable levels of HDL-specific cell association to that of 125I-rHDL containing CE only, suggesting that FC had no auxiliary effect on cell association (results not shown).

DISCUSSION

In order to examine the mechanisms involved in the processing of HDL by the kidneys, in vitro experiments were undertaken using the immortalized HKC-8 human proximal tubule cell line. The cell line has been shown to maintain normal expression of tubule markers and biochemical properties and to display a normal epithelial monolayer morphology with a well-developed brush border for prolonged periods [24]. Fluorescent studies were performed to track the binding and uptake of Cy3–HDL to HKC-8 cells. The study showed that at 4 °C HDL bound to the HKC-8 cell surface but was not internalized. The exact nature of this association is unknown but may represent both specific binding to receptors and/or non-specific cell-surface association. Subsequent
made by Glass et al. [2], wherein apoA-I was immunohistochemically identified in the brush border and densely stained granules in the apical regions of the proximal tubule in frozen sections of rat kidney. Peterson et al. [5] also showed $^{125}$I-HDL to accumulate in apical vesicles, vacuoles and lysosome-like dense bodies and viewed these as components of the endocytic apparatus of the proximal tubule cells.

Competitive binding assays showed that $^{125}$I-HDL can efficiently bind to the HKC-8 cells, as evident from the saturable and high-specific-binding component of the total $^{125}$I-HDL cell association. Several laboratories have demonstrated $^{125}$I-HDL binding to the membranes of the renal cortex in various species, including those of rat, porcine and human origin [9,14,25]. However, the findings in these studies differ enough to suggest that more than one HDL-binding receptor may be present in the kidneys. van Tol and colleagues [14] have identified a low-affinity non-saturating and a high-affinity saturating rat HDL binding to partially purified rat kidney membranes or kidney homogenates. Senault and co-workers [25] have described a two-component association of porcine $^{125}$I-HDL with basolateral membranes of the porcine renal cortex; a high-affinity, lower-capacity interaction associated with a 95 kDa receptor and a low-affinity, higher-capacity interaction that appeared to be a non-specific lipid–lipid interaction. Kozyraki and co-workers [9] identified a high-affinity interaction between human $^{125}$I-HDL and cubulin, a 460 kDa receptor purified from the human as well as the rabbit renal cortex. The interaction was Ca$^{2+}$-dependent, and required apoA-I [9]. Cubulin appears to be localized exclusively in the apical membranes of the renal cortex [10]. Cellular uptake studies of fluorescent phospholipid-labelled HDL or $^{125}$I-HDL suggested that HDL undergoes cubulin-mediated endocytosis and delivery to lysosomes [8,9].

The lipid and apoprotein composition of HDL directly affects the charge and conformation of apoA-I and thereby determines the overall surface charge of an HDL particle [18,26]. HDL surface charge also appears to affect the plasma clearance of this lipoprotein [18] in a manner that appears to implicate a renal involvement [27]. Data suggest that HDL composition may directly affect the renal handling of this lipoprotein. In order to evaluate the effects of lipid composition of HDL on its association with HKC-8 cells, incubations were undertaken with rHDL particles that varied in their lipid and apoprotein composition. Lipid-free $^{125}$I-apoA-I bound relatively poorly to the HKC-8 cells, in comparison with that observed with native $^{125}$I-HDL. Pure $^{125}$I-apoA-I has also been shown to compete less effectively for HDL-binding sites than $^{125}$I-HDL, in human enterocytes [20]. Enterocytes, making up the small intestinal epithelia, display numerous structural and functional similarities to renal epithelial cells [28]. However, the addition of PC (phosphatidylcholine) to apoA-I (rHDL) did not have a marked effect on the association with the HKC-8 cells, in contrast to that previously observed with human enterocytes [20]. To determine if the lipid or protein components of HDL affect the HDL particle association with the HKC-8 cells, incubations were undertaken with rHDL particles that varied in their lipid and apoprotein composition. Lipid-free $^{125}$I-apoA-I bound relatively poorly to the HKC-8 cells, in comparison with that observed with native $^{125}$I-HDL. Pure $^{125}$I-apoA-I has also been shown to compete less effectively for HDL-binding sites than $^{125}$I-HDL, in human enterocytes [20]. Enterocytes, making up the small intestinal epithelia, display numerous structural and functional similarities to renal epithelial cells [28]. However, the addition of PC (phosphatidylcholine) to apoA-I (rHDL) did not have a marked effect on the association with the HKC-8 cells, in contrast to that previously observed with human enterocytes [20]. To determine if the lipid or protein components of HDL affect the HDL particle association with the HKC-8 cells, the HDL-apolipoproteins and -lipids were purified and then reconstituted into novel rHDL with PC and apoA-I. The HDL-lipid rHDL particle displayed an enhanced HDL-specific cell association and one comparable with that for the native $^{125}$I-HDL. In contrast, the HDL-apolipoprotein particle displayed a 5-fold lower cell-association than that for native $^{125}$I-HDL. These results demonstrate that the HDL-lipid composition plays an important role in the association of this lipoprotein with the HKC-8 renal proximal tubule cells.

While apoA-I may be important for the initial association of HDL with the HDL receptors [13], the HDL-lipid composition may play a role in determining the affinity of this interaction by
uniquely regulating apoA-I conformation. Numerous studies show that HDL-lipid composition has major effects on the conformation of apoA-I and the stability and charge of the apoA-I-containing HDL particle [29–32]. A detailed characterization of rHDL particles prepared from a variety of pure lipids showed no increase in apoA-I binding to the HKC-8 cells, but instead showed a similar association to that observed for the POPC–apoA-I rHDL complex. In contrast, inclusion of CE in the membrane lipids markedly enhanced the association of HDL to the HKC-8 cells. This study suggests that renal proximal tubule cells are capable of binding and internalizing HDL particles (D. L. Sparks and D. Breznan, unpublished work).

The exact fate of the native or rHDL particles after their association with the HKC-8 cells is, as yet, unclear. In the present study, all labelled ligands appeared to undergo only minimal degradation, when tracked intracellularly or in the cell media. Western blots of the media or cell extract confirmed this view and showed no evidence of apoA-I fragments (results not shown), Hammad et al. [8] identified significant catabolism of HDL-apolipoproteins in mouse yolk-sac endoderm-like cells, following cubilin-mediated endocytosis. Kozyraki et al. [9] also detected degradation of HDL in rat yolk sac epithelial cells. Degradation in these studies may therefore be species- and or tissue-specific. HKC-8 cells, in contrast, do not readily degrade human apoproteins. These cells instead appear to have a unique pathway to salvage lipoproteins from the urinary filtrate. Preliminary studies in this laboratory show that HDL particles are readily transcytosed across a polarized monolayer of HKC-8 cells (D. L. Sparks and D. Breznan, unpublished work).

In summary, our study demonstrates that the human HKC-8 proximal tubule cells are capable of binding and internalizing HDL particles. Cell incubations show a saturable and specific uptake of HDL by the cells in a manner suggestive of a receptor-mediated pathway. The lipid composition of the HDL particles appears to play a major role in this HDL-specific association, as some components, notably CE, of the HDL-lipid fraction markedly enhance the association of HDL to the HKC-8 cells. This study suggests that renal proximal tubule cells are physiologically capable of salvaging HDL from the glomerular filtrate and that the process is sensitive to the structural properties of an HDL particle.

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