The expression of native and oxidized LDL receptors in brain microvessels is specifically enhanced by astrocytes-derived soluble factor(s)

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Received 27 March 2002; revised 15 May 2002; accepted 15 May 2002

First published online 27 May 2002

Edited by Guido Tettamanti

Abstract Ex vivo rat brain microvessels express receptors for native as well as for oxidized low-density lipoproteins. In brain microvessels-derived endothelial cells, the expression levels of both receptors were enhanced by co-cultivation with rat astrocytes, even in the absence of actual contact between the two cell types, suggesting a soluble factor(s)-based mechanism of induction. No modulation effect could be evidenced in a heterologous cellular system. Since both receptors were found to be expressed also in astrocytes, these cells are likely to contribute substantially to the lipoprotein management at the blood–brain barrier and in the brain compartment.

Key words: Astrocytes co-culture; Blood–brain barrier; Brain microvessels; Endothelial cells; Lipoprotein receptors; Oxidized lipoproteins

1. Introduction

The importance of the superfamily of low-density lipoprotein (LDL) receptors [1–4] in the cerebral compartment has been emphasized by the evidence, in several neurodegenerative disorders, of an altered lipoprotein(s) metabolism [5–7]. Although most investigations have focused on the metabolic pathways within the brain compartment, the endothelial lining of brain microvessels, which is the anatomic and functional equivalent of the blood–brain barrier (BBB) and thus regulates the uptake of nutrients and of specific macromolecules from the general circulation [8,9], is likely to be somehow involved in such metabolic derangement(s).

A specific receptor for native LDL (LDL-R) has been shown, both at the protein [10] and at the mRNA levels [11], to occur in brain endothelial cells. As in other tissues, LDL-R is unable to bind modified lipoproteins, for which there are instead, in several cell types including brain microvessels, appropriate 'scavenger receptors' [3,11–13]. A third class of receptors for moderately oxidized LDL, namely LOX1-R, has been identified and cloned in the last few years [4,14]. Little is however known about its cellular distribution in the different tissues and, in particular, in brain. The endothelium of brain microvessels is reportedly somewhat different, on the other hand, from other endothelia [8], presumably as a consequence of its interaction with adjacent cells, in primis astrocytes [9,15]. In vitro, most of its peculiarities can indeed be induced (or their loss can be prevented) by co-culturing brain endothelial cells together with astrocytes [16]. In such co-cultures, an up-regulation of LDL-R has been documented [17,18], whereas no data about LOX1-R modulation have been published.

The aim of this work was to study the expression pattern of both LDL-R and LOX1-R in brain microvessels endothelial cells and its modulation by astrocytes.

2. Materials and methods

2.1. Chemicals

Random hexamers and oligo-d$(T)_{16}$ were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). M-MuLV reverse transcriptase (cloned), Super Taq (Thermus thermophilus DNA polymerase), human placental ribonuclease inhibitor, buffer for reverse transcription, buffer for PCR and ΦX/HaeIII marker were obtained from HT Biotechnology Ltd. (Cambridge, England). Oligonucleotides used as primers were synthesized by M-Medical Genenco (Florence, Italy). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Isolation of brain microvessels and cell culture

Rat brain microvessels (RBM) were isolated from rat (*Rattus norvegicus*) brain cortex, as previously described [19]. Primary in vitro cultured endothelial cells from RBM (RBM-EC) were obtained from capillary fragments and cultured as previously reported [19]. At the 10–12th day, the cells exhibited typical endothelial features and were positive for von Willebrand factor. Aorta was used as source of in vitro cultured bovine aorta endothelial cells (BAEC) as described [20]. Primary cultures of rat (*Rattus norvegicus*) astrocytes (RA) were obtained from corpus callosum of 5 day old rats, as described [21]. The

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Abbreviations: BAEC, in vitro cultured bovine aorta endothelial cells; BBB, blood–brain barrier; LDL, low-density lipoprotein; LDL-R, low-density lipoprotein receptor; LOX1-R, moderately oxidized low-density lipoprotein receptor; RA, in vitro cultured rat astrocytes; RBM, ex vivo rat brain microvessels; RBM-EC, in vitro cultured endothelial cells from rat brain microvessels

0014-5793/02/$22.00
PII: S0014-5793(02)02857-0

final RA cell population exhibited full immunoreactivity toward glial fibrillary acidic protein. Co-cultures of RA and RBM-EC were performed in multiwell plates, using tissue culture inserts with 3.0 μm diameter membrane pores, under two different sets of conditions. In the former system, named 'without contact', RA were plated on the bottom of the multiwells plate; when they had grown to confluency, RBM-EC were then seeded (25–30 000/cm^2) on the upper side of the precoated insert membrane and cultured, up to confluence, for 8–10 days. In the alternative 'with contact' system, instead, the RA were plated on the underside of the insert membrane, whose upper side was then seeded, as in the other system, with RBM-ECs.

We defined the above described RA and RBM-EC co-cultures as homologous and the co-cultures of RA and BAEC as heterologous; the heterologous co-cultures were performed as above described for homologous. All cell cultures were collected by trypsinization and usually stored at −80°C before RNA extraction.

2.3. RNA extraction and cDNA synthesis

Total RNA extraction from microvessels or from cells was performed by the acid guanidinium/thiocyanate/phenol-chloroform procedure [22]. Reverse transcription was performed with 1 μg of total RNA by using, in each sample, either 150 pmol of random hexamers or 50 pmol of oligo-d(T)$_{16}$ (preferred for semiquantitative assays), with 80 U per sample of M-MuLV reverse transcriptase at 42°C for 1 h, in the manufacturer's assay buffer (20 μl final volume) plus 20 U of human placental ribonuclease inhibitor per sample and 300 μM dNTPs, followed by heat inactivation at 94°C for 5 min. In subsequent amplification reactions, 2–5 μl of these RT samples were used for each PCR panel. Using control samples without reverse transcriptase treatment, any genomic DNA contamination was excluded (data not shown).

2.4. PCR strategy and semiquantitative assay

In Table 1 are summarized the primers used for amplification. In all reactions 10 pmol of each primer and 0.5 U of Super Taq DNA polymerase were used. PCR was performed in a final volume of 25 μl in the manufacturer's assay buffer plus 175 μM dNTPs. For semiquantitative assays we performed a scalar number of cycles on a fixed quantity of cDNA [23], the results being then normalized by the independent amplification of β- or γ-actin, used as control genes; the coefficient of variation between actin signals relative to different experimental conditions was, in the linearity range of the assay, less than 10%. After an initial denaturation step of 3 min at 94°C, we performed, on a Perkin Elmer Cetus Thermal Cycler mod. 480 for each sample panel, 23, 26, 29, 32 and 35 cycles (to assess the linearity range of the assay) of 1 min at 94°C, 1 min at 62°C, 1.5 min at 72°C, with a final 7 min step at 72°C. Electrophoresis of 15 μl of the PCR reaction (out of 25 μl) was performed under standard conditions. All gels were photographed with a POLAROID camera DS-34 using POLAROID b/w 667 films. The quantification was performed with a CCD imaging system [24], using a KODAK Megaplus (mod. 1.4) camera for scanning of the films, and a BIO IMAGE (Genomic Solutions) computerized densitometer for image acquisition. The ratio of the densitometry of specific receptor signal with that of corresponding actin signal and the average ratio of at least three values falling in the linearity range of the assay were calculated. The results were expressed as the percentage of highest mean signal ratio (set to 100%).

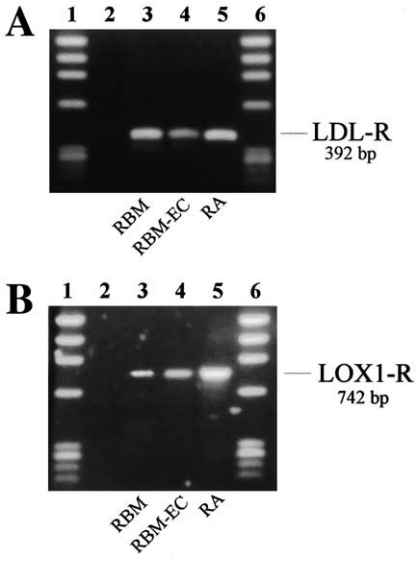

Fig. 1. Both LDL-R (A) and LOX1-R (B) are expressed in RBM, RBM-EC and in RA. Lane 2 = negative control; lanes 1 and 6 = ΦX/HaeIII marker. These amplifications cannot be assumed to reflect quantitative differences.

3. Results

The expression of LDL-R (Fig. 1A) and of LOX1-R (Fig. 1B) was evidenced in RBM (lane 3), RBM-EC (lane 4) and RA (lane 5). These experiments had not been planned for a semiquantitative approach, and the differences in the signal intensities should therefore not be taken into account; in particular the high intensity of LDL-R signal in RBM-EC (Fig. 1A, lane 4) depends on the high number of cycles performed, being a correct densitometric evaluation performed by scalar number of cycles reported in Fig. 2 (19% of maximal signal). In Fig. 2 are reported the expression levels of LDL-R and of LOX1-R in RA and RBM, as well as RBM-EC cultured alone or in contact with RA. Both receptors were expressed at higher levels in RA (lanes 7) than in RBM (lanes 8). In the RBM-EC there was a further decrease, more marked for LDL-R than for LOX1-R, in the expression of both receptors (lanes 9). The apparent absence of LDL-R signal in RBM-EC (Fig. 2A, lane 9) depends on the low number of cycles performed in the panel shown in the figure, being a correct densitometric evaluation (reported as % of maximal signal in the figure) performed also by a higher number of cycles. Co-culture with RA produced, in RBM-EC, a considerable enhancement

Table 1
Primers used for the amplification

Receptor	s	Primer sequence	amp	dim
LDL-R [34]	f	5′-TGCACTCCATCTCCAGCATCG-3′	B+R	392 bp
	b	5′-GAGGCAGCTTCTCATGTCCTTGG-3′		
LOX1-R [35]	f	5′-GCAAACTCTTCAGGTCCTTGTCC-3′	R	742 bp
	b	5′-GGAAATTGAGCAGAAAGCATCC-3′		
β-actin [36]	f	5′-ACCCAGGCATCGCTGACAGGATGC-3′	R	216 bp
	b	5′-CCGCCTAGAAGCATTTGCGGTGCACG-3′		
LOX1-R [37]	f	5′-AATCCAAGAAACTAATGGAACTTCACCGCC-3′	B	597 bp
	b	5′-TCAACCAGTAGCAGGCATTTCCCTGG-3′		
γ-actin [38]	f	5′-TTCCAGCAGATGTGGATCAG-3′	B	342 bp
	b	5′-AGCCGCATATAACTAGGGGT-3′		

The kind of receptor, strand specificity ('**s**', with f = forward; b = backward), primer sequence, use in amplification ('**amp**') of bovine (B) or rat (R) sequences, and dimension ('**dim**') of amplified products are indicated.

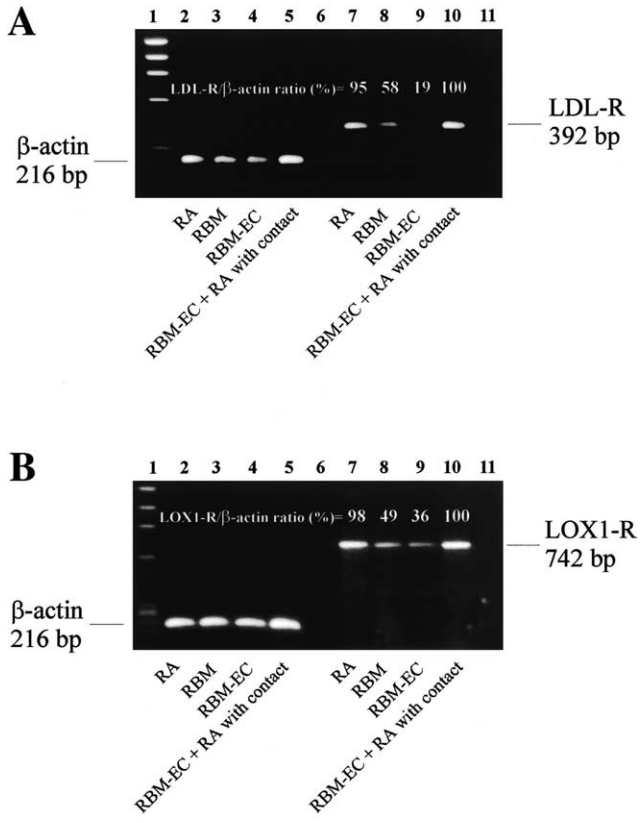

Fig. 2. Both LDL-R (A) and LOX1-R (B) expression is enhanced by RA in RBM-EC. Lanes 6 and 11 = negative controls; lane 1 = ΦX/HaeIII marker. The panels showed are two (chosen as examples) of those performed by the scalar number of cycles approach; the densitometric evaluation of RT-PCR products obtained in the semiquantitative assays is reported (see Section 2).

of the expression of both receptors (lane 10). This effect, though depending on the presence of astrocytes, did not require a physical contact between the two kinds of cells: as shown in Fig. 3, the signal intensities of both LDL-R (lanes 6–8) and LOX1-R (lanes 10–12) were higher in the presence of RA than in their absence, independently from RBM-EC/RA contact. These results were confirmed by a semiquantitative densitometric analysis, reported in Figs. 2 and 3 as receptor/β-actin densitometric ratio (%). The normalization of receptor signal densitometry by actin signal densitometry, by the scalar number of cycles approach, removed minor variations between actin expression in different samples, more evident in some panels (those performed at lower number of PCR cycles, one of which showed in Fig. 2A) but less evident in other panels (those performed at higher number of PCR cycles, one of which showed in Fig. 2B). The enhancement of expression of both receptors by RA was not observed in a heterologous system of co-cultures of BAEC/RA (Fig. 4): the signals of both LDL-R (lanes 5 and 6) and LOX1-R (lanes 8 and 9) in BAEC were similar, independently from RA presence either without BAEC/RA contact (Fig. 4) than with contact (data not shown).

4. Discussion

In brain microvessels there is a consistent expression of LDL-R, both as protein [10] and as mRNA (our results). As suggested by Dehouck and co-workers [18], astrocytes are likely to be responsible for this peculiar expression of LDL-R in the cerebrovascular district. On the other hand the expression of this receptor is markedly down-regulated in other vascular districts [25], as for example in aorta endothelium, presumably due to the combined effects of the high cellular density and of the continuous exposure to circulating serum lipoproteins. In this work we found that the expression pattern of LOX1-R was quite similar to that of LDL-R: a decreased expression when shifting from RBM to RBM-EC (Fig. 2) with this effect not only prevented but even overcorrected, presumably by an induction mechanism, if RBM-EC were co-cultured with RA (Figs. 2 and 3). For both receptors, repression mechanism(s) in aorta endothelium and activation mechanism(s) in brain microvessels endothelium appear to act at transcriptional level. The role of BBB micro-environment in the up-regulation of both receptors in endothelial cells of brain microvessels appears crucial, with results pointing to astrocytes as the main (if not unique) responsible cellular component. It is reasonable to assume that the final expression level of both receptors in endothelial cells of brain microvessels is defined by the equilibrium between inhibitory (e.g. cell–cell contact and/or high lipoprotein levels) and stimulatory (astrocytes induction) signals.

A number of previous experimental results have indeed evidenced that several BBB properties are induced by astrocytes. The induction of the protein(s) responsible for some of these properties (γ-glutamyltranspeptidase, transferrin receptor,

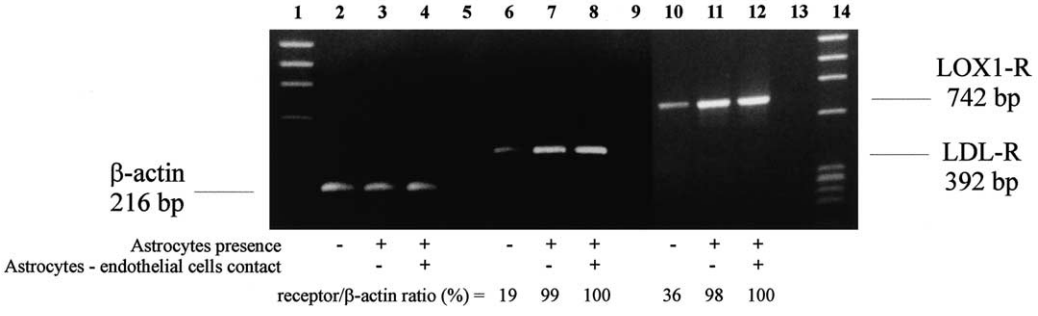

Fig. 3. The enhancement of both LDL-R and LOX1-R expression in RBM-EC does not require a physical contact with RA. Lanes 5, 9 and 13 = negative controls; lanes 1 and 14 = ΦX/HaeIII marker. The panels showed are two (chosen as examples) of those performed by the scalar number of cycles approach; the densitometric evaluation of RT-PCR products obtained in the semiquantitative assays are indicated (see Section 2). If the RBM-EC were co-cultivated with RA (astrocytes presence, +) or cultivated alone (astrocytes presence, −) is indicated, as well as if the RBM-EC/RA contact was allowed (astrocytes–endothelial cells contact, +) or not allowed (astrocytes–endothelial cells contact, −).

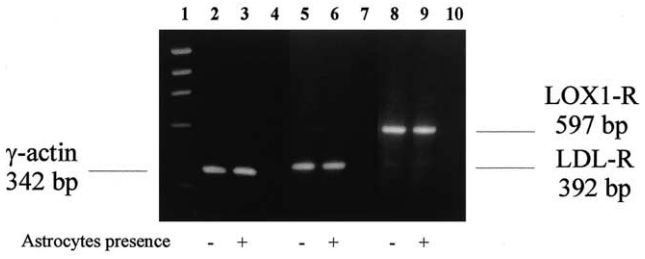

Fig. 4. The expression of both LDL-R and LOX1-R in a heterologous (BAEC/RA) co-culture system is not modulated by RA. Lanes 4, 7 and 10 = negative controls; lane 1 = ΦX/HaeIII marker.

P-glycoprotein [16], as well as those leading to tight junctions formation [9,16]) has been shown to require a physical contact between astrocytes and endothelial cells; some other properties, on the contrary – such as the brain-type glucose transporter, [16,26]; LDL-R [18]; manganese superoxide dismutase [27]; immunoglobulin-like surface glycoprotein HT7, UEA-1 lectin-binding sites, angiotensin receptors [28] – are apparently elicited by soluble factor(s) produced by astrocytes. In our experimental system, the induction by RA of LDL-R and of LOX1-R did not require a close cellular contact of the RA with the cerebral endothelial cells (Fig. 3). These results strongly point to soluble factor(s) produced by RA as the main signal responsible for the modulation of both receptors expression at the BBB level.

Recent experimental results have evidenced the production by astrocytes of cytokines and growth factors (tumor necrosis factor-α, interleukin-1β [27]; vascular endothelial growth factor [29]; basic-fibroblast growth factor [30]; interleukin-6 [31]; monocyte-chemoattractant protein-1 [32,33]). Some of these macromolecules can presumably act on brain microvessels endothelial cells to enhance the expression of both LDL-R and LOX1-R, but further studies are needed to characterize the specific soluble factor(s) involved in the interaction between RA and RBM-EC. This interaction is likely to represent a quite specific signalling pathway since no effect could be evidenced on the expression of both receptors in a heterologous co-culture (BAEC/RA) system (Fig. 4). The finding of this high specificity of interaction is in agreement with previous works on LDL-R [18] and on glucose uptake [26]. It should however be pointed out that astrocytes have been reported to have the capacity of inducing some BBB properties also in non-neural endothelial cells [9,16].

The detection, in RA, of a high level of expression of both LDL-R and LOX1-R (Figs. 1 and 2) stresses a direct role of these cells also in the maintenance of the lipid composition of the cellular and extracellular environment of the brain. Astrocytes appear therefore as one of the possible recipients for native and modified LDL that cross the BBB. LDL-R has been clearly shown [17] to be directly involved in native LDL transcytosis across the BBB. Some experimental results have on the other hand evidenced an uncoupling, in primary cultures of brain endothelial cells, between the binding site(s) for acetylated LDL and the intracellular degradative pathway of these modified lipoproteins [13], suggesting a transcytosis pathway also for these modified LDL. The up-regulation of LOX1-R in BBB and its expression in RA suggest that also this receptor might have a functional role in the transcytosis of moderately oxidized LDL through the BBB.

Acknowledgements: This work was made possible by financial support from the University of Rome ('Progetti di Ateneo') and from the Italian Ministry of University and of Scientific and Technological Research ('Progetti di Interesse Nazionale 1997'). The skillful technical assistance of Mr. Vincenzo Peresempio is gratefully acknowledged.

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