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Undetectable apolipoprotein *A-I* gene expression suggests an unusual mechanism of dietary lipid mobilisation in the intestine of *Cyprinus carpio*

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Summary

High density lipoprotein (HDL) has been shown to play an important role in the dietary lipid mobilisation in the carp. In spite of this, previous studies have failed to demonstrate the synthesis of the major protein component of HDL, apolipoprotein A-I (apoA-I), in the proximal intestine of the carp. Therefore, the aim of the present study was to evaluate the expression of apoA-I throughout the entire intestine. Curiously, no transcription of the *apoA-I* gene could be detected either by northern blot or RT–PCR assays in the intestinal mucosa, in clear contrast with the abundant cytosolic immunoreactive apoA-I detected in almost all intestinal segments, which suggests a different origin for this protein. In addition, the detection of specific, but low affinity, binding sites for apoA-I in the carp intestinal brush-border membranes (BBM), and the strong interaction with BBM, which is highly dependent on temperature, points to an important contribution of membrane lipids in apoA-I binding to the intestinal mucosa. This idea was reinforced by the ability of carp apoA-I to associate with multilamellar phospholipid vesicles.

Key words: apoA-I, internalisation, lipid absorption, carp, *Cyprinus carpio.*

Introduction

In mammals, intestinal absorption of fat and its subsequent secretion from the enterocyte as chylomicrons is a multi-step process that includes the uptake of lipolytic products, re-esterification and translocation of cellular lipid pools, synthesis and post-translational modification of various apolipoproteins (Levy et al., 2000; Kumar and Mansbach, 1999). In teleost fish, intestinal absorption of fats is basically comparable to the mammalian process, however, some differences in the type, size and destiny of the lipoprotein secreted have been reported (Babin and Vernier, 1989). Export of the lipoprotein particles apparently occurs only *via* the portal route in carp, only *via* the lymph in trout, or *via* both in tench and perch (Babin and Vernier, 1989). In the carp intestine, conflicting results have been reported with respect to the type of lipoprotein particles being secreted. Particles resembling very low density lipoprotein (VLDL) have been observed by electron microscopy inside enterocytes (Noaillac-Depeyre and Gas, 1974). In contrast, Iijima and coworkers (Iijima et al., 1990a,b) showed that shortly after fatty acids have been generated by hydrolysis of triglycerides in the intestinal lumen or reesterified to triglycerides in the enterocytes, they appear mainly associated with HDL in the blood and not with chylomicrons or VLDL. These results are in agreement with the observation that HDL and its major apolipoprotein (apoA-I) are the most abundant plasma protein fractions in some teleost fish (Amthauer et al., 1989; Babin and Vernier, 1989), and also with several studies showing that HDL serves as the major transport protein for fatty acids, such as palmitic acid in fish (Metcalf et al., 1999; De Smet et al., 1998).

In mammals and birds both the liver and the intestine constitute the major sites for *apoA-I* gene expression (Lamon-Fava et al., 1992; Oku et al., 1997). In contrast, the liver has always been identified as the main tissue involved in apoA-I synthesis in teleosts, with a more variable expression in the intestine (Kondo et al., 2001; Llewellyn et al., 1998; Powell et al., 1991). In a previous study we showed the presence of apoA-I in the lumen and inside the epithelial cells of the proximal intestine, however, we were unable to detect apoA-I synthesis using tissue slices from the same intestinal region (Vera et al., 1992). In the same study, we demonstrated that after oral administration of biotinylated-HDL, labelled apoA-I was found in an intact form associated with plasma HDL, indicating the transfer of intact apoA-I from the intestinal lumen to the blood. The luminal apoA-I detected in the carp intestine could be of biliary and/or hepatic origin (Vera et al., 1992). This apparent contradiction led us to study the localisation of apoA-I protein and its gene expression along the entire intestine of the carp.

It remains to be established if luminal apoA-I detected in the carp intestine is of biliary or hepatic origin. The present study aims to characterise *apoA-I* gene expression in the intestine of carp. The localisation and potential source of apoA-I protein present in carp enterocytes is also studied.

Materials and methods

Chemicals

Chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA), unless otherwise specified. The reagents and enzymes used in molecular biology techniques were obtained from Gibco-BRL (Rockville, MA, USA), except for Taq DNA polymerase and ribonuclease inhibitor (RNasin®) that were purchased from Promega (Madison, WI, USA). The antisera against carp apoA-I was prepared in rabbit and characterised in our laboratory (Amthauer et al., 1989).

Fish

Common carp *Cyprinus carpio* L. were caught in the Cayumapu River and maintained in an outdoor tank with running river water. Fish weighing 800–1200 g were acclimatised for at least 3 weeks at 20±2°C (average summer temperature) with a photoperiod of 14 h:10 h light:dark, and fed to satiation twice every day. Fish were anaesthetised in a bath containing 50 mg l^{-1} of benzocaine and then sacrificed by decapitation. The entire intestine was removed and dissected into seven segments according to the method of Villanueva et al. (1997), and differentially processed for immunohistochemistry, RNA isolation or brush border membrane (BBM) preparation.

Immunohistochemistry

Fresh slices of carp intestine were immediately fixed in Bouin's fixative for 4 h, dehydrated and embedded in paraffin. Serial cross sections (5 μm) were deparaffinised in xylene and rehydrated. Briefly, slides were blocked for 30 min with 1% (w/v) BSA in phosphate-buffered saline (PBS) and incubated for 1 h in a wet chamber with preimmune serum or a specific rabbit polyclonal antiserum against carp apoA-I (dilution 1:5000). After several washings with PBS, slides were incubated with a 1:1000 dilution of alkaline phosphatase conjugated with goat anti-rabbit IgG antibody. Enzyme activity was detected with Nitro Blue Tetrazolium/4-bromo-5-chloro-3-indolylphosphate solution as dye substrates for alkaline phosphatase. The desired signal level was achieved after 15–20 min of incubation. Serial slides of intestine treated exactly the same way but missing the primary antiserum were used as negative controls of the reaction. Liver sections were used as positive controls.

RNA isolation, northern blot and RT–PCR analyses

Slices (1 cm long) were removed from each intestinal segment, opened longitudinally and washed in sterile saline. Epithelial cells were collected by scraping the mucosa with a sterile glass microscope slide. Total RNA was extracted using the Trizol Reagent (Gibco-BRL) according to the manufacturer's instructions. RNA was quantified by absorbance at 260 nm and used immediately or stored precipitated in ethanol at –70°C until use. Total RNA (30 μg per sample) was incubated in 1× Mops buffer, 2.2 mol l^{-1} formaldehyde and 50% deionised formamide for 10 min at 65°C, mixed with formaldehyde loading buffer [50% (v/v) glycerol, 1 mmol l^{-1} EDTA pH 8.0, 0.25% (w/v) Bromophenol Blue and 0.25% (w/v) Xylene Cyanol] and separated by electrophoresis in 1% (w/v) agarose gel containing 6% (v/v) formaldehyde. Samples were run in duplicate; one for ethidium bromide staining and confirmation of the integrity of the RNA; the other for transfer to nylon membranes (ICN Biomedicals, Inc. Costa Mesa, CA, USA). Transfer was performed according to the manufacturer's instructions and prehybridised for 3 h at 42°C in a solution containing 50% deionised formamide, 6× SSC (900 mmol l^{-1} NaCl, 90 mmol l^{-1} sodium citrate), 5× Denhardt's, 0.5% SDS and 100 μg ml^{-1} of yeast tRNA. Hybridisation was carried out in the same solution containing the ^{32}P-labelled carp apoA-I DNA insert (2×10^8 c.p.m. μg^{-1}) corresponding to a partial cDNA clone (GenBank accession number AJ308993), for 15 h at 42°C. After hybridisation, the membranes were washed twice in 2× SSC containing 0.1% SDS (65°C, 10 min) and then twice in 0.2× SSC/0.1% SDS (65°C, 20 min). Total liver RNA was used as a positive control to evaluate *apoA-I* expression by northern blot and RT–PCR.

The RT–PCR analyses were performed essentially as described by Concha et al. (2003). Briefly, liver and intestine total RNA (5 μg each), treated with amplification grade deoxyribonuclease I, were incubated with antisense primer (5′-cccttctccatctgctccctataa-3′), RNasin® (Promega), dNTP mixture (2.5 mmol l^{-1} of each nucleotide), enzyme buffer and 200 U of Superscript II reverse transcriptase (Gibco-BRL) at 42°C for 1 h. Reverse transcriptase was omitted in the negative control. After enzyme inactivation, the antisense primer and the following sense primer (5′-ctccacggctactttcagaacg-3′) were used to amplify a 428 bp target of the carp apolipoprotein *A-I* gene. The reaction mixture (50 μl) contained 1 μl of the reverse transcription reaction, 0.2 μmol l^{-1} of each primer, 200 μmol l^{-1} of each dNTP, 2.5 mmol l^{-1} $MgCl_2$ and 2 U of Taq DNA polymerase in a standard PCR buffer (10 mmol l^{-1} Tris-HCl, pH 9.0, 50 mmol l^{-1} KCl and 0.1% Triton X-100). The thermocycler (MJ Research, Hercules, CA, USA) was programmed as follows: initial denaturation (94°C, 3 min), followed by 30 cycles (94°C, 55°C and 72°C, for 1·min each) and a final extension step at 72°C for 5 min. The amplification product was then separated on 1.5% (w/v) agarose gel. As no amplification product was obtained in any of the intestinal segments, the amplification of the β-*actin* gene was used as an additional internal control to ensure the integrity of the intestinal RNA preparations. The primers, 5′-ggacctgtatgccaacactg-3′ (sense) and 5′-gtcggcgtgaagtggtaaca-3′ (antisense) that allow discrimination of amplification product derived from cDNA and gDNA were used according to the method of Sarmiento et al. (2000), essentially using the same conditions described above expect for the annealing temperature (50°C).

ApoA-I isolation and radiolabelling

Carp plasma HDL was isolated by affinity chromatography as described by Amthauer et al. (1989). After HDL delipidation with ethanol–ether (3:2 v/v) mixture at –10°C, the

apolipoproteins were purified by filtration chromatography on a Sephacryl S-200 column (1.5×90 cm) pre-equilibrated with buffer 10 mmol l^{-1} Tris–HCl, pH 8.6, 1 mmol l^{-1} EDTA, 8 mol l^{-1} urea (Amthauer et al., 1989).

ApoA-I was radiolabelled with ^{125}I using the IODO-GEN iodination reagent (Pierce, Rockford, IL, USA) according to the manufacturer's recommendations. Briefly, 50 µg of apoA-I were incubated for 15 min at 4°C in a IODO-GEN pre-coated polypropylene tube containing 0.5 mCi of $Na^{125}I$ in 100 µl of 100 mmol l^{-1} sodium phosphate buffer (pH 7.0). The reaction was stopped by addition of 200 µl of 100 mmol l^{-1} sodium phosphate buffer (pH 7.0) and the radioactive protein was separated from free iodine on Sephadex G-50, pre-equilibrated with sodium phosphate buffer containing 0.1 mg ml^{-1} BSA.

ApoA-I binding assays

Brush border membrane vesicles (BBMV) were isolated from carp intestinal mucosa and characterised before their use in the binding assays as described by Amthauer et al. (2000). Prior to the binding assays the test tubes were blocked by incubation with 1 mg ml^{-1} BSA for 1 h at 0°C to avoid unspecific binding of ^{125}I-apoA-I to the test tube wall. Binding assays were carried out at 25°C for 30 min in a final volume of 25 µl, containing 25 mmol l^{-1} Tris-HCl, pH 7.5, ^{125}I-apoA-I and 0.6 mg ml^{-1} of membrane protein. After incubation, the reaction mixture was cooled on ice and layered onto 400 µl of a sucrose cushion containing 25 mmol l^{-1} Tris-HCl, pH 7.5, and 250 mmol l^{-1} sucrose. Bound and unbound ^{125}I-apoA-I were separated by centrifugation at 14 000 ***g*** for 30 min at 4°C. The supernatant was discarded and the pellet containing ^{125}I-apoA-I bound to BBMV was quantified with a gamma counter.

Peripheral and integral membrane isolation

To obtain the peripheral membrane and integral membrane protein fractions, fresh BBMV or the pellet obtained after the binding assay were suspended in 50 µl of 50 mmol l^{-1} Na_2CO_3, pH 11.5, 10 mmol l^{-1} EDTA and incubated for 15 min on ice (Hu et al., 1997). The integral membrane protein fraction was recovered by centrifugation for 30 min at 14 000 ***g***, where the supernatant corresponds to the peripheral membrane fraction. Aliquot portions of both fractions were diluted in Laemmli's sample buffer and fractionated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Radiolabelled proteins were detected by autoradiography. For western blot analysis the proteins separated by SDS-PAGE were transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) using a semi-dry blotter unit (Amthauer et al., 2000). Membranes were blocked with 5% (w/v) non-fat dry milk in PBS/Tween-20 (0.1% v/v). ApoA-I was detected by incubation with a rabbit polyclonal antiserum against carp apoA-I (Amthauer et al., 1989) diluted 1:25 000, followed by incubation with alkaline phosphatase-conjugated antibody (Gibco-BRL) diluted 1:3000. Finally, alkaline phosphatase activity was developed by incubating the membrane at room temperature for 20 min in 0.1 mol l^{-1} Tris-HCl, pH 9.5, containing 0.1 mol l^{-1} NaCl, 5 mmol l^{-1} $MgCl_2$, 0.16 mg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate and 0.33 mg ml^{-1} Nitro Blue Tetrazolium.

Dimyristoylphosphatidylcholine multilamellar vesicles solubilisation assay

The solubilisation of dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles (mLV) by apoA-I was assayed according to the technique of Pownall et al. (1978). Essentially, 0.1 mg apoA-I was added to 1 ml of the reaction mixture containing 0.01 mol l^{-1} Tris-HCl, pH 7.4, 8.5% KBr, 0.01% sodium azide, 0.01% EDTA and DMPC mLV 0.5 mg ml^{-1}, preincubated at 24°C for 10 min. The cuvette content was mixed within 10 s by repeated aspiration and release, and vesicle solubilisation (clearance) was monitored as a decrease in absorbance at 325 nm using a Hewlett Packard Model 8453 Diode Array Spectrophotometer. Slight differences in initial absorbance for each time course were corrected by expressing all the values as a percentage of the initial absorbance at 325 nm.

Results

Immunolocalisation of apoA-I in carp intestine

Here we report the analysis of the distribution of apoA-I along the entire intestine by immunohistochemistry. As expected for a plasma protein, abundant apoA-I was immunodetected in the lamina propria, which is rich in blood vessels (Fig. 1). ApoA-I was also present in the cytoplasm of enterocytes (Fig. 1A–F), indicating that apoA-I could be synthesised in carp enterocytes along the intestine. The intestinal epithelium consists of a monolayer of columnar cells and the majority of the apoA-I was found close to the apical pole of these cells, which would suggest that it could be being secreted into the lumen or being endocytosed. Very faint intracellular apoA-I staining was observed in the last segment, whereas the surface of the intestinal folds was intensely labelled (Fig. 1G). The absence of any signal in the tissue slices incubated only with the secondary antibody ruled out the presence of active endogenous alkaline phosphatase or non-specific binding of this antibody (Fig. 1H). The identity of the immunoreactive protein was confirmed by western blotting, where a band with the same mobility as that of carp apoA-I was immunodetected in intestinal washings and BBM fractions of carp enterocytes (Figs 2B, 5A).

ApoA-I gene expression in carp intestine

No *apoA-I* transcript was detected by northern blot analysis in the intestinal segments, in clear contrast with the intense hybridisation signal observed with total liver RNA (Fig. 3A). As shown in Fig. 3B, the integrity and the amount of RNA loaded were similar in all cases. More sensitive RT–PCR analyses confirmed that *apoA-I* is not expressed in carp enterocytes (Fig. 3C). Total RNA from carp liver was used as positive control for the RT–PCR reaction as it has been demonstrated previously that *apoA-I* gene is expressed in this tissue (Concha et al., 2003). As shown in Fig. 3C, the expected

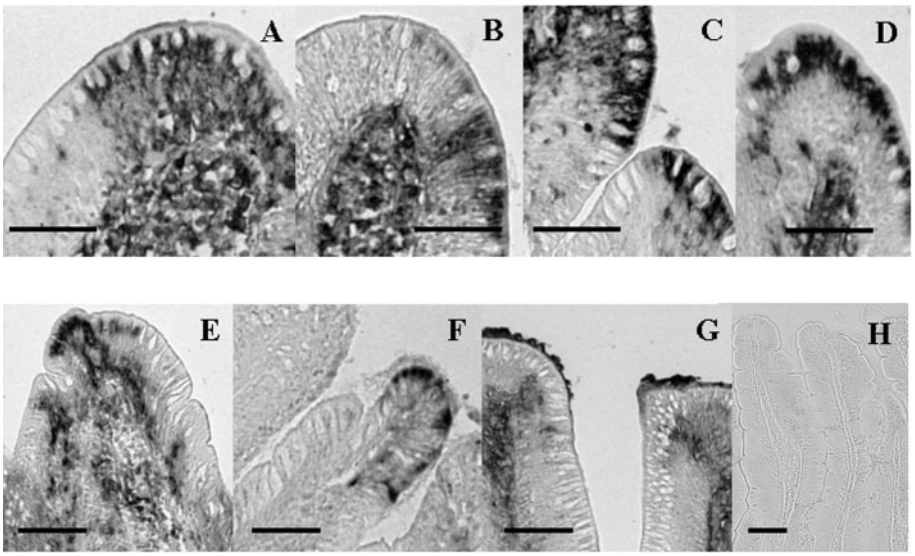

Fig. 1. Immunohistochemical localisation of apoA-I in the carp intestinal epithelium. (A–G) Thin sections (5 μm) of each of carp intestinal segments 1–7, respectively. (H) A negative control incubated only with the conjugate secondary antibody. Scale bars, 50 μm.

428 bp amplification product was found in the positive control, but no amplification was obtained in any of the intestine RNA preparations. However, a 282 bp product corresponding to the specific carp β-*actin* cDNA was obtained from the same intestinal RNA preparations (Fig. 3C). These findings are in agreement with our previous results showing no apoA-I synthesis in the carp intestine (Vera et al., 1992) and suggest that apoA-I detected in the intestinal mucosa could be of biliary and/or hepatic origin.

ApoA-I interaction with brush border membrane

The first step for the putative transepithelial transport of apoA-I should be the interaction of the protein with the brush border membrane (BBM) of the enterocytes. To test if apoA-I is transported across the epithelium by receptor-mediated endocytosis we assayed the ability of apoA-I to bind to isolated carp intestinal BBMV. As shown in Fig. 4A, in the range of

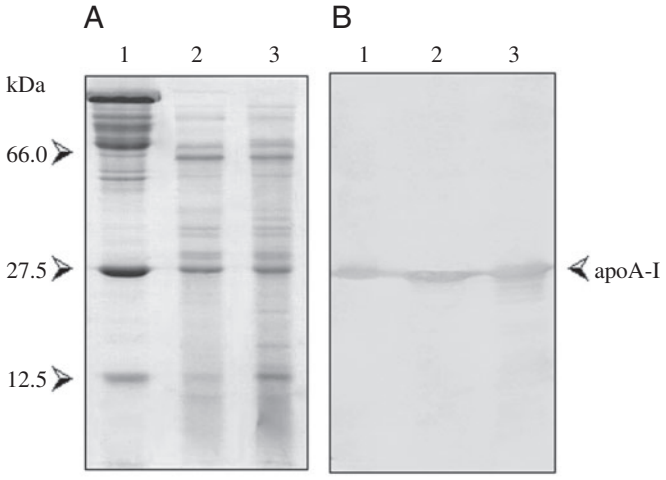

Fig. 2. Immunodetection of apoA-I in carp intestine. (A) SDS–PAGE separation of plasma proteins (lane 1) and proteins in the lumen of different intestinal samples (lanes 2, 3). (B) Western blot analysis of the gel in A. All lanes were loaded with 20 μg of proteins.

the concentrations tested, a linear increase of BBMV-bound ^{125}I-apoA-I was observed when the same amounts of BBMV were incubated with increasing apolipoprotein concentration. Addition of an excess of cold protein dramatically reduced the amount of bound ^{125}I-apoA-I showing that the binding is specific. There was no saturation at relatively high concentrations of ^{125}I-apoA-I (230 nmol l^{-1}), indicating that there are no high affinity binding sites for apoA-I in the BBM. Given that the amphipatic nature of apoA-I favours its direct interaction with lipid bilayers, the strength of the interaction of ^{125}I-apoA-I with BBM was analysed by a classical procedure designed to differentiate between peripheral and integral membrane-associated proteins. Briefly, the BBMV were washed with carbonate/EDTA buffer after incubation with ^{125}I-apoA-I as described above. After centrifugation, apoA-I was analysed by autoradiography in both supernatant and precipitate fractions. As shown in Fig. 5B, ^{125}I-apoA-I was found equally distributed in both fractions. Surprisingly, endogenous apoA-I already present in the isolated BBMV exhibited the same distribution after washing with carbonate/EDTA buffer (Fig. 5A). These results indicate that under these conditions approximately 50% of the bound apoA-I behaves like an integral membrane protein by exhibiting a strong interaction with the membrane. To confirm whether or not the apoA-I binding to BBMV is attributable to its interaction with membrane phospholipids, the binding assay was performed at different temperatures. The results shown in Fig. 4B indicate that apoA-I binding to BBMV increases almost exponentially with temperature, clearly favouring its interaction with membrane lipids rather than with specific binding sites (i.e. receptor). Accordingly, apoA-I effectively solubilised DMPC mLV, clearly indicating its capacity to interact directly with phospholipids (Fig. 6).

Discussion

In all vertebrate species analysed to date, apolipoprotein A-I, the major protein component of HDL, has been shown to be produced in both liver and intestine. However, the relative contribution of both tissues in the total apoA-I synthesis is highly variable among different species (Lamon-Fava et al., 1992). In rat, the intestine and liver contribute in similar proportion to the total apoA-I synthesis, and the intestinal apoA-I is incorporated preferentially into nascent chylomicrons

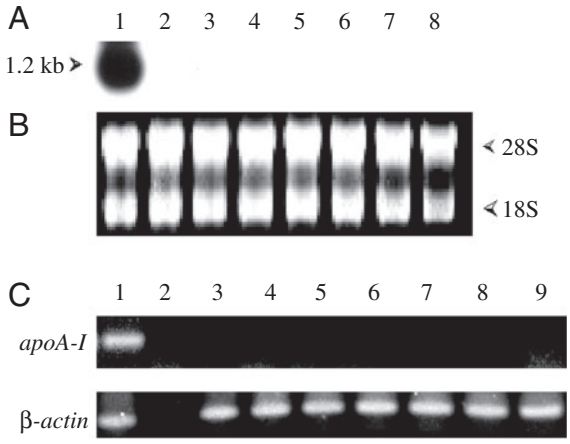

Fig. 3. *apoA-I* gene expression in carp intestine. (A) Total RNA (30 μg) extracted from liver and each of the intestinal segments were analysed by northern blot. Lane 1, liver RNA; lanes 2–8, all segments of intestine, from proximal to distal. (B) Ethidium bromide-stained total RNA (30 μg) from liver (lane 1) and all intestinal segments (lanes 2–8). (C) RT–PCR products for *apoA-I* (top) and β-*actin* (bottom) gene expression. Lanes 1, 2, liver RNA with and without reverse transcriptase, respectively; lanes 3–9, intestinal RNA from all segments.

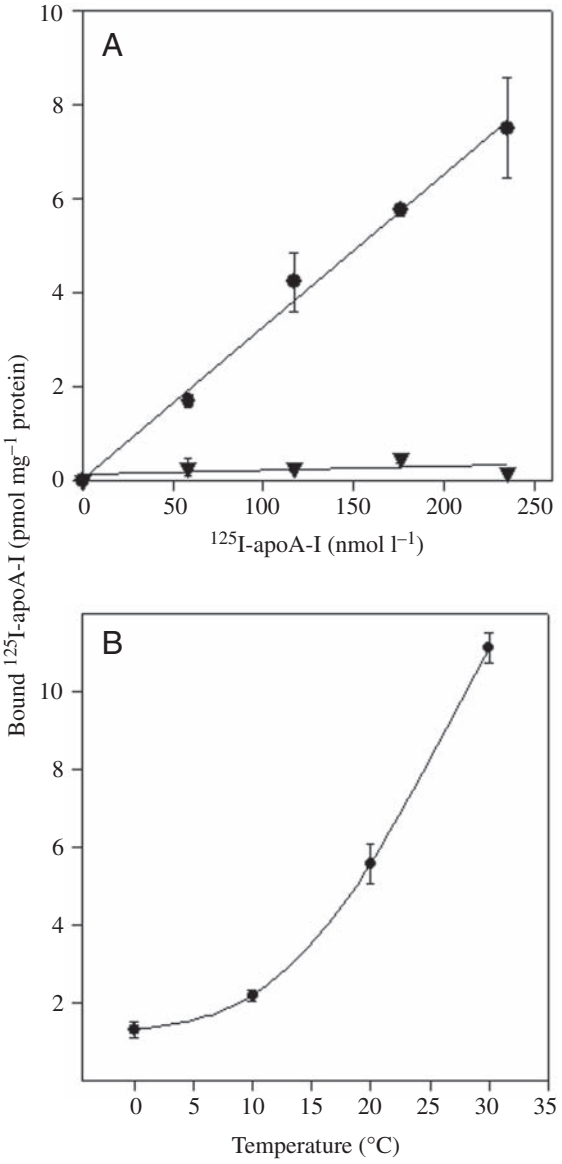

Fig. 4. Concentration and temperature effect on apoA-I binding to brush border membrane vesicles (BBMV). (A) BBMV were incubated with varying concentrations of ^{125}I-apoA-I. The amount of bound apoA-I was determined as described in Materials and methods. Total apoA-I binding (circles) and non-specific binding (triangles) was determined by adding 100 times the molar ratio of unlabelled apoA-I to the binding assay. Values are means ± S.E.M. of triplicate determinations. (B) BBMV were incubated with 200 nmol l^{-1} ^{125}I-apoA-I at different temperatures. Values are means ± S.E.M. of triplicate determinations.

(Windmueller and Wu, 1981; Wu and Windmueller, 1979). In rabbit, apoA-I is mainly synthesised in the intestine and the level of liver *apoA-I* mRNA is approximately 500-fold lower than the reported level of *apoA-I* mRNA in rat and human livers (Chao et al., 1984). In teleost fish, the relative contribution of liver and intestine in global apoA-I synthesis also varies among species. For example, similar levels of *apoA-I* are expressed in liver and intestine of Atlantic salmon (Powell et al., 1991), whereas in gilthead sea bream and Japanese eel a much weaker expression is observed in intestine (Llewelyn et al., 1998; Kondo et al., 2001). To the best of our knowledge the present study is the first to report the lack of *apoA-I* gene expression in the intestine of a vertebrate animal evaluated under physiological conditions. Importantly no differences in the expression pattern were seen between male and female fish or in fish of different sizes captured from the wild immediately prior to analysis (data not shown).

The possibility that the lack of *apoA-I* expression could be attributed to the presence of inhibitors of the reverse transcriptase in the RT–PCR reaction or degradation of the mRNAs in the preparations obtained is ruled out by the successful amplification of a housekeeping transcript (β-*actin*) in the same preparations. Likewise, it is unlikely that the probes and the primers, which were designed to be complementary to carp liver *apoA-I* cDNA (Concha et al., 2003), were inappropriate for detecting an intestinal transcript. In the rainbow trout, two slightly different apoA-I transcripts have been detected, only one of which corresponds to the major transcript in normal hepatic tissue (Delcuve et al., 1992). The other one seems to be restricted to tumoural tissues. Similarly, Japanese eel also expresses two different mRNA for apoA-I, but the only transcript present in intestine corresponds to the major liver form (Kondo et al., 2001). The present study therefore corroborates the findings of Vera et al. (1992) and strongly reinforces the view that there is complete lack of *apoA-I* expression in the carp intestine under the physiological conditions tested. Given the important role that apoA-I plays in the mobilisation of the lipids derived from the diet, this is a novel and intriguing finding.

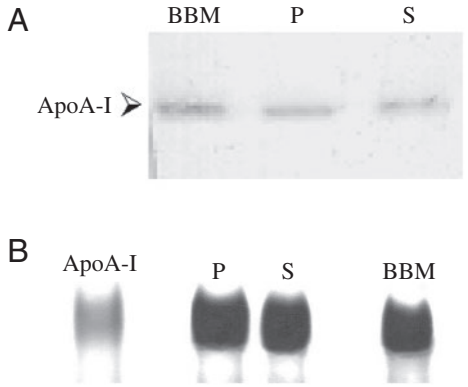

Fig. 5. Interaction of apoA-I with brush border membrane vesicles (BBMV). (A) ^{125}I-apoA-I bound to BBMV; S, washed peripheral membranes; P, integral membrane fractions. Fractions were separated on a 12% SDS polyacrylamide gel and the protein detected by autoradiography. (B) Endogenous content of apoA-I in BBMV and distribution in the peripheral membrane (S) and integral membrane (P) fractions analysed by western blot.

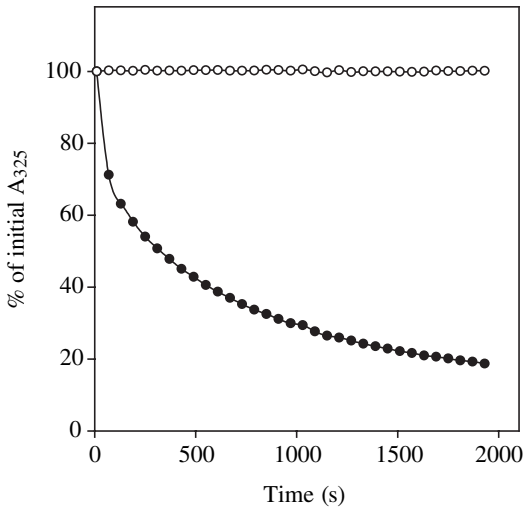

Fig. 6. Dimyristoylphosphatidylcholine multilamellar vesicle (DMPC mLV) solubilisation by apoA-I. DMPC mLV were incubated in the absence (open circles) or presence (closed circles) of apoA-I under the conditions described in Materials and methods. The solubilisation kinetics of the multilamellar vesicles was followed by measuring the decrease in absorbance at 325 nm.

Despite the evidence against *apoA-I* expression in the carp intestine presented here, we demonstrate the presence of intracellular and luminal apoA-I in all segments of this tissue. Its identity was confirmed by western blotting. As it is very unlikely that apoA-I is synthesised in the intestine, it must have originated from biliary or hepatic secretion. Some evidence for this is the presence of apoA-I in the carp bile (Vera et al., 1992) and also vesical mucosa (data not shown). The biliary system of carp liver has an unusual structure: the bile canaliculus is formed by deep invagination of the cell membrane of one hepatocyte (Kalashnikova and Kazanskaia, 1986). This study suggests that, in periportal hepatocytes, newly synthesised proteins could be secreted both to blood capillaries and to bile canaliculus. Thus, it is possible that apoA-I could reach the intestinal lumen through the bile during the lipid absorption process.

The presence of intracellular apoA-I in carp enterocytes in the absence of local synthesis seen in the present study, and the transepithelial transport of intact apoA-I previously reported (Vera et al., 1992), may be explained by the existence of specific binding sites for carp apoA-I in BBM. According to results presented here, these sites would not correspond to high affinity receptors as no saturation was reached at relatively high concentrations (230 nmol l^{-1}) of the ligand apoA-I. Not withstanding, these sites could be low affinity receptors, e.g. scavenger receptor class B type I (SR-BI), which display a K_d in the micromolar range for free and HDL-associated apoA-I (Schulthess et al., 2000). In mammals, the SR-BI receptor participates in selective sterol and phospholipid uptake from the donor particle HDL in intestinal BBM (Werder et al., 2001). In the present study, specific antiserum against the murine SR-BI, failed to detect a protein of molecular mass in the range of monomeric mammalian SR-BI (≈80 kDa), although two faint bands of >150 kDa were seen after western blotting of carp BBM proteins (data not shown). Therefore, the presence of this protein in carp enterocytes cannot be ruled out. It seems that apoA-I or HDL binding to SR-BI would be involved in cholesterol and phospholipids transfer but not in apoA-I endocytosis/transcytosis (Werder et al., 2001). Internalisation of a wide variety of intact proteins across the intestinal epithelium has been demonstrated for several teleost fish (Moriyama et al., 1990; Hertz et al., 1992; Vera et al., 1992). In the carp, a specific receptor protein for the endocytosis tracer horseradish peroxidase has been identified by ligand blotting (Amthauer et al., 2000; Concha et al., 2002) but no specific protein could be identified as a putative receptor for apoA-I, utilising a similar approach (data not shown).

Surprisingly, the present study revealed unusually strong interaction of an important fraction of labelled apoA-I (≈50% of the BBMV-bound apoA-I) that behaves like an integral membrane protein. Human apoA-I has a central domain capable of penetrating the bilayer of phospholipids vesicles (Córsico et al., 2001). This indicates that apoA-I could interact directly and strongly with membranes through protein insertion in the bilayer. In the present study, we demonstrated a similar behaviour for carp apoA-I through its ability to solubilise DMPC mLV. Also a dramatic increase of apoA-I binding to BBMV was found at temperatures above 10°C. The amount of bound apoA-I more than doubled with each 10°C increment between 10 and 30°C. The insertion process of a protein into a membrane bilayer is very temperature dependent, and occurs only above a threshold temperature (Meijberg and Booth, 2002). Below 20°C membrane lipids are essentially in a highly ordered, gel-like, phase of low fluidity, whereas at higher temperatures they are in a liquid crystal (‘fluid’) phase (Mamdouh et al., 1996). Observations in carp favour the notion that apoA-I binding to BBM is dependent on interaction with lipids rather than to specific receptor proteins.

In summary, the above results demonstrate that in spite of the important role proposed for HDL in the early mobilisation of dietary lipids (free fatty acids and triglycerides) in the carp intestine (Iijima et al., 1990a,b) and the abundant apoA-I immunodetected in carp enterocytes, no local expression of the apoA-I gene could be detected. These apparently contradictory results could reflect the existence of a unique recycling system in which an important fraction of the apoA-I synthesised by the liver and/or biliary system is subsequently released to the bile during the fat absorption process and later on internalised by endocytosis in the enterocytes. Although we could not detect high affinity binding sites for free apoA-I in intestinal BBMV, the unusually strong interaction of apoA-I with BBMV suggests the possibility of a constitutive endocytosis pathway rather than a receptor-mediated process.

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