FEBS 24463 FEBS Letters 487 (2000) 293–297

Identification of binding proteins for cholesterol absorption inhibitors as components of the intestinal cholesterol transporter

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Received 17 October 2000; revised 27 November 2000; accepted 27 November 2000

First published online 13 December 2000

Edited by Shozo Yamamoto

Abstract To identify protein components of the intestinal cholesterol transporter, rabbit small intestinal brush border membrane vesicles were submitted to photoaffinity labeling using photoreactive derivatives of 2-azetidinone cholesterol absorption inhibitors. An integral membrane protein of M_r 145.3 ± 7.5 kDa was specifically labeled in brush border membrane vesicles from rabbit jejunum and ileum. Its labeling was concentration-dependently inhibited by the presence of cholesterol absorption inhibitors whereas bile acids, D-glucose, fatty acids or cephalexin had no effect. The inhibitory potency of 2-azetidinones to inhibit photolabeling of the 145 kDa protein correlated with their in vivo activity to inhibit intestinal cholesterol absorption. These results suggest that an integral membrane protein of M_r 145 kDa is (a component of) the cholesterol absorption system in the brush border membrane of small intestinal enterocytes.

Key words: Intestinal cholesterol absorption; Cholesterol transporter; 2-Azetidinone; Transport protein; Cholesterol transport inhibitor; Photoaffinity labeling; Small intestine

1. Introduction

Intestinal absorption of cholesterol is a major determinant for body cholesterol homeostasis and specific inhibitors of intestinal cholesterol absorption may become major drugs of the future to prevent atherosclerosis and cardiovascular diseases. The molecular mechanism responsible for intestinal cholesterol absorption is poorly understood and discussed controversially [1–3]. Originally a passive process was assumed [4] but several findings support the involvement of a protein-mediated specific process: (i) Cholesterol but not closely related plant sterols like β-sitosterol or campesterol are absorbed by the small intestine [5]. (ii) Cholesterol transport from mixed micelles to intestinal brush border membrane vesicles (BBMV) is protease-sensitive [6]. (iii) Highly potent compounds of different chemical structures have been identified as specific inhibitors of intestinal cholesterol absorption [7–15]. Hauser and co-workers have suggested an intestinal cholesterol transporter based on their in vitro studies with intestinal BBMV [6,16,17] and several proteins have been described in the past as possible components of intestinal cholesterol absorption [16–18]. Their role, however, for intestinal cholesterol absorption is still far from clear and remains to be elucidated. The first step of intestinal cholesterol absorption is the interaction of cholesterol with the brush border membrane of small intestinal enterocytes. Recent studies have demonstrated a specific binding of cholesterol absorption inhibitors to the brush border membrane of enterocytes in duodenum, jejunum and ileum [12,19]. We therefore attempted to identify the respective binding protein(s) for cholesterol absorption inhibitors by photoaffinity labeling with photoreactive analogues of 2-azetidinone-derived cholesterol absorption inhibitors. In the present manuscript we describe the first molecular identification of specific binding proteins for cholesterol absorption inhibitors which are probably involved in intestinal cholesterol absorption. An integral membrane protein of M_r 145 kDa in the enterocyte brush border membrane exerts the characteristics of the hitherto unknown intestinal cholesterol transporter.

2. Materials and methods

2.1. Materials

The cholesterol absorption inhibitors shown in Fig. 1 were synthesized at Aventis Pharma Deutschland GmbH according to published procedures [7–9]. The photoreactive analogues C-1 and C-2 were synthesized by acylation of aminobenzyl derivatives at R4 with 4-azido[3,5-^{3}H]benzoyl-*N*-hydroxysuccinimide ester (NEN Du Pont de Nemours) or [^{3}H]acetanhydride leading to radiolabeled C-1 and C-2 with specific radioactivities of 45.7 and 3.41 Ci/mmol, respectively. Photoreactive bile acid derivatives carrying a diazirino function at position 3 or 7 were synthesized as described [20,21]. Acrylamide, *N*,*N*′-bismethylenacrylamide, materials for electrophoresis and marker proteins for the determination of molecular masses were purchased from Serva (Heidelberg, Germany) or Sigma (München, Germany). Scintillators (Quickszint 501 and 361) and the tissue solubilizer Biolute S were from Zinsser Analytic GmbH (Frankfurt, Germany). Protein was determined with a Bradford assay kit from Bio-Rad (München, Germany).

2.2. Animals

Male New Zealand white rabbits weighing 4–5 kg (Harlan Winkelmann, Borchen, Germany) were kept on Altromin® standard diet C 2023 (Altromin®, Lage, Germany) ad libitum. For cholesterol challenge the animals obtained Altromin® standard diet C 2023 enriched with 3% (w/w) cocoa fat and 0.2% (w/w) cholesterol ad libitum for a period of 10 days. Animals were kept at 18–20°C on a 12 h dark/light rhythm. Rabbit intestinal BBMV from jejunum and ileum were prepared by the Mg^{2+} precipitation procedure and characterized as described earlier [22].

2.3. Inhibition of cholesterol absorption

Intestinal cholesterol absorption was determined by a modification

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PII: S0014-5793(00)02372-3

of the method of Zilversmit and Hughes [23]. Male NMRI mice (Charles River Deutschland GmbH, Sulzfeld, Germany) kept on regular chow (Altromin®, Lage, Germany) were starved for 12 h. 0.5 ml of a solution of 0.5% methylcellulose/5% Solutol (BASF, Ludwigshafen, Germany) as vehicle with or without 3 mg of the respective cholesterol absorption inhibitor were applied by gavage to each animal followed by 0.25 ml of Intralipid® (Pharmacia and Upjohn, Erlangen, Germany) solution containing 0.24 μCi [^{14}C]cholesterol and 0.25 μCi [^{3}H]sitostanol. The animals (five mice per group) were kept on metabolism cages and feces were collected. After 24 h the animals were killed and the radioactivity in the feces and the liver was determined by combustion analysis.

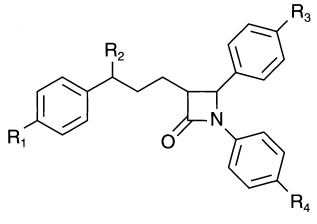
2.4. Photoaffinity labeling and binding studies

Photoaffinity labeling was carried out in a Rayonet RPR-100 photochemical reactor (The Southern Ultraviolet Company, Hamden, CT, USA) equipped with four RPR 2537 Å lamps. BBMV (100–200 μg of protein) were incubated at 20°C for 5 min in the dark with the appropriate concentrations of C-1 or C-2 in a total volume of 200 μl in 10 mM Tris/HEPES buffer (pH 7.4)/100 mM NaCl/100 mM mannitol followed by irradiation at 254 nm for 20 s (C-1) or 60 s (C-2). Subsequent to photoaffinity labeling, BBMV were washed twice with the above buffer, proteins were precipitated and analyzed by SDS–gel electrophoresis [22]. For Western blotting, proteins were transferred to nitrocellulose membranes (0.2 μm trans-blot transfer medium from Bio-Rad) from SDS–gels in a trans-blot cell (Bio-Rad) with 25 mM Tris/192 mM glycine (pH 8.3)/33% (v/v) methanol. Blotting was performed of 300 mM for 3 h followed by 400 mÅ for 0.5 h. Immunodetection was performed with antibodies against the scavenger receptor SR-BI (Novus, Cambridge, UK) or the C-terminus of the rabbit ileal Na^{+}/bile acid cotransporter [24] by using the Western light chemiluminescence detection system from Serva (Heidelberg, Germany). Radioactively labeled polypeptides were detected either by liquid scintillation counting after slicing of the gels into 2 mm pieces and hydrolysis of proteins with 0.25 ml of tissue solubilizer Biolute S or by fluorography [22]. Binding of radiolabeled C-1 and C-2 to rabbit small intestinal BBMV was determined by incubation of BBMV with C-1 or C-2 in the concentration range 1 nM to 10 μM in the absence or presence of 1–50 μM competing ligands for 60 min at 20°C followed by centrifugation and measurement of membrane-associated radioactivity by liquid scintillation counting.

3. Results

3.1. Rationale for the design of photoreactive cholesterol absorption inhibitors

Binding proteins for photoreactive analogues of 2-azetidinone-derived cholesterol transport inhibitors (Fig. 1) in BBMV from small intestinal cells probably are specific components of the uptake machinery for cholesterol across the enterocyte brush border membrane for the following reasons:



Compound	R_1	R_2	R_3	R_4
S 3302 (≙ Racemate of SCH 48461)	H	H	OCH_3	OCH_3
SCH 58235	F	OH	OH	F
S 6503	H	OH	OCH_3	F
C–1	H	OH	OCH_3	$CH_2NH-CO-C_6H_4-N_3$
C–2	H	OH	OCH_3	$CH_2NH-CO-CH_3$

Fig. 1. Chemical structure of 2-azetidinone cholesterol absorption inhibitors.

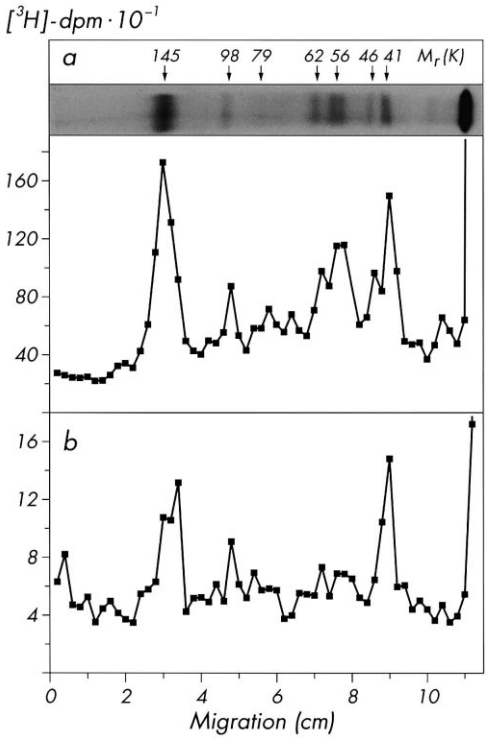


Fig. 2. Photoaffinity labeling of rabbit small intestinal BBMV with photoreactive cholesterol absorption inhibitors. BBMV isolated from rabbit ileum (200 μg of protein, 2 mg/ml) were photolabeled with 0.212 μM [^{3}H]C-1 or 2.93 μM [^{3}H]C-2 as described in Section 2. Labeled membrane proteins were analyzed by SDS–gel electrophoresis on 7.5% gels followed either by fluorography or by slicing of the gel lanes into 2 mm pieces and detection of radioactivity by liquid scintillation counting of hydrolyzed proteins. a: After labeling with [^{3}H]C-1. Top: Fluorographic detection of radioactivity. Bottom: Detection by slicing of the gel and liquid scintillation counting. b: After labeling with [^{3}H]C-2.

1. 2-Azetidinones are specific inhibitors of intestinal cholesterol absorption [7–12] in different animal species including rabbits [25] and humans [26].
2. 2-Azetidinone- as well as sterol glycoside-derived cholesterol transport inhibitors act at the brush border membrane of enterocytes from duodenum, jejunum or ileum blocking the movement of cholesterol into villi of enterocytes [19].
3. The in vivo potency of cholesterol transport inhibitors correlates with their binding affinity to enterocyte brush borders [27].
4. 2-Azetidinone-derived cholesterol transport inhibitors show clear structure–activity relationships [7–9,27] indicating a specific interaction with protein components.

Structural changes in position R4 of 2-azetidinones are well tolerated and small residues of R4 completely retain pharmacological activity as cholesterol absorption inhibitors [9]. We therefore introduced radiolabeled photoreactive groups into position R4 obtaining with C-1 a nitrene-generating azido derivative whereas the [^{3}H]acetyl-aminobenzyl derivative C-2 should be suited for direct photoaffinity labeling [28]. The

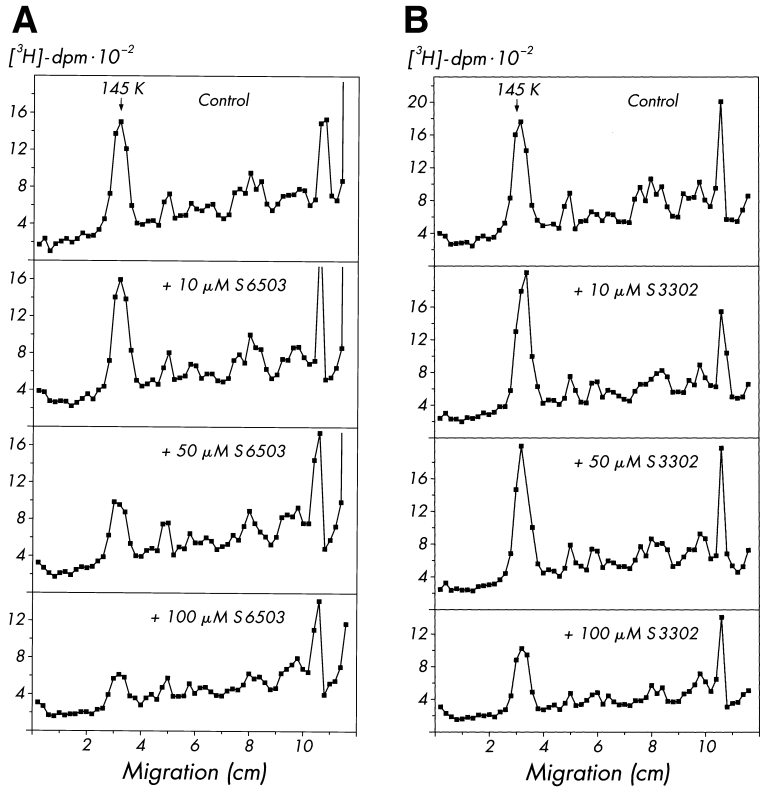


Fig. 3. Effect of cholesterol absorption inhibitors on photoaffinity labeling of rabbit small intestinal BBMV by the photoreactive cholesterol absorption inhibitor [^{3}H]C-1. BBMV obtained from rabbit ileum (200 μg of protein, 2 mg/ml) were photolabeled with 0.212 μM [^{3}H]C-1 in the absence or presence of the indicated concentrations of the cholesterol absorption inhibitors S 6503 or S 3302 followed by SDS–gel electrophoresis and detection of radioactivity by liquid scintillation counting. A: Labeling in the presence of S 6503. B: Labeling in the presence of S 3302.

high potency of 2-azetidinone cholesterol reabsorption inhibitors such as SCH 58235 with an ED_{50} value of 0.0005 mg/kg in the rhesus monkey [11] suggests a low abundance of the cholesterol transporter in BBMV. Consequently, the photoaffinity probes C-1 and C-2 were synthesized with high specific radioactivities of 45.7 and 3.41 Ci/mmol, respectively. The suitability of radiolabeled C-1 and C-2 as photoaffinity probes was demonstrated by covalent crosslinking to albumin upon UV irradiation at 254 nm (data not shown). Incubation of BBMV with radiolabeled C-1 or C-2 demonstrated the metabolic stability of the photoprobes indicating that photoaffinity labeling of small intestinal BBMV with these derivatives should allow the identification of the protein(s) responsible for intestinal cholesterol absorption.

3.2. *Photoaffinity labeling of rabbit small intestinal BBMV with radiolabeled cholesterol absorption inhibitors [^{3}H]C-1 and [^{3}H]C-2*

Photoaffinity labeling of BBMV from rabbit jejunum or ileum with C-1 led to a labeling of membrane proteins of M_r 145.3 ± 7.5, 98.5 ± 1.6, 79 ± 1.4, 61.8 ± 2.5, 55.5 ± 1.4, 46, 41.4 ± 1.3 and 35.2 ± 2.2 kDa (n = 6), the 145 kDa protein being mostly labeled (Fig. 2). C-2 resulted in a similar labeling pattern with a prominent labeling of the 145 and 41 kDa proteins. Variation of the incubation time up to 60 min or photoaffinity labeling in the frozen state did not significantly change the labeling pattern. The scavenger receptor SR-BI has been suggested as a receptor mediating absorption of dietary cholesterol in the intestine [16]. None of the photolabeled binding proteins, however, comigrated with SR-BI after immunostaining with SR-BI antibodies nor could be precipitated by SR-BI antibodies excluding the possibility that SR-BI is the target protein for cholesterol absorption inhibitors. The molecular weights and described functions make the involvement of other members of the scavenger receptor family in intestinal cholesterol absorption unlikely [29]. Additionally, none of the radiolabeled bands showed immunoreactivity with the ileal Na^+-dependent ileal bile acid transporter [24]. The labeling of the 62, 56, 46 and 41 kDa was dependent on the vesicle preparation. Solubilization experiments with nonionic or zwitterionic detergents and sodium carbonate identified the labeled proteins of M_r 145, 98, 56 and 35 kDa as integral membrane proteins. Identical labeling patterns were obtained with vesicles from rabbit jejunum and rabbit ileum with a slightly higher labeling of the 145 kDa protein in the ileum. The identification of identical binding proteins for cholesterol absorption inhibitors in the jejunum and the ileum is in line with the findings that the entire small intestine has the capacity to absorb cholesterol [30–32].

3.3. *Specificity of the binding proteins for cholesterol absorption inhibitors*

To identify the prime candidate for the intestinal cholesterol transporter, differential photolabeling experiments were performed with high and low affinity inhibitors for intestinal cholesterol absorption. A difference in their in vivo potency should be reflected in a different extent of inhibition on photoaffinity labeling of the specific binding protein(s). S 3302 is the racemate of the cholesterol absorption inhibitor SCH 48461 potently inhibiting intestinal cholesterol absorption in rabbits,

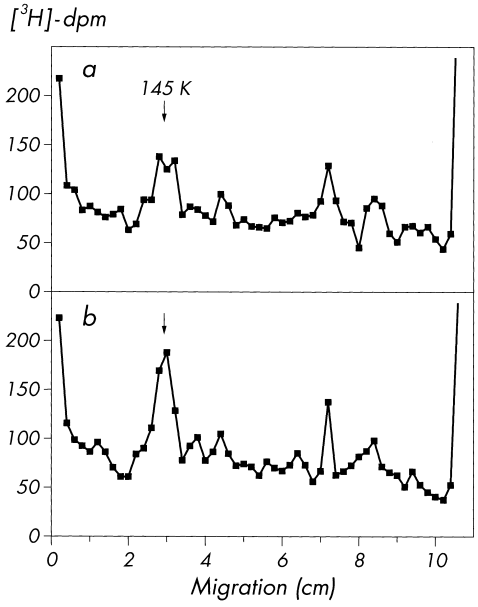


Fig. 4. Influence of cholesterol feeding on photoaffinity labeling of rabbit small intestinal BBMV by the photoreactive cholesterol absorption inhibitor [^{3}H]C-2. BBMV (200 μg of protein, 2 mg/ml) isolated from rabbits kept for 10 days on a standard diet (a) or a high cholesterol diet (b) were photolabeled with 2.93 μM [^{3}H]C-2 followed by SDS–gel electrophoresis and detection of radioactivity by liquid scintillation counting.

hamsters, dogs, rhesus monkeys and humans [25,26]. Introduction of a (3S)-hydroxy group in position R2 significantly increases the efficacy of 2-azetidinones [8] explaining the higher in vivo potency of SCH 58235 compared to SCH 48461 [8,11,12]. In vivo studies with NMRI mice revealed the higher in vivo efficacy of the (3S)-hydroxy group containing compounds S 6503 – a R3-methoxy derivative of SCH 58235 – and photoprobe C-2 compared to S 3302. Table 1 shows that the intestinal absorption of [^{14}C]cholesterol as measured by an increase in fecal excretion of [^{14}C]cholesterol was strongly inhibited by S 6503 and C-2 and moderately by S 3302. In contrast, fecal excretion of [^{3}H]sitostanol was not significantly affected indicating their specific effect on intestinal cholesterol absorption only. The radioactivity found in the livers 24 h after application as a measure for intestinal absorption of [^{14}C]cholesterol was accordingly strongly inhibited by S 6503 and C-2 and weakly by S 3302.

Photoaffinity labeling in the presence of increasing concentrations of S 6503 and S 3302 led to a specific inhibition in the extent of labeling of the 145 kDa protein whereas labeling of the other proteins was not significantly changed (Fig. 3). Unspecific effects by optical shielding can be ruled out due to the comparable extinction coefficients of both compounds. S 6503 was in accordance with its higher in vivo activity more potent compared to S 3302 to inhibit photolabeling of the 145 kDa protein. By repeated experiments with different vesicle preparations and detection of radioactivity by liquid scintillation counting and fluorography, IC_{50} values of 71 ± 18 μM for S 6503 and 129 ± 11 μM for S 3302 ($n = 4$) were determined. Fluorographic detection revealed that the labeling of the M_r 62 and 56 kDa were also inhibited by the presence of 2-azetidinones. At present it remains unclear whether these polypeptides are closely associated to the 145 kDa protein or whether the 145 kDa protein is a dimer of the 56/62 kDa proteins similar to the dimerization of the ileal Na^+/bile acid cotransporter [24]. None of the azetidinone-derived cholesterol absorption inhibitors had any influence on the intestinal transporters for bile acids, glucose, oligopeptides, alanine or fatty acids nor on photoaffinity of the ileal Na^+/bile acid cotransporter [22,24]. Vice versa, none of the substrates for these transporters influenced photoaffinity labeling of the 145 kDa protein by C-1 or C-2. In an in vitro binding assay measuring the effect of substrates on the binding of radiolabeled C-2 to intestinal BBMV, S 6503 was more potent compared to S 3302 to inhibit binding of radiolabeled C-1 or C-2 to BBMV, whereas bile acids, glucose, β-lactam antibiotics or fatty acids had no effect.

3.4. *Effect of cholesterol feeding on photoaffinity labeling of rabbit small intestinal BBMV by photoreactive cholesterol absorption inhibitors*

Repa et al. [33] demonstrated that intestinal cholesterol absorption is decreased concomitantly with an increase in the expression of the ABC-1 transporter protein which pumps cholesterol out of the enterocyte. The interrelationship of ABC-1 with the uptake system for cholesterol is unknown. We therefore investigated whether cholesterol feeding had an influence on photoaffinity labeling of BBMV with cholesterol absorption inhibitors. Rabbits are prone to the development of profound atherosclerosis and severe hyperlipidemia by cholesterol feeding [34]. Preliminary studies showed that photoaffinity labeling of BBMV obtained from rabbits ($n = 3$) treated for 10 days with a high cholesterol diet revealed a slightly higher labeling of the 145 kDa protein compared to BBMV from rabbits fed with regular chow (Fig. 4).

4. Discussion

Intestinal absorption of cholesterol is a major regulator of serum cholesterol levels and body cholesterol homeostasis. Under normal conditions 50–70% of cholesterol is absorbed by the small intestine, the absorption rate greatly varying

Table 1
Effect of 2-azetidinone cholesterol absorption inhibitors on cholesterol absorption in NMRI mice

	Control	3 mg S 6503/mouse	3 mg S 3302/mouse	3 mg C-2/mouse
Fecal excretion of [^{14}C]cholesterol	0.23 μCi	0.60 μCi (+160.8%)	0.33 μCi (+43.5%)	0.59 μCi (+156.5%)
Fecal excretion of [^{3}H]sitostanol	0.77 μCi	0.68 μCi (−11.6%)	0.75 μCi (−2.6%)	0.72 μCi (−6.5%)
Retention of [^{14}C]cholesterol in the liver	0.163 μCi	0.047 μCi (−71.2%)	0.151 μCi (−7.4%)	0.063 μCi (−61.3%)

Male NMRI mice (five animals per group) were dosed 0.5 ml of a solution of 0.5% methylcellulose/5% Solutol® without or with 3 mg of cholesterol absorption inhibitor by gavage followed by 0.25 ml of Intralipid® solution containing 0.24 μCi [^{14}C]cholesterol and 0.25 μCi [^{3}H]sitostanol. After 24 h the amount of radioactivity excreted with the feces and retained in the liver was determined. The values represent the summarized radioactivity of the five animals per treatment group. For each group two independent determinations of radioactivity in feces or liver were performed.

between 29 and 80%. Since the molecular mechanism of intestinal cholesterol absorption is unknown, a rational design of optimized cholesterol absorption inhibitors is not possible yet. Owing to the ubiquitous distribution of cholesterol in biological membranes and its physicochemical properties to incorporate into the lipid bilayer of biological membranes, photoaffinity labeling approaches with photoreactive cholesterol analogues probably will lead to unspecific intramembrane labeling of many membrane proteins making an unequivocal identification of the putative cholesterol transporter difficult and unlikely. Therefore we made use of the specificity of 2-azetidinones to inhibit intestinal cholesterol absorption and identified specific binding proteins for cholesterol absorption inhibitors by photoaffinity in small intestinal BBMV by photoaffinity labeling. An integral membrane protein of M_r 145 kDa exerts the characteristics for an intestinal cholesterol transporter:

1. Photoaffinity labeling of the integral membrane protein of M_r 145 kDa was concentration-dependently inhibited by unlabeled 2-azetidinone cholesterol absorption inhibitors.
2. The inhibitory potency of 2-azetidinone cholesterol absorption inhibitors on photoaffinity labeling of the 145 kDa protein correlated with their in vivo potency and as their potency to inhibit binding of radiolabeled C-2 to rabbit small intestinal BBMV.
3. Inhibition of labeling of the 145 kDa protein was specific for cholesterol absorption inhibitors. None of the substrates for the intestinal transporters for bile acids, glucose, alanine, β-lactam antibiotics/oligopeptides or long-chain fatty acids exerted any effect.
4. The extent of labeling of the 145 kDa protein was increased in BBMV obtained from rabbits having received a cholesterol-rich diet for 10 days.

These results strongly indicate that an integral membrane protein of M_r 145 kDa in the brush border membrane of rabbit small intestinal cells is responsible for the critical step of intestinal cholesterol absorption, the transport across the enterocyte brush border membrane. Whether this protein acts as a solute transporter translocating monomeric cholesterol across the enterocyte brush border membrane or is a receptor triggering uptake by endocytosis or other mechanisms remains to be elucidated.

Acknowledgements: The authors thank Meike Scharnagl and Vanessa Wambach for excellent secretarial assistance and preparing the manuscript.

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