The uptake of cholesterol at the small-intestinal brush border membrane is inhibited by apolipoproteins

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Abstract The uptake of free and esterified cholesterol at the brush border membrane is protein-mediated. Here we show that this sterol uptake is effectively inhibited by exchangeable serum apolipoproteins. Binding of the apolipoprotein to the brush border membrane mediates the inhibitory effect. Evidence is presented to show that the structural motif responsible for the inhibition is the amphipathic α-helix.

Key words: Cholesterol; Intestinal absorption; Brush border membrane; Inhibition; Apolipoprotein

1. Introduction

We have shown that the absorption of free and esterified cholesterol in the small intestine is protein-mediated [1–3]. Using small-intestinal brush border membrane vesicles (BBMV), intact enterocytes and Caco-2 cells, ample experimental proof is now available for a transporter in the brush border membrane (BBM) [4,5]. This is at variance with the widely accepted view documented in review articles and text books that dietary lipids diffuse passively along a concentration gradient from the lumen of the small intestine to the cytosol of enterocytes. Here we show that serum apolipoproteins, their lipidated forms and amphipathic α-helical peptides inhibit sterol uptake by BBMV. This inhibition will have important implications for the development of compounds and methods to reduce the uptake of dietary fats, particularly cholesterol.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (PC) and dimyristoyl PC were purchased from Lipid Products (Nutfield, Surrey, UK), sodium dextran sulfate, Phenyl Sepharose 6 Fast Flow, Q-Sepharose Fast-Flow, Sephadex G-50, PBE 94 and Polybuffer 74 for chromatofocusing from Pharmacia (Dübendorf, Switzerland), cholesterol (purity >99%) from Fluka, cholesteryl oleate (purity >98%) from Sigma (Buchs, Switzerland), bicinchoninic acid protein assay reagent and monoclonal anti-human apolipoprotein (apo) A-I antibodies from Pierce (Lausanne, Switzerland). [4-^{14}C]cholesterol (~50 Ci/mol) and [1α,2α(n)-^{3}H]cholesteryl oleyl ether (37 Ci/mmol) were obtained from Amersham (UK). Sheep serum was obtained from the Basel Institute of Immunology (Basel, Switzerland) and stored at −80°C prior to use.

2.2. Preparation of donor and acceptor particles

BBMV as the acceptor membrane were routinely prepared from rabbit duodenum and jejunum according to [6]. As a quality control some physicochemical properties of the BBMV were determined routinely as described in [4] and [6]. Furthermore, the stability and integrity of the BBMV were checked by determining the specific activity of marker enzymes and the Na^+-dependent glucose uptake [6]. Small unilamellar vesicles (SUV) of egg PC containing 1 mol% cholesterol or 1 mol% cholesteryl oleate and a trace amount of [4-^{14}C]cholesterol or [1α,2α(n)-^{3}H]cholesteryl oleyl ether, respectively, were used as donor particles and made according to [7]. Both donor and acceptor particles were dispersed in Tris-NaCl buffer (0.05 M Tris, pH 7.4, 0.15 M NaCl, 0.02% NaN_3).

2.3. Isolation and purification of inhibitors of sterol uptake

We observed that sheep serum inhibits the protein-catalysed absorption of free and esterified cholesterol by BBMV. This striking result prompted the isolation and purification of the inhibitory factor. Lipoprotein-depleted sheep serum produced by sequential ultracentrifugation according to [8] was inactive; all the inhibitory activity was found in the lipoprotein fraction. When sheep serum was fractionated with dextran sulfate and Mn^{2+} according to [9], most of the activity was recovered in the HDL fraction. This fraction was further purified by hydrophobic interaction chromatography using a Phenyl Sepharose 6 Fast Flow column (6.5×2.8 cm) equilibrated with 0.05 M Tris-HCl, pH 7.4, 2 M NaCl. Nearly all of the inhibitory activity applied to the column was bound. After washing out the flow-through proteins, bound proteins were eluted with 0.15 M NaCl (fraction 1), with H_2O (fraction 2) and 15% ethanol (fraction 3, Table 1). The specific inhibitory activity of fraction 2 eluted with water was increased by a factor of 15 relative to the HDL fraction. Fraction 2 was further purified by chromatofocusing on polybuffer exchanger 94. The activity was eluted with a pH gradient from 7 to 3.8 [10] yielding a single symmetric peak centered at pH 5.4 (Fig. 1). The specific activity of this fraction was increased by a factor of 65 relative to sheep serum. For amino acid sequencing the purified protein was subjected to SDS–15% PAGE, transferred to a PVDF membrane, and sequenced on an Applied Biosystem 477A protein sequencer.

Pure human apo A-I and apo A-II were prepared from human high-density lipoprotein (HDL) by delipidation [11] and ion exchange chromatography on Q-Sepharose [12]. The purity of apo A-I and apo A-II was checked by SDS–PAGE with 8–25% gradient gels using a Phast Electrophoresis system (Pharmacia). Both proteins gave single bands on overloaded gels. Prior to use, the proteins were solubilized in 3 M guanidine HCl and dialyzed against the Tris-NaCl buffer. Human apo A-IV was a gift of Dr. P. Tso of LSU Medical Center and pure by SDS–PAGE. Human HDL_3 (density d=1.125–1.21 g/ml) was isolated from fresh plasma of normallipidemic donors by sequential centrifugation [8]. The HDL_3 preparation was pure by electrophoresis in 0.5% agarose gels. A reconstituted discoidal HDL particle was obtained by interacting lipid-free apo A-I with an unsonicated dimyristoyl PC dispersion at 24°C according to [13].

The peptides Ac-18A-NH_2 (CH_3CO-DWLKAFYDKVAEKL-KEAF-NH_2) and Ac-18S-NH_2(CH_3CO-DWLAKDYFKKALVEE-FAK-NH_2) [14–16] were synthesized by solid-phase synthesis with the N-terminus being acetylated and the C-terminus amidated and

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Abbreviations: Apo, apolipoprotein; BBM, brush border membrane; BBMV, brush border membrane vesicle(s); HDL, high-density lipoprotein; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PC, phosphatidylcholine; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; SUV, small unilamellar vesicle(s)

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supplied by Anawa Biomedical Services and Products (Zürich, Switzerland). The purities were better than 95% as assessed by HPLC. The molecular weights of the two peptides determined by mass spectrometry were consistent with the calculated values.

2.4. Lipid uptake by BBMV

The kinetics of sterol uptake by BBMV were measured as described before [1–4] (see legend of Fig. 3). The inhibitor concentration IC_{50} required to produce 50% inhibition was determined as follows: the rate of sterol uptake by BBMV was determined by incubating donor (0.05 mg total lipid/ml) and acceptor (BBMV at 2 mg protein/ml) for 20 min at 23°C with and without inhibitor. Sterol uptake by BBMV in the absence of inhibitor was taken as 100%, and the reduced uptake activity measured in the presence of inhibitor was expressed as percent inhibition. This yielded dose–response curves relating percent inhibition to the total inhibitor concentration (cf., Fig. 4). The data points of these dose–response curves were fitted by a modified Hill equation [17]:

$$y = 1/[1 + (IC_{50}/x)^n]$$

where y is percent of inhibition, x the total inhibitor concentration and n the Hill coefficient. The programs MacCurveFit (Kevin Raner Software, Victoria, Australia) and Excel (Microsoft) were used on a Macintosh computer.

2.5. Analytical methods

The molecular mass of the peptides and proteins was determined by matrix-assisted laser desorption ionization mass spectrometry using time-of-flight mass analysis (Voyager Elite mass spectrometer from PerSeptive Biosystem). Phospholipid concentrations were determined according to [18] and protein concentrations by the bicinchoninic acid method [19].

3. Results

3.1. Isolation, purification and identification of the inhibitor

We discovered serendipitously that sheep serum inhibited effectively sterol uptake by BBMV. Consequently, the inhibiting factor was isolated from sheep serum and purified to homogeneity. The main peak obtained by chromatofocusing at pH 5.4 (Fig. 1) gave a single band on SDS–15% PAGE using silver staining with an apparent molecular mass of 28 kDa (Fig. 2A). The sequence of the first 29 NH_2-terminal amino acids (Fig. 2B) was highly homologous to human (69%), rabbit (73%), rat (83%) and bovine apo A-I (90%). The molecular mass of the purified inhibitor determined by mass spectrometry was 27.57 kDa. The isoelectric point of the protein determined by chromatofocusing was 5.40 (Fig. 1). These values are in excellent agreement with published data on human and rabbit apo A-I [20]. Immunoblot analysis of the purified inhibitor using monoclonal antibodies against human apo A-I revealed a clear-cut immunological cross-reactivity between the purified inhibitor and human apo A-I. Lipid analysis indicated that the purified inhibitor contained phospholipid (0.45 mg phospholipid/mg protein) and cholesterol (0.26 mg cholesterol/mg protein). We conclude that the inhibitor isolated from sheep serum is a partially delipidated HDL, (thereafter referred to as sheep HDL) [21].

Table 1
Hydrophobic chromatography of HDL on Phenyl Sepharose

Fractions	Elution of inhibitory activity		
	(au)	(%)	(au/mg protein)
Flow-through	2.8×10^4	2	80
Fraction 1	3.1×10^4	3	430
Fraction 2	9.6×10^5	81	2.7×10^4
Fraction 3	1.6×10^5	14	4.1×10^4

The HDL fraction applied to the column contained 0.6 g protein and an activity of 1.1×10^6 arbitrary units (au).

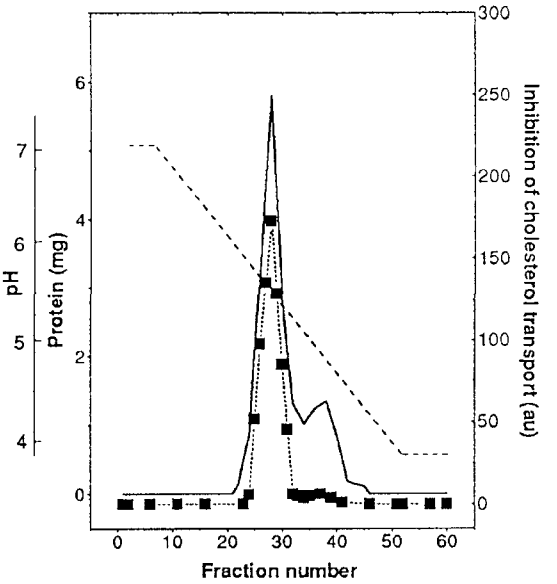

Fig. 1. Chromatofocusing of the partially purified inhibitor on polybuffer exchanger 94. Protein (35 mg) of fraction 2 (Table 1) was applied to a 20 ml polybuffer exchanger column (25.5×1 cm) equilibrated with 0.025 M imidazole-HCL buffer, pH 7.3. The inhibitory activity bound to the column was eluted at 0.5 ml/min with a linear pH gradient from 7 to 3.8 generated with polybuffer 74. Fractions of 8 ml were collected and analysed for protein and inhibitory activity. Solid line: protein content; dashed line: pH gradient; dotted line and squares: inhibitory activity in arbitrary units (au).

3.2. Sterol uptake by small-intestinal BBMV in the absence and presence of human apo A-I

The kinetics of cholesteryl oleate uptake by BBMV in the absence and presence of human apo A-I are shown in Fig. 3. The kinetic curve in the absence of apo A-I was adequately fitted by the sum of two exponential functions while that in the presence of apo A-I was fitted best by a single exponential decay (see dotted lines, Fig. 3). Pseudo-first-order rate constants k (half-times $t_{1/2}$) derived from curve fitting gave the following values: in the absence of apo A-I the values were $k_1 = 0.46 \pm 0.04$ min^{-1} ($t_{1/2} = 1.5$ min) and $k_2 = 0.66 \pm 0.1$ h^{-1} ($t_{1/2} = 1.1$ h) for the initial fast phase and the second slow phase, respectively. In the presence of apo A-I the single exponential decay was characterized by $k_1 = 0.36 \pm 0.06$ h^{-1} ($t_{1/2} = 1.93$ h). In the absence of apo A-I the final equilibrium x_∞ obtained from curve fitting was 3% entirely consistent with the experimental value (Fig. 3). So the effect of the inhibitor is to abolish the initial fast phase of sterol uptake and to shift the final equilibrium x_∞ from 3 to 37% (Fig. 3).

Cholesteryl oleate was used as a representative sterol and cholesteryl oleyl ether as its nonhydrolyzable analogue. Ester and ether compounds behaved identically with respect to the uptake kinetics [2]. As shown before [2], the kinetics of cholesterol uptake were similar to those of cholesteryl oleate, and this was also true for the kinetics of sterol uptake in the presence of apo A-I. With cholesterol there was a small but still measurable contribution to the uptake from passive diffusion. With the more hydrophobic cholesteryl oleate this contribution was negligible and in this case no cor-

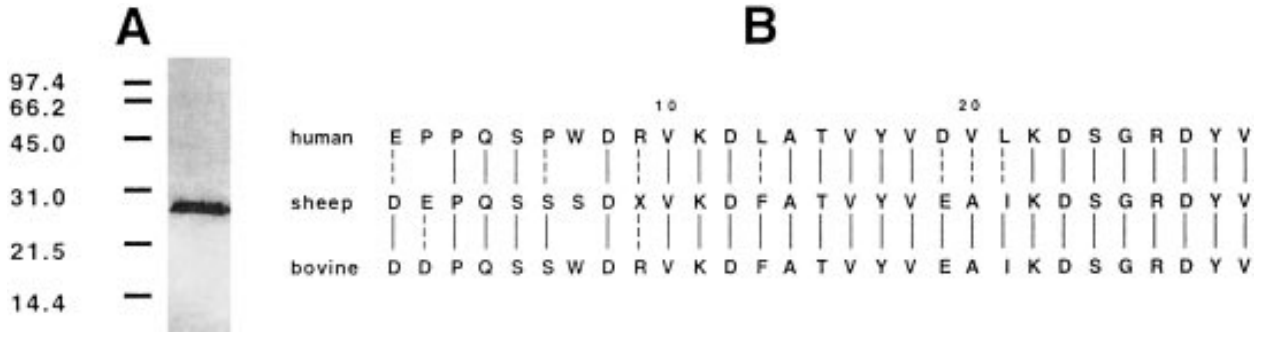

Fig. 2. A: SDS–15% PAGE pattern of the inhibitory activity isolated and purified from sheep serum. SDS–PAGE was carried out using the Mini-Protean II dual slab cell from Bio-Rad according to the Bio-Rad instruction manual. Proteins were made visible by silver staining. Bio-Rad low-range protein standards were used and the positions of apparent molecular masses in kDa are marked. B: The sequence of the first 29 NH_2-terminal amino acids of the inhibitory protein isolated and purified from sheep serum is compared to the NH_2-terminal sequences of human and bovine apo A-I. Conservative replacements are indicated by dashed lines.

rection had to be made. For this reason the discussion of the data for cholesteryl oleate presented in Fig. 3 is straight-forward.

The inhibiting effect of apo A-I is presented as a dose-response curve and compared to the effect of other inhibitors in Fig. 4. The dose–response curves for the inhibition of free and esterified cholesterol uptake measured for apo A-I, apo A-II, HDL_3 and Ac-18A-NH_2 were identical within experimental error (cf., IC_{50} values, Table 2). The data in Fig. 4 demonstrate that sterol uptake by BBMV is effectively and completely inhibited by apo A-I concentrations of $\sim$100 μg/ml. Other serum apolipoproteins and the synthetic peptide Ac-18A-NH_2 were also effective though to a different extent (Table 2). The lipidated forms of apo A-I such as sheep HDL, human HDL_3 and the discoidal lipoprotein particles reconstituted from human apo A-I and dimyristoyl PC also effectively inhibited sterol uptake.

Control experiments were carried out to show that apo A-I as a detergent-like molecule neither changes the average size of the donor vesicles nor does it solubilize BBMV at concentrations used in this study.

4. Discussion

4.1. Mechanism of inhibition

The mechanism(s) by which lipid molecules cross the BBM remains to be elucidated, partly because of the unavailability of specific inhibitors. Here, we report that sterol uptake by BBMV is effectively and completely inhibited by apo A-I and the lipidated forms of apo A-I such as HDL and discoidal lipoprotein particles reconstituted from apo A-I and dimyristoyl PC. In principle, apo A-I could inhibit sterol uptake by interacting with either the donor SUV or the acceptor BBMV. With regard to the donor, apo A-I could change the structure of the SUV and prevent sterol transfer. This is clearly not the case. We studied the interaction of apo A-I with egg PC SUV using gel filtration on Sepharose CL-4B. The binding parameters derived were in good agreement with published values [22]. Under the conditions of lipid uptake (Fig. 3) only about 2% of apo A-I was bound to the SUV, and the size distribution of egg PC SUV remained unchanged as determined by gel filtration on Sepharose CL-4B. Furthermore, apo A-I-containing SUV of egg PC still give passive cholesterol transfer [23].

With regard to the acceptor, since apo A-I is an amphipathic protein, and thus a detergent, it could solubilize BBMV. This is not the case. At apo A-I concentrations used in our lipid uptake measurements (Fig. 3) the average size and size distribution of the BBMV remained unaltered. Clear-cut evidence to this effect was obtained by light scattering and freeze-fracture electron microscopy. The structural integrity of these BBMV was maintained despite the fact that apo A-I does bind to BBMV. Binding could be demonstrated unambiguously by using ^{14}C-labeled apo A-I. The inhibition produced by apo A-I (Fig. 3) was largely reversible. Thorough washing of BBMV, which had lost their protein-mediated sterol uptake ability due to exposure to apo A-I, restored the protein-mediated sterol uptake to about 80–90%. Apo A-I binds to the BBMV and this binding leads to inhibition.

Table 2
IC_{50} values for inhibition of cholesterol and cholesteryl oleate uptake by small-intestinal BBMV

Protein, peptide	IC_{50}*			
	Cholesteryl oleate		Cholesterol	
	(μg/ml)	(μM)	(μg/ml)	(μM)
Human apo A-I	11 ± 1	0.4 ± 0.04	10 ± 1	0.35 ± 0.04
Human apo A-II	8 ± 2	0.5 ± 0.1	10 ± 2	0.6 ± 0.1
Human apo A-IV	32 ± 4	0.7 ± 0.1		
Human apo A-I in discoidal HDL particles	25 ± 5	0.9 ± 0.2		
Human HDL_3	29 ± 2	1.0 ± 0.1	28 ± 1	1.0 ± 0.05
Sheep HDL	8 ± 2	0.3 ± 0.1		
Ac-18A-NH_2	33 ± 2	15 ± 1	30 ± 2	14 ± 1

*Values are the averages of three separate experiments in duplicate. In the cases of lipoprotein particles concentrations are expressed in terms of apo A-I using a molecular mass of 28 000.

4.2. Structural requirement of the inhibitor

The data summarized in Table 2 indicate that the inhibitory effect is not specific for apo A-I because human apo A-II, and apo A-IV also inhibited sterol uptake. The inhibitory effect appears to be characteristic of the class of exchangeable apolipoproteins. Since these apolipoproteins all have different amino acid sequences, the inhibition cannot be due to a specific amino acid sequence occurring in one of these apolipoproteins. A structural motif that is common to this class of proteins is the amphipathic α-helix [15,24]. The hypothesis that the amphipathic α-helix is the structural principle underlying the inhibition is readily subjected to test. To this end the amphipathic peptide Ac-18A-NH_2 (CH_3CO-DWLKAFYDK-VAEKLKEAF-NH_2) was designed to have minimal homology to naturally occurring amphipathic α-helices [14]. It possesses the characteristics of class A amphipathic α-helices as present in apo A-I: the positively charged Lys are present at the polar–nonpolar interface, the negatively charged Asp and Glu are present at the centre of the polar face and the polar and nonpolar faces have approximately equal surface areas [14,15,22]. The addition of the acetyl group at the NH_2-terminus and amidation at the C-terminus were reported to produce a large increase in the helicity of the peptide both in solution and when bound to lipids [15]. Thus Ac-18A-NH_2 is an idealized amphipathic, α-helical peptide that was shown to mimic some properties of apo A-I [14]. As evident from Fig. 4 and Table 2, Ac-18A-NH_2 is an effective inhibitor of sterol uptake at the BBM. That the amphipathic α-helix is the structural principle underlying the inhibition is supported by the observation that the peptide Ac-18S-NH_2 (CH_3CO-DWLAKDYFKKALVEEFAK-NH_2) was inactive. This peptide is scrambled Ac-18A-NH_2: it has the same amino acid composition as 18A, but the amino acid sequence is randomized to eliminate the amphipathic character of the helical peptide [16].

The finding reported here may be physiologically relevant. Small-intestinal epithelial cells are short-lived (48–72 h) and eventually shed into the lumen of the small intestine. Since these cells are one of the major sites of apo A-I biosynthesis, it is feasible that apo A-I released from shed cells inhibits cholesterol uptake at the BBM. To answer the question of the physiological relevance would require the knowledge of the effective apo A-I concentration in the small-intestinal lumen.

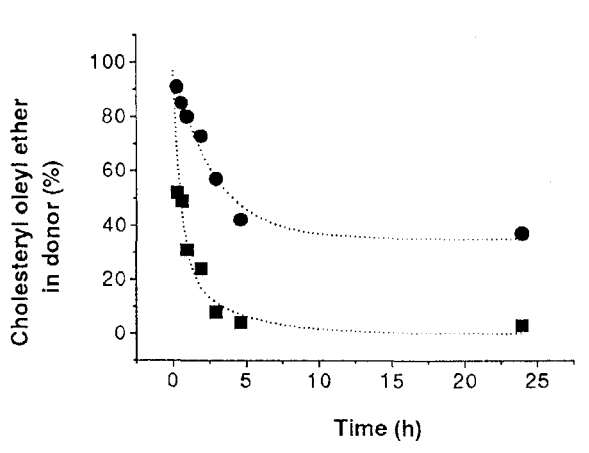

Fig. 3. The kinetics of cholesteryl oleate uptake by BBMV. Egg PC SUV as the donor containing 1 mol% cholesteryl oleate trace-labeled with [^{3}H]cholesteryl oleyl ether (0.05 mg total lipid/ml) were incubated with BBMV (5 mg protein/ml; 3 mg total lipid/ml) at 23°C in the absence (■) and presence of human apo A-I (●) at 45 μg protein/ml. Both donor and acceptor were dispersed in Tris-NaCl buffer.

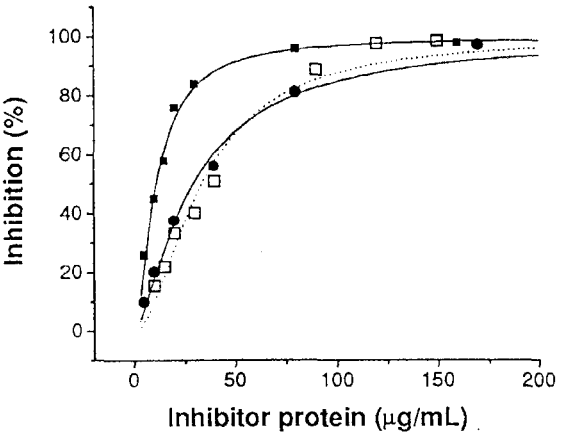

Fig. 4. Inhibition of cholesterol and cholesteryl oleate uptake by BBMV expressed as percent as a function of increasing amounts of different inhibitors. The sterol uptake activity of BBMV measured in the absence of inhibitor was taken as 100% and the loss in this activity observed in the presence of inhibitor is expressed as percent inhibition. Human apo A-I (■); human HDL_3 (●) and peptide Ac-18A-NH_2 (□). The standard deviation of three different measurements in duplicate was $<5\%$; therefore error bars were omitted. The solid and dotted lines were obtained by curve fitting using a modified Hill equation [17].

Elucidation of the mechanism of inhibition of sterol uptake at a molecular level has to await the systematic study of structure–activity relations using different apolipoproteins as well as model peptides. The inhibition of sterol uptake at the BBM holds great potential for controlling cholesterol absorption in vivo. Our discovery raises the prospect of future development of drugs targeted against the sterol transporter in the BBM. Such agents would have wide applications in the control of atherosclerosis.

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