

Expression of receptors for native and chemically modified low-density lipoproteins in brain microvessels

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Abstract Despite the importance of cholesterol metabolism in the central nervous system, only relatively few studies have dealt with the cerebral uptake and transport of lipids into the brain compartment. These functions are mediated by the endothelium of brain microvessels, which forms the anatomical basis of the blood-brain barrier. By a reverse transcriptase PCR study of messenger RNA expression we could show, in bovine brain microvessels, the presence of transcripts of native low-density lipoprotein receptor and of both type I and II scavenger receptors. Brain microvessels therefore appear to play an active role in the uptake of native and modified low-density lipoproteins.

Key words: Scavenger receptor; Low-density lipoprotein receptor; Blood-brain barrier; Chemically modified lipoprotein; Cholesterol metabolism; Atherosclerosis

1. Introduction

The low-density lipoprotein receptor (LDL-R) mediates the recognition and cellular uptake of plasma low-density lipoprotein (LDL). However, the LDL-R, which binds both apoprotein B-100 and apoprotein E, fails to recognize these apoproteins after their chemical modification, namely after their transformation into acetylated LDL (AcLDL) or oxidized LDL (OxLDL). These chemically modified LDLs are instead recognized by separate scavenger pathway(s), mediated by scavenger receptors (SCAV-Rs), which can also bind a broad but limited group of highly or abnormally negatively charged macromolecules and appear, from *in vitro* and *in vivo* studies, to be involved in the development of early atherosclerotic lesions (for review see: [1–4]).

The SCAV-R bovine sequences have been cloned and characterized as two distinct although closely related (type I and II) cDNAs [5,6]. The data on SCAV-Rs are, however, still incomplete in terms of tissue specificity and of developmental appearance. SCAV-Rs have been found, mainly by ligand

blotting and/or by ¹²⁵I-labeled or fluorescent modified LDL uptake, to be present in macrophages [5–7], in various kinds of vascular endothelial cells (Ecs) [5,6,8–10], in fibroblasts and in smooth muscle cells (SMCs) [11,12]. Their presence has been reported in brain by some authors [13] but not by others [5].

Despite the importance of cholesterol metabolism in central nervous system (CNS), only a few studies have dealt with the cerebral uptake and transport of lipids into the brain. These functions are mediated by the endothelium of brain microvessels, whose morphological and functional characteristics differ from those of peripheral endothelia. The presence of tight junctions between the Ecs of brain microvessels and the low rate of pinocytosis and vesicular transport showed by these cells appear to be in fact the structural basis for its identification with the blood-brain barrier (BBB), which prevents passive diffusion of compounds from the blood into the brain and contributes to the maintenance of the homeostasis of brain interstitial fluid [14,15]. In particular, despite some evidence for the presence of LDL-R on the Ecs of brain capillaries [16], little is known about the presence of SCAV-Rs, and the conclusions appear uncertain.

Thus, while it has been reported that modified LDLs are not taken up *in vivo* by brain microvessels [8], very conflicting results have been obtained *in vitro*. In particular, although cultured cells from major cerebral arteries have been shown to be able to take up AcLDL [17], only a few authors [10,18,19] have demonstrated AcLDL uptake by microvascular Ecs isolated from bovine or rat brain, in contrast with others who failed to demonstrate such an activity on AcLDL and even on unmodified LDL [9]. Even those authors who have reported specific uptake of AcLDL by bovine cerebral Ecs *in vitro* have omitted to describe any sub-type distinction or data concerning the preferential transcription of these receptors. More recent studies [20] have shown, on the other hand, that the existence of a binding site for AcLDL, on cerebral Ecs *in vitro*, is not efficiently coupled to a degradative pathway.

The present paper reports the expression of SCAV-Rs type I and II as well as of LDL-R in bovine brain microvessels, assessed by a reverse transcriptase PCR (RT-PCR) assay, indicating the presence of different LDL uptake systems, *in vivo*, at the BBB level.

2. Materials and methods

2.1. Materials

Random hexamers and oligo-d(T)₁₆ were obtained from Perkin

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Abbreviations: AcLDL, acetylated low-density lipoprotein; BBB, blood-brain barrier; CNS, central nervous system; Ecs, endothelial cells; LDL, low-density lipoprotein; LDL-R, low-density lipoprotein receptor; OxLDL, oxidized low-density lipoprotein; RT, reverse transcriptase; SCAV-R, scavenger receptor; SMCs, smooth muscle cells

Elmer Cetus Co. Super RT (AMV reverse transcriptase), Super Taq (*Thermus thermophilus* DNA polymerase), human placental ribonuclease inhibitor, buffer for reverse transcription, buffer for PCR and Φ XI/HaeIII marker were obtained from HT Biotechnology Ltd. Oligonucleotides used as primers were synthesized by PRIMM. The restriction enzyme *Hph*I was obtained from New England Biolabs. All other chemicals were obtained from Sigma Chemical Co.

2.2. Isolation and purity assay of brain microvessels

All glassware used for transport or treatment of bovine brains was previously sterilized at 180°C for 8 h; whenever possible, disposable plasticware was used. All manipulations were performed with gloves and in ice. Fresh bovine brains were obtained from the local slaughterhouse and immediately transported to the laboratory. Meninges were removed, and only the cortex was used. The capillaries were isolated using a previously described method [21,22], which produced preparations without significant contamination by perivascular cells. However, in order to reach the highest level of preparation purity and to exclude definitively any eventually remaining contamination by perivascular microglia, astrocytes or pericytes, the microvessel preparations were treated, after the mechanical isolation procedure, with crude collagenase at 500 or 5000 U/mg of microvessel protein as described [22,23]. On small fractions of every preparation, alkaline phosphatase (EC 3.1.3.1) and γ -glutamyltranspeptidase (EC 2.3.2.2) were routinely assayed, as a test for purity and reproducibility, using the methods of Torack and Barnett [24] and of Orlowski and Meister [25], respectively. The microvessel preparations were enriched in γ -glutamyltranspeptidase and in alkaline phosphatase with respect to gray matter (Table 1), according to previous data [21,26]. These enzymatic activities were decreased only partially following treatment with crude collagenase at 500 U/mg of microvessel protein; even when the isolated microvessels were treated with a 10-fold higher concentration of collagenase (5000 U/mg of microvessel protein) or subjected to longer incubation (data not shown), there were no further modifications of the enzymatic activities tested (Table 1 and [22]). This confirmed the close association existing between γ -glutamyltranspeptidase and alkaline phosphatase with the brain Ecs, as reported previously [20,22,23], and demonstrated that the residual activity was unlikely to be due to contamination by other cells but is characteristic of Ecs of brain microvessels. Collagenase treatment at 500 U/mg and phase-contrast light microscopy observation were performed as routine steps in this work for sample purity achievement and assessment.

The isolated brain microvessels to be used for total RNA extraction were then collected in sterile Petri dishes without buffer and stored at -80°C .

2.3. DNA extraction, RNA extraction and cDNA synthesis

DNA extraction, from bovine blood, was performed using a standard procedure [27], i.e. by lysis with 0.5% SDS and subsequent treatment with proteinase K (100 $\mu\text{g}/\text{ml}$), followed by phenol extractions and ethanol precipitation. 1 μg of DNA was used for subsequent PCR.

Total RNA extraction, from isolated brain microvessels, was performed by the acid guanidinium/thiocyanate/phenol-chloroform procedure [28]. Quantitation and purity of preparations were assessed by reading the 260/280 nm absorbances, while RNA integrity was checked according to standard electrophoretic procedures [27]. Reverse transcription was performed with 1 μg of total RNA, by using either 150 pmol of random hexamers or 50 pmol of oligo-d(T)₁₆ per sample, with 25 units per sample of Super RT at 42°C for 1 h, followed by heat inactivation at 94°C for 5 min. Total reaction volume was of 20 μl per sample, in the following assay buffer: 50 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM DTT, 10 mM MgCl₂, human placental ribonuclease inhibitor 20 units per sample, 200 μM each of dGTP, dATP, dCTP and dTTP. In subsequent amplification reactions, 7 μl of this volume were used for each PCR sample.

2.4. PCR strategy and restriction analysis

Starting from a cDNA mixture obtained by reverse transcription of brain microvessels mRNAs, the bovine LDL-R cDNA [29,30] was amplified by using BTLDL19 (5'-TGCACTCCATCTCCAGCATC-G-3') as forward primer and BTLDL3 (5'-GAGGCAGCTTCTCATGTCCTTGG-3') as backward primer (Fig. 1A); an amplified fragment of 392 bp was obtained. For amplification of bovine SCAV-R cDNAs, we chose a couple of primers allowing amplification of the common part of SCAV-Rs type I [5] and type II [6], namely

BTSCAVRP2 (5'-TGATGGAACGCATGAGCAACATGG-3') as forward primer and BTSCAVRM2 (5'-TGGTCCTGGGAATCCTCGAATCC-3') as backward primer (Fig. 1B). This couple of primers produced an amplified fragment of 626 bp, which could originate from the presence, in the total RNA preparation, of type I and/or type II mRNA of SCAV-R(s), as this couple of primers anneal at the ends of the common part of the cDNA sequence of these receptors. In order to distinguish between the two types of SCAV-Rs, we also performed amplifications using the same previously described forward primer BTSCAVRP2 coupled either with the backward primer BTSCAVRM3 (5'-CATCTTCGTCGTGCGAACAGGC-3'), which is specific for the cysteine-rich domain VI of type I (producing a 1002 bp amplified fragment), or with the alternative backward primer BTSCAVRM2B (5'-TCGGTTTGGAGGAGCCACAAGG-3'), which is specific for the 3'-UTR of type II (producing a 864 bp amplified fragment) (Fig. 1B). With the aim of assessing the specificity of amplified fragments both of LDL-R and of SCAV-R(s) and of studying the synonymous replacement of T by C at position 750 in type II, we used the restriction enzyme *Hph*I which is expected to produce different restriction patterns in the cDNAs of the type I and II receptors (Fig. 1B). In this regard, digestions were carried out on 5 μl of amplified PCR products, in a final volume of 50 μl , with 15 units of enzyme for 3 h at 37°C, using the buffer provided by the manufacturer. Restriction analysis was performed on the amplified fragments or, eventually, on re-amplified fragments purified from the gel and recovered with Amicon filters (Micropure 0.22+Microcon 50). As a positive control of retrotranscription and amplification we used γ ACT1 (5'-TTCCAGCAGATGTGGATCAG-3') as forward primer and γ ACT2 (5'-AGCCGCATATACTAGGGGT-3') as backward primer, which amplify the γ -actin bovine mRNA producing an amplified fragment of 342 bp [31]. In all reactions we used 40 pmol of each primer and 1 unit of Super Taq DNA polymerase. PCR was performed in a final volume of 50 μl in 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.01% gelatin, 200 μM each of dGTP, dATP, dCTP and dTTP. After an initial denaturation step of 3 min at 94°C, we performed, on a Perkin Elmer Cetus Thermal Cycler model 480, 30 cycles of 1 min at 94°C, 1 min at 60°C, 1.5 min at 72°C, followed by 7 min at 72°C.

2.5. Gel electrophoresis and size assignment

Electrophoresis was performed in 1.8% (w/v) agarose gel in 1 \times TBE (90 mM Tris-borate, 1 mM EDTA pH 8.0) with 0.5 $\mu\text{g}/\text{ml}$ of ethidium bromide, loading on each lane 15 μl from the PCR reaction (out of 50 μl), supplemented with 5 μl of loading buffer (0.1% Orange G, 20% (w/v) Ficoll, 10 mM EDTA pH 7.2). All gels were photographed with a Polaroid MP4 Land Camera using Polaroid 667 films. These films were scanned by with a Kodak Megaplug camera (model 1.4) and images acquired by a Bio Image (Millipore) computerized densitometer. This procedure allowed both confirmation of the size of the amplified products of PCR, and characterization of the restriction analysis fragments, by comparison with standard marker Φ XI/HaeIII. Original images were exported from Bio Image in TIFF format, imported into Corel Draw software (Corel Corp.), labeled and printed with a Scitex Inkjet printer (model Iris 3047).

3. Results

By means of cDNA templates synthesized by reverse transcription from total RNA of bovine brain microvessels, followed by PCR performed with the primer pair BTLDLB19 and BTLDL3, we detected an amplified product of 392 bp corresponding to the LDL-R mRNA (Fig. 2, lane 5). RT-PCR performed using the primers BTSCAVRP2 and BTSCAVRM2 showed a band of 626 bp which originated from the presence, in the total RNA preparation of bovine brain microvessels, of type I and/or type II mRNA of SCAV-R(s) for modified LDLs, this pair of primers having been chosen because they anneal at the ends of the common part of the cDNA sequence of these receptors (Fig. 2, lane 8). A PCR product of 342 bp was obtained, as expected, using primers γ ACT1 and γ ACT2 which correspond to the γ -actin

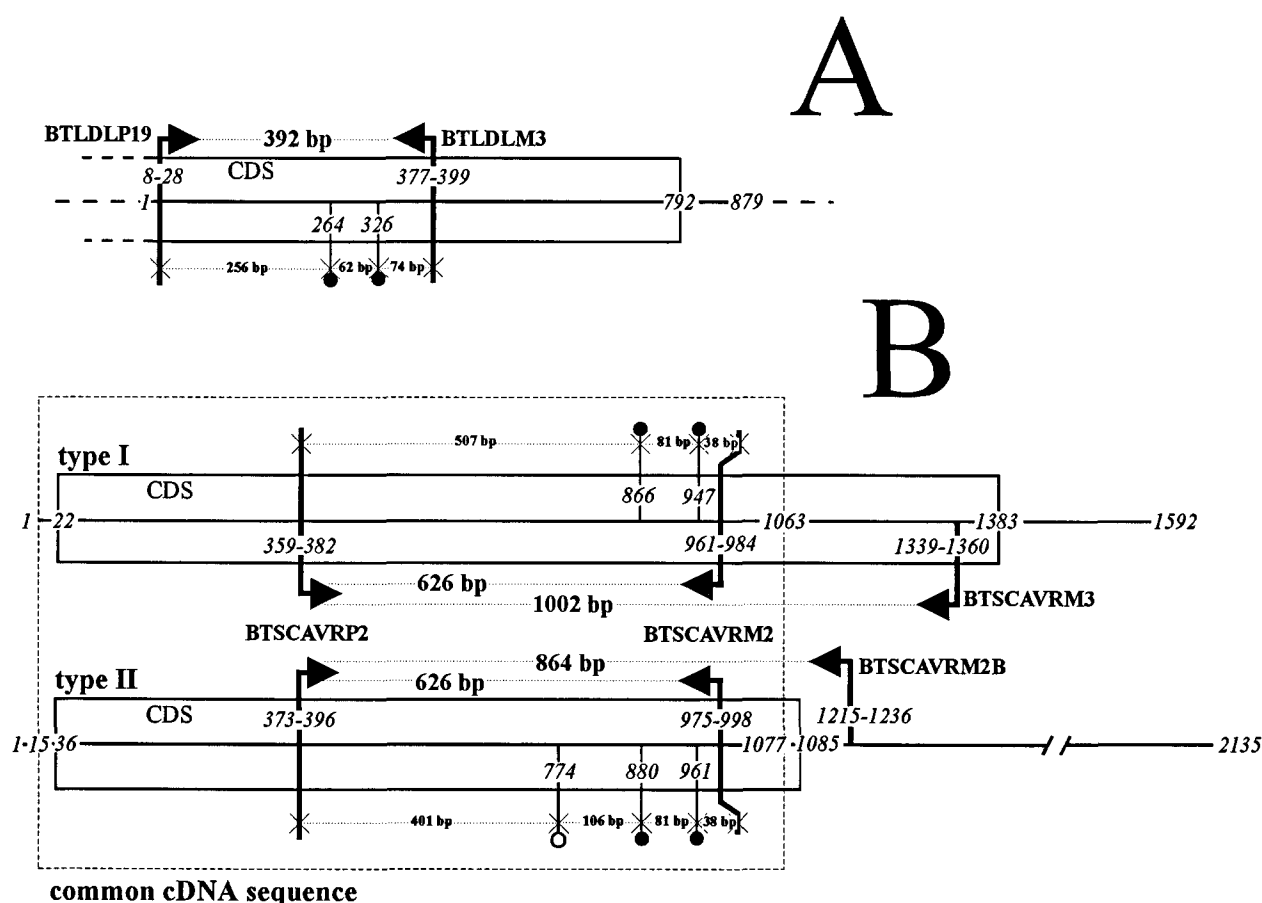


Fig. 1. Structures of LDL-R and of SCAV-Rs and choice of suitable PCR primers. (A) Structure of the 3' half cDNA of bovine LDL-R, according to Russell et al. [30]. (B) Structure of bovine SCAV-R cDNAs according to Kodama et al. [5] for type I and to Rohrer et al. [6] for type II. The solid lines represent the coding domain sequence (CDS). The dashed box indicates the common sequence between types I and II. Italic numbers denote the nucleotide positions. The location of primers used for PCR is shown by bold arrows, near the indication of the nucleotide range of each primer. Filled circles indicate *HphI* restriction enzyme sites relative to the regions amplified by BTLDDL19 and BTLDDL3, or BTSCAVRP2 and BTSCAVRM2 couples of primers; the open circle indicates the hypothetical additional restriction site for *HphI* originating from the synonymous replacement of T by C in type II SCAV-R; larger bold numbers are dimensions of the amplified fragments; smaller bold numbers denote dimensions of *HphI* expected restriction fragments from amplified fragments.

housekeeping gene (Fig. 2, lane 2). Collagenase treatment failed to affect the presence or abundance of the amplified fragment of the LDL-R (Fig. 2, lane 6), of the SCAV-R(s) (Fig. 2, lane 9), as well as of the γ -actin gene (Fig. 2, lane 3).

Similar patterns of amplified products, relative to the LDL-R and to the SCAV-R(s), were obtained when RT was performed by the use of either oligo(dT) or random hexamers (Fig. 3A, lanes 2,3,7,8). Using genomic DNA as template in PCR, the pair of primers BTLDDL19 and BTLDDL3 amplified a band of higher molecular weight (about 920 bp) than using the RNA template in RT-PCR (Fig. 3A, lane 6). This differential amplification (between cDNA and genomic DNA) enabled us to overcome the problem of genomic DNA inter-

ference in RT-PCR. In addition, PCR performed on genomic DNA, using the primers BTSCAVRP2 and BTSCAVRM2, failed to produce any amplified fragment (Fig. 3A, lane 11).

With the aim of distinguishing between the two types of SCAV-Rs, we also performed selective amplifications using either the couple of primers BTSCAVRP2 and BTSCAVRM3, which produced an amplified fragment of 1002 bp specific for type I (Fig. 3B, lane 2), or the couple of primers BTSCAVRP2 and BTSCAVRM2B which produced an amplified fragment of 864 bp specific for type II (Fig. 3B, lane 4). We could conclude that both type I and II SCAV-R mRNA are present in bovine brain microvessels, since both amplified products could be obtained.

Table 1
Enzymatic activities in gray matter and in isolated brain microvessels after collagenase treatment

Enzyme	Gray matter	Isolated microvessels collagenase treatment (U/mg of microvessel protein)		
		None	500	5000
Alkaline phosphatase	18 \pm 7 (8)	182 \pm 39 (7)	142 \pm 8 (7)	138 \pm 10 (6)
γ -Glutamyltranspeptidase	10 \pm 0.5 (7)	2148 \pm 410 (6)	1325 \pm 57 (7)	1350 \pm 40 (7)

The enzymatic activities were determined on the gray matter homogenate and on isolated microvessels treated, after the isolation procedure, with different collagenase concentrations. The enzyme levels were determined in triplicate for each preparation (the number of preparations is reported in parentheses). The mean values (\pm S.D.) of enzymatic activities are reported as mU/mg protein per min.

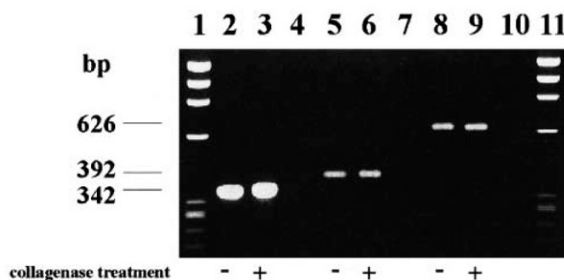


Fig. 2. RT-PCR amplified products from bovine brain microvessels before (–) and after (+) collagenase treatment (500 U/mg of microvessel protein). (Lanes 2,3) Amplified products obtained using primers γ ACT1 and γ ACT2, respectively, specific for bovine γ -actin sequence; (lanes 5,6) amplified products obtained using primers BTLDLP19 and BTLDLM3, specific for bovine LDL-R sequence, respectively; (lanes 8,9) products obtained using primers BTSCAVRP2 and BTSCAVRM2, specific for the cDNA common portion of bovine SCAV-R types I and II, respectively; (lanes 4,7,10) respective negative controls. The amplified products shown were obtained by PCR performed on cDNAs synthesized by oligo(dT) reverse transcription of total RNA. A Φ X/HaeIII marker is shown in lanes 1 and 11.

The specificity of all RT-PCR products was evaluated by digestion with the *Hph*I restriction endonuclease which, in addition, recognizes a T→C transition at nucleotide 750 in the SCAV-R(s) cDNA. This substitution was initially considered type II specific [6], further evidence suggesting however the possibility of polymorphism of a single gene transcript [11,32]. *Hph*I digestion of the 626 bp amplified fragment, obtained with the BTSCAVRP2 and BTSCAVRM2 primers, produced three fragments of 507, 81 and 38 bp, as expected from the presence of a T at nucleotide 750 (Fig. 4). In fact, the presence of a C at nucleotide 750 should produce an additional cut at location 401 (Fig. 1B), the 507 bp band therefore being substituted by two bands of 401 and 106 bp. This result, together with the specific amplification of type II SCAV-R, obtained using primers BTSCAVRP2 and BTSCAVRM2B, confirmed that the synonymous replacement of T by C at position 750 is most probably an allelic variation rather than a sequence-specific feature of type II SCAV-R. This finding agrees with previous evidence of a common gene coding for both type I and II human SCAV-Rs [33].

4. Discussion

We have demonstrated the presence, in bovine brain microvessels *in vivo*, of mRNAs for LDL-R as well as for type I and II SCAV-Rs. Our findings agree with previous evidence on the activity, in the brain microvasculature, of enzymes of lipoprotein metabolism [34], and demonstrate the presence, in CNS, of complete systems for lipid uptake and for *de novo* synthesis.

Contrasting data had thus far been collected concerning the expression, in total brain as well as in cerebral microvessels, of receptorial systems involved in recognition of native or modified LDL. In particular, the presence of SCAV-Rs in total brain had been detected in bovine brain by some authors [13] but not by others, and Northern blot analysis performed with specific probes for SCAV-Rs type I [5] or type II [6] had failed to show any expression in bovine brain. The discrepancy between these negative results in total brain and our positive findings in cerebral microvessels can reasonably be ascribed

to quantitative differences in the relative amounts of mRNA for SCAV-Rs, as well as to the higher sensitivity of RT-PCR with respect to Northern blot analysis. In addition to quantitative effects, discrepancies in SCAV-Rs detection could also be due to the necessity of other concomitant patterns for the expression and/or for the functionality of these mRNAs. Several differences between Ecs of brain capillaries vs. other blood vessels have indeed been attributed to the specific environment leading to the formation of a BBB [35]. Interactions of brain Ecs with other cell types (e.g. astrocytes [36]) have been reported to affect the expression of both LDL-R and SCAV-Rs. Indeed, in confluent brain capillary Ecs, the LDL-R escapes the down-regulation mechanisms occurring in vascular Ecs of large vessels [16,36]. The expression of LDL-R and of SCAV-Rs is therefore likely to be modulated by cellular interaction occurring in brain microvessels, with possible variations in this interaction according to the experimental design, particularly *in vitro*.

Our data show that the uptake of both native and modified LDL by cerebral microvessels is likely to play a physiological role in the local clearance of native or modified lipoproteins from the circulation and in the lipid supply to the CNS compartment. The expression of SCAV-Rs, the LDL-R one (specifically in brain Ecs), and the subsequent internalization of native or modified LDLs are not down-regulated by high levels of intracellular cholesterol. Due to this escape from the common regulatory feedback, it may be speculated that the expression of these receptors can mediate an increase in the local concentration of LDL-carried cholesterol and in its availability to the other cells involved in pathological atherosclerotic processes. In cerebral microvascular Ecs, which reportedly behave differently from other Ecs under conditions of high cell-cell contacts, the relative fluxes of cholesterol through the native or modified LDL uptake pathways are likely to depend on the equilibrium between the different

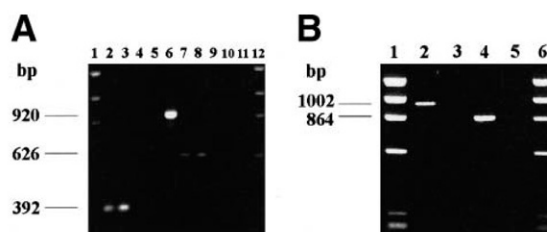


Fig. 3. Expression of LDL-R and of type I and II SCAV-Rs in brain microvessels. (A) Amplified products obtained by PCR performed on cDNAs synthesized from total RNA by reverse transcription with either oligo(dT) (lanes 2,7) or with random hexamers (lanes 3,8). As a check for DNA contamination, PCR was also performed from total RNA without the reverse transcription step (lanes 5,10). As a check for possible genomic DNA amplification, PCR was also performed on genomic DNA from bovine blood (lanes 6,11). (Lanes 2–6) Results obtained using primers BTLDLP19 and BTLDLM3, specific for bovine LDL-R. (Lanes 7–11) Results obtained using primers BTSCAVRP2 and BTSCAVRM2, specific for the cDNA common portion of bovine SCAV-R types I and II. (Lanes 4,9) Respective negative controls. A Φ X/HaeIII marker is shown in lanes 1 and 12. (B) Amplified products obtained by reverse transcription with oligo(dT) (Lane 2 (and 3 as negative control)) Results obtained using primers BTSCAVRP2 and BTSCAVRM3, specific for the bovine SCAV-R type I. (Lane 4 (and 5 as negative control)) Results obtained using primers BTSCAVRP2 and BTSCAVRM2B, specific for the bovine SCAV-R type II. A Φ X/HaeIII marker is shown in lanes 1 and 6.

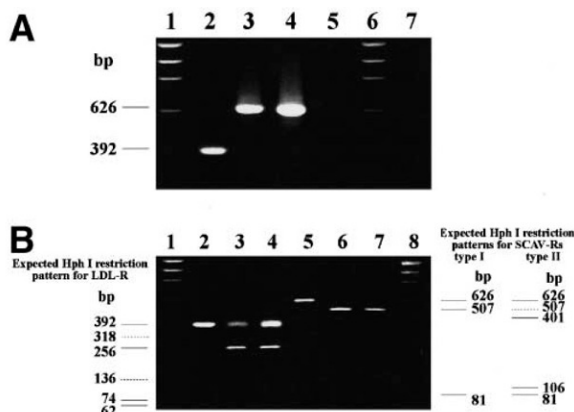


Fig. 4. *HphI* restriction analysis. (A) Purified amplification products relative either to the 3'-half of bovine LDL-R, obtained using primers BTLDL19 and BTLDL3 (lane 2) or to the cDNA common portion of bovine SCAV-Rs, obtained using primers BTSCAVRP2 and BTSCAVRM2 (lane 3,4); (lanes 5,6) respective negative controls; (lanes 1,6) Φ X/*HaeIII* marker. (B) Restriction analysis performed with *HphI* enzyme on the purified products shown in (A). The observed restriction patterns are reported for the amplified product relative to LDL-R (lanes 3,4), with the expected restriction pattern shown on the left and for the amplified product relative to the SCAV-Rs (lanes 6,7), with the expected restriction patterns for either type I or II shown on the right. Solid lines indicate the expected products of a complete digestion, dashed lines the expected products of partial digestion. The undigested amplification products relative to the LDL-R (lane 2) and to the SCAV-Rs (lane 5) are also reported as references. A Φ X/*HaeIII* marker is shown in lanes 1 and 8.

down- and up-regulating stimuli acting on the expression of LDL-R and SCAV-Rs, which in vivo remain to be assessed. Although the uncontrolled cellular uptake of cholesterol is likely to increase the risk of atherosclerosis, indicating a crucial role for LDL-R and SCAV-Rs in brain, the exact nature of involvement of these receptors remains to be established. A previous work [20], demonstrated by 125 I-AcLDL binding, the uptake of modified LDL by cerebral Ecs in vitro, which was not efficiently coupled with intracellular degradation. The authors hypothesized that the low degradation rate of AcLDL could be the reflection of a possible transcellular route of the internalized ligands for SCAV-Rs. This hypothesis, although yet to be directly verified, would raise the possibility of lipoprotein permeation through BBB, with pericytes assuming the task of binding native or modified LDL released from brain microvessel Ecs.

Our results demonstrate that a complete receptorial system for lipid uptake is present in BBB and strongly suggest that the scavenger pathway plays a central role also in this vascular district. This peculiarity can hypothetically be utilized for modified LDL-mediated drug-targeting [9] to this particular endothelium, e.g. by inserting inhibitors of cholesterol biosynthesis into modified LDLs or in suitably modified liposomes [37].

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