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Research Report

Transcriptional profiles of receptors and transporters involved in brain cholesterol homeostasis at the blood–brain barrier: Use of an in vitro model

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ABSTRACT

Brain is the most cholesterol rich organ of the whole body and recent studies suggest a role for the blood–brain barrier (BBB) in cerebral cholesterol homeostasis. Low density lipoprotein receptor (LDLR)-related receptors and ATP-binding Cassette (ABC) transporters play an important role in peripheral sterol homeostasis. The purpose of this study was to determine the mRNA expression profiles of ABC transporters (ABCA1, 2, 3, 7 and ABCG1) and low density lipoprotein receptor (LDLR)-related receptors (LDLR, vLDLR, LRP1, LRP2 and LRP8) in BBB endothelium using an in vitro co-culture model of bovine brain capillary endothelial cells (BCECs) and rat glial cells. All transcripts tested are expressed by BCECs and in capillary extract, except vLDLR. Glial cells influence ABCG1, A1, 2, 7 and LRP1 transcription, suggesting a role in cerebral lipid supply/elimination through the modulation of the expression of these transporters and receptors by these cells. Altogether, these results highlight the importance of glial input in the BBB transport phenotype for sterol homeostasis in the central nervous system, and confirm the importance of the BBB in this process.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia and affects up to 15 million individuals worldwide. Abnormal biosynthesis and accumulation of the neurotoxic amyloid β (Aβ) peptide in senile plaques is suggested to play a central role in the pathogenesis of AD and investigating mechanisms involved in these processes remains essential to elucidate origin and evolution of this disease. In this regard, it was demonstrated under in vitro conditions that cholesterol promotes the proteolytic cleavage of the cerebral amyloid precursor protein (APP) to Aβ peptide suggesting that cellular cholesterol content may influence AD (Bodovitz and Klein, 1996; Simons et al., 1998). Moreover, many epidemiological studies highlight a close relationship between vascular risk factors, including hypercholesterolemia, and AD (Shobab et

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Abbreviations: ABCA, ATP-binding cassette subfamily A; ABCG, ATP-binding cassette subfamily G; AD, Alzheimer's disease; Aβ peptide, amyloid β peptide; Apo, Apolipoprotein; BBB, blood–brain barrier; BCECs, brain capillary endothelial cells; CNS, central nervous system; HDL, high density lipoprotein; LDL, low density lipoprotein; LDLR, LDL receptor; LRP, LDL-related protein; LXR, Liver X activated Receptor; P-gp, P-glycoprotein; RT-PCR, reverse transcription-polymerase chain reaction; SCARB1, scavenger receptor class B member 1; tPA, tissue-type plasminogen activator

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al., 2005). Indeed, rabbits and mice when fed with long-term cholesterol-enriched diets showed an elevated serum cholesterol level, and an increase in Aβ peptide formation and deposition was observed (Ghribi et al., 2006; Hooijmans et al., 2007; Refolo et al., 2000; Sparks et al., 2002). Lastly, in mice, treatment with statins (inhibitors of cholesterol synthesis) significantly decreased plasma cholesterol, brain Aβ peptide production or deposition (Refolo et al., 2001). In humans, statins lowered the risk of AD and cause a reduced deterioration of cognitive functions, reinforcing the relationship between plasma cholesterol and AD (Jick et al., 2000; Sparks et al., 2005).

How serum cholesterol influences AD remains unknown especially as brain is isolated from the whole body by the blood–brain barrier (BBB), a dynamic interface formed by brain capillary endothelial cells (BCECs). This barrier is known to restrict cholesterol exchanges between brain and blood circulation, but the mechanisms involved in this transport remain poorly understood. One of the principal roles of the BBB is to supply the brain with essential nutrients, and to mediate the efflux of many waste products from the brain. These processes are possible because BCECs express some specific receptors or transporters to their luminal (plasma) and/or abluminal (cerebral) membranes.

In our laboratory, we have developed an in vitro BBB model consisting of a co-culture of bovine BCECs and rat glial cells that closely mimics the in vivo situation (Cecchelli et al., 1999, 2007; Dehouck et al., 1990). Using this model, we previously demonstrated that the low density lipoprotein receptor (LDLR) is involved in LDL cholesterol transcytosis across the BBB towards the abluminal side with ensuing uptake by astrocytes. We have also demonstrated that a lipid requirement of astrocytes increases the expression of endothelial cell LDLR (Dehouck et al., 1994, 1997). This mechanism has recently been exploited for targeted delivery of proteins across the BBB. Indeed, Spencer and colleagues demonstrated in transgenic mice, that it is possible to deliver the lysosomal enzyme glucocerebrosidase and a secreted form of the GFP protein to the central nervous system (CNS) by fusing the LDLR-binding domain of an apolipoprotein (Apo) to these proteins (Spencer and Verma, 2007). The current number of members of this family in mammals has grown over ten receptors playing an important role in lipid transport/metabolism. Indeed, the major members including LDLR, very LDLR (vLDLR), LRP1 (LDLR-related protein 1), 2 (also called megalin) and 8 (also called Apolipoprotein E receptor 2), can bind and internalize lipoproteins, especially ApoE-containing lipoproteins (Andersen and Willnow, 2006). Altogether, these data strongly suggest that LDLR, and probably the other members of this family, may mediate the transport of cholesterol across the BBB for neuronal and/or glial use. As the brain contains the second greatest concentration of the cholesterol transporter ApoE, and because subcellular localization of these receptors remains unknown, a possible involvement in cerebral cholesterol efflux cannot be excluded.

The characterization of this efflux activity remains essential because no degradation mechanism for cholesterol is reported within mammalian brain. On the other hand, recent studies report that porcine, rat and human BCECs express several efflux transporters belonging to the ATP-binding Cassette (ABC) transporters family, reinforcing the hypothesis of BBB involvement in brain cholesterol efflux (Ohtsuki et al., 2004; Panzenboeck et al., 2002).

The 48 members of this superfamily are divided in seven subfamilies named ABCA to ABCG (Kim et al., 2008). Growing evidences suggest that some of them are involved in sterol transport processes. ABCA1 and 7 mediate ApoE- and ApoA1-dependent cholesterol release from cells (Chan et al., 2008; Ikeda et al., 2003; Panzenboeck et al., 2002). ABCA2, ABCA3 and ABCA7 have been reported to be induced by cholesterol (Kaminski et al., 2000, 2001; Klucken et al., 2000). ABCG1 is the founding member of the subfamily G and it seems also involved in cellular cholesterol efflux. Recent studies on the formation of plasma high density lipoprotein (HDL) indicate that ABCA1 mediates an initial lipidation of lipid-poor or lipid-free ApoA1, whereas ABCG1 is responsible for the further lipidation of the particles that have been partially lipidated by ABCA1 (Hirsch-Reinshagen and Wellington, 2007).

In absence of any bovine specific antibodies available against the lipoproteins receptors and cholesterol transporters, we established the first transcriptional profiles of these receptors and transporters at the BBB endothelium level using reverse transcription-polymerase chain reaction (RT-PCR) technique in a co-culture of BCECs and glial cells. This system, developed in our laboratory, has been intensively used as a relevant in vitro model of the BBB (Cecchelli et al., 2007) and allows the characterization of the glial influence on transporters/receptors expression as previously reported (Berezowski et al., 2004; Descamps et al., 2003; Fenart et al., 1998).

2. Results

2.1. *Expression of lipoprotein receptors in BCECs*

As it is well known that glial cells are necessary to induce many properties of the BBB, including an increase in the tightness of endothelial tight junctions, a reduced permeability and expression of different transporters or receptors (Abbott et al., 2006; Berezowski et al., 2004; Fenart et al., 1998), we analyzed the transcriptional profile of transporters and receptors involved in lipid homeostasis in absence (E) or in presence (Coc) of glial cells. The influence of glial cells on BBB properties is demonstrated by a decrease in permeability in co-cultures as shown in Fig. 1 ($Pe_E = 1.03 \pm 0.08 \times 10^{-3}$ cm/min versus $Pe_{Coc} = 0.49 \pm 0.04 \times 10^{-3}$ cm/min) and by an up-regulation of specific transporters expression like the P-glycoprotein (P-gp) (data not shown and Berezowski et al., 2004; Fenart et al., 1998).

We first investigated the transcriptional expression of the major receptors of the LDLR family (LDLR, vLDLR, LRP1, LRP2 and LRP8). Primers and conditions used to amplify these mRNA are listed in Table 1. As shown in Fig. 2, all the five mRNA are expressed in BCECs. Only LRP1 was significantly down-regulated by 0.59 in presence of glial cells. LRP2, LRP8, LDLR and vLDLR expressions gave the same signal in both culture conditions, with no significant differences. The stable β-actin levels confirmed similar amounts of template in each condition. To analyse the expression of these receptors in vivo, we performed RT-PCR experiments on freshly isolated

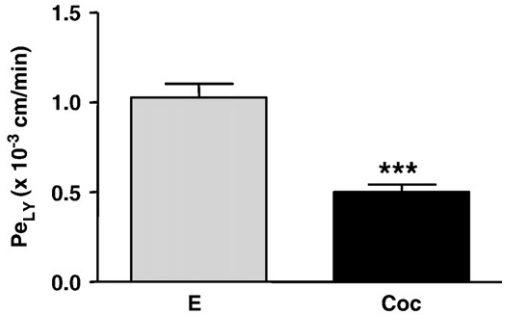


Fig. 1 – Influence of glial cells population on BBB permeability to Lucifer Yellow (Pe_{LY}). Bovine BCEC monolayer (E) were cultivated in solo for 12 days or co-cultivated with glial cells (Coc). Results represent the mean ± SE of four experiments pooled from three filters. Permeability was determined as described in the experimental procedure section. $^{***}p<0.001$ versus BCEC monolayer (E) control (paired t-test).

bovine capillaries (BC). Except vLDLR, all the transcripts were detected. As BC preparation contains pericytes embedded in the basal lamina (Risau et al., 1992), it is necessary to take into account their presence for the possible PCR signals observed from BC preparation. All the transcripts tested were detected in pericytes and in particular LRP1 for which an intense signal was measured (12.86 fold versus (E) condition).

2.2. *Expression of ABC transporters of the subfamilies A and G in BCECs*

To investigate a possible role of glial cells in cerebral lipid supply/elimination, we also analyzed four ABC transporters of the subfamily A (such as ABCA1, 2, 3 and ABCA7) and one of the subfamily G (ABCG1) which are suspected to mediate cellular cholesterol efflux (see for review Kim et al., 2008). These five transporters were expressed in BCECs and capillaries whereas ABCA2 and ABCG1 were absent in pericytes (Fig. 3). In BCECs, ABCA3 transcript signal was not influenced by glial cells while ABCG1, ABCA2 and ABCA7 signals were significantly increased by 2.18, 14.41 and 1.90 respectively in co-cultured BCECs. By contrast, ABCA1 mRNA was twice less expressed in this condition. It is interesting to note that very weak signals of ABCA1 was obtained in pericytes in comparison with (E) condition (0.29 fold versus (E) condition).

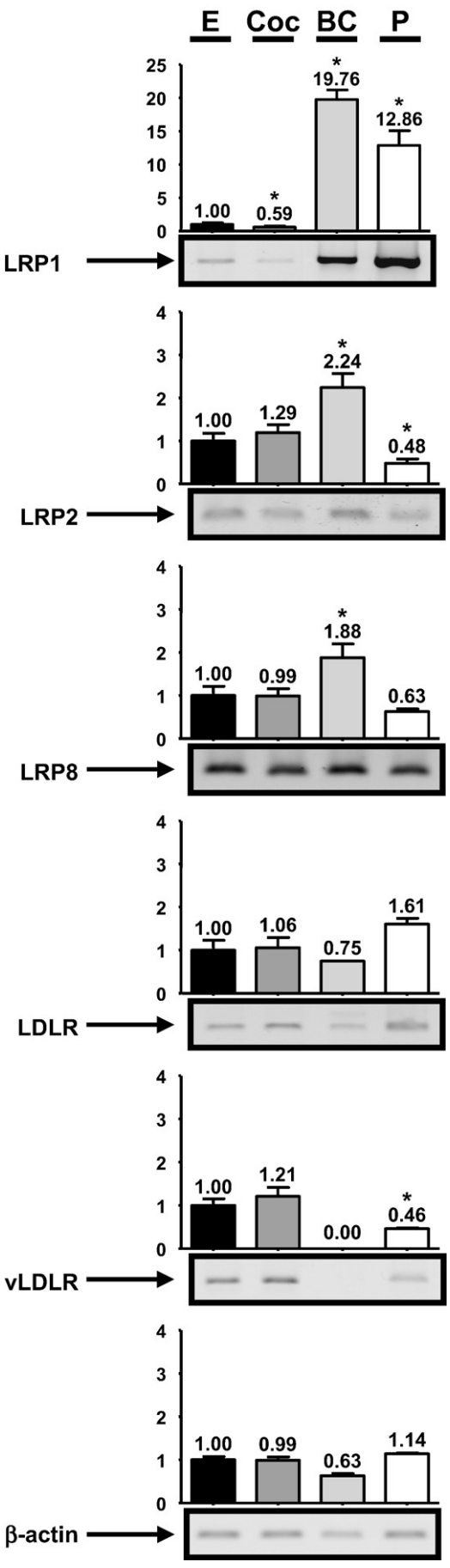
2.3. *Expression of ApoE, ApoA1 and SCARB1 in BCECs*

As ApoE is the major lipoprotein of LDL and since ABC transporters mediate cholesterol release to ApoE and/or ApoA1 particles, we investigated transcriptional expression of both apolipoproteins. All transcripts were amplified in BCECs and in cerebral capillaries (Fig. 4). Glial cell population did not influence the signal obtained in BCECs. In pericytes, a strong signal was observed for ApoE whereas ApoA1 was very weakly detected.

Table 1 – DNA primers and conditions used to amplify receptors, transporters and apolipoproteins mRNA

mRNA	Species	Accession number	F/R	Sequence	Size (bp)	Ta (C°)
ABCG1	*Homo sapiens*	BC029158	F	5′-gAggAAgAAAggATACAAGACC-3′	330	55
			R	5′-gTCAgTATCTCCTTgACCATTT-3′		
ABCA1	*Bos taurus*	NM_001024693	F	5′-gTgTCTCgCCTgTTCTCAg-3′	540	55
			R	5′-gAAACATCACCTCCTgCCg-3′		
ABCA2	*Homo sapiens*	AF178941	F	5′-AgATTggCCAgTTTgACAAgAT-3′	259	50
			R	5′-gACCTTggTCAggTTCTCAATC-3′		
ABCA3	*Bos taurus*	NM_001113746	F	5′-gAAgATACAgTCAgAgAACgTg-3′	343	55
			R	5′-TgTAACTgAAgCgTAAgTggTA-3′		
ABCA7	*Bos taurus*	XM_589159	F	5′-CTATgTggACgATACgTTCCTg-3′	380	55
			R	5′-ggCAgATAgAgTACgAAgTAgg-3′		
LRP1	*Mus musculus*	NM_008512	F	5′-gCATCCTgATCgAgCACCTg-3′	531	60
			R	5′-gCCAATgAggTAgCTggTgg-3′		
LRP2	*Bos taurus*	XR_028042	F	5′-TTgCCTTTgACTggATCAATAA-3′	109	55
			R	5′-ggAACTTgggCTATCAgAgTgT-3′		
LRP8	*Bos taurus*	AY364441	F	5′-TATAgTCATCTTCCATgAgCTg-3′	216	55
			R	5′-AACgTCgTAgTTgAggTAgACT-3′		
LDLR	*Bos taurus*	AJ551281	F	5′-ACATTgTCCTTTTCCACAAC-3′	193	50
			R	5′-gACTCTgTgAggCAgCTACT-3′		
vLDLR	*Bos taurus*	NM_174489	F	5′-gATAggAAAACCTgTggAgATA-3′	463	55
			R	5′-CgTCTAgggTAgCTACTgAAAT-3′		
SCARB1	*Bos taurus*	NM_174597	F	5′-CTTATgTgTACAgggAgTTCAg-3′	312	55
			R	5′-AgTACTgATTgATgAggTggAT-3′		
ApoE	*Bos taurus*	NM_173991	F	5′-TAAgATACgCCAgCAgCTAGA-3′	351	55
			R	5′-gCCATgAAgCTTgAgAAATC-3′		
ApoA1	*Bos taurus*	NM_174242	F	5′-CACATCCCTTCAAgATgAAAgC-3′	219	55
			R	5′-gTTTCAggTTgAgCTgTTTTCC-3′		
β-Actin	*Rattus norvegicus*	NM_031144	F	5′-gAAgTACCCCATTgAACACg-3′	177	55
			R	5′-ggTCTCAAACATgATCTggg-3′		

From left to right: cDNA targeted for amplification, species, accession numbers of cDNA from NCBI database, Forward (F), Reverse (R) primer designation, 5′ to 3′ primer sequence, size in bp of amplification and annealing temperature in °C used to amplify cDNA.



The transcript of SCARB1, a receptor mediating cellular cholesterol transfer to apolipoproteins, was found in BCECs and in pericytes and capillaries (Fig. 4). Like ApoE and ApoA1, SCARB1 gave the same signal of comparable intensity in both BCECs culture conditions.

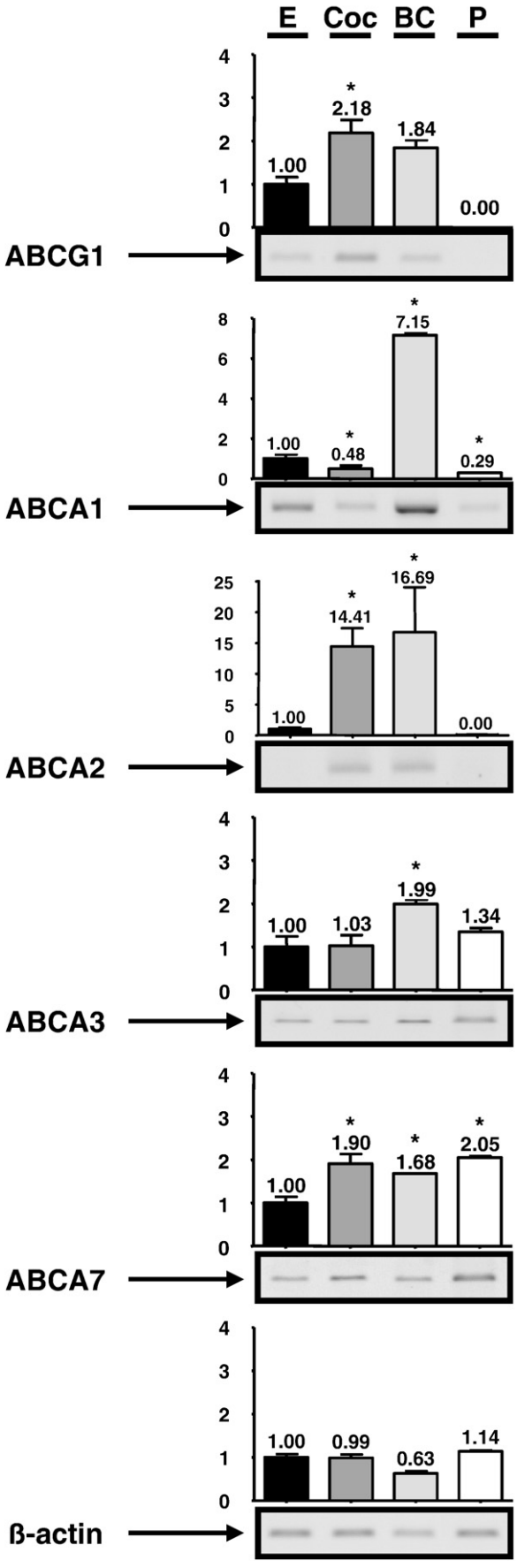
3. Discussion

The importance of BBB in brain cholesterol homeostasis is highlighted by recent studies demonstrating that this barrier is very important in cholesterol exchanges between blood and brain and in brain cholesterol metabolism (Dehouck et al., 1994, 1997; Panzenboeck et al., 2002, 2006). To establish the transcriptional profiles of major cholesterol transporters and lipoproteins receptors in BBB endothelium, we used a BBB in vitro model reproducing the main characteristics of the BBB in vivo (Cecchelli et al., 1999, 2007; Dehouck et al., 1990).

3.1. *Receptors of the LDLR family*

First, we have examined the expression of the major receptors of the LDLR family: LRP1, LRP2, LRP8, LDLR and vLDLR. These receptors are important in cholesterol transport/metabolism because they can bind and internalize lipoproteins. All of these receptors are expressed in the brain, particularly in regions prone to amyloid deposition. They are also very important for the development and functional integrity of the CNS (Andersen and Willnow, 2006). As demonstrated by our results, these receptors are expressed in BCECs. At the level of BBB, the role of these receptors in transcytosis is highlighted by their involvement in transport of neurotoxic Aβ peptide and its precursor APP across the BBB (Jaeger and Pietrzik, 2008; Shibata et al., 2000; Van Uden et al., 2002). Moreover, in our BBB model, LDLR was shown to be involved in LDL transcytosis (Dehouck et al., 1994, 1997) and LRP1 in melanotransferrin (P97) and tissue-type plasminogen activator (tPA) transcytosis (Benchenane et al., 2005; Demeule et al., 2002). We demonstrated that transcriptional expression of LRP1 only was significantly restricted when glial cells are present. As this receptor mediates P97, tPA and Aβ peptide transcytosis across the BBB, we suggest that these cells may influence CNS delivery/elimination of these molecules. These results highlight a possible role for glial cells in Aβ peptide transport across the BBB. As no direct contact occurs between glial cells and BCECs, we hypothesize that these cells release diffusible factors to induce BBB properties as previously

Fig. 2 – Lipoprotein receptors expression detected by RT-PCR in bovine BCEC monolayer (E), in BCECs co-cultured with glial cells (Coc), in bovine capillary extracts (BC) and in pericytes (P). Analysis was performed using primers and conditions given in Table 1. The expression of the β-actin was determined as a simple quantity control. For (E), (Coc) and (P) conditions, densitometry values represent the mean ± SE-fold intensity of four experiments (each pooled from three filters for (E) and (Coc) conditions) normalized to β-actin intensity. *$p < 0.05$ versus BCEC monolayer (E) control (one-way ANOVA, Tukey test).



demonstrated (Berezowski et al., 2004; Dehouck et al., 1994; Descamps et al., 2003). The relatively high level of LRP1 measured in capillary fraction and in pericytes strongly reinforces the recently reported role of pericytes in Aβ peptide internalization and/or transport across the BBB (Wilhelmus et al., 2007).

All receptors tested were expressed in pericytes and in brain capillary extracts, except vLDLR. This apparent discrepancy could be explained by the possible influence of neurons or an effect of culture condition as previously reported in astrocytes and endothelial cells cultures (Berezowski et al., 2004; Decleves et al., 2000).

3.2. *Transporters of the ABC family*

We have also focused our analysis on five transporters (ABCA1, 2, 3, 7 and ABCG1) belonging to the ABC family and for which there is growing evidence suggesting they may contribute to brain lipid homeostasis (Kim et al., 2008). ABCA1 is the most studied member of the subfamily A as this gene is genetically linked with Tangier disease characterized by an absence of plasma HDL and a severely impaired ability of cholesterol to be removed from peripheral tissue (Oram and Vaughan, 2000). ABCG1 seems also involved in cellular cholesterol efflux. Recent studies on the formation of plasma HDL indicate that ABCA1 mediates an initial lipidation of lipid-poor or lipid-free ApoA1, whereas ABCG1 is responsible for the further lipidation of the particles that have been partially lipidated by ABCA1 (Hirsch-Reinshagen and Wellington, 2007).

While expression of ABCA1 was recently demonstrated in another BBB model (Panzenboeck et al., 2002), the presence of ABCG1 remained undetermined. We showed that both transporters were expressed in BCECs and in capillary extracts and that ABCG1 mRNA was absent in pericytes. ABCA1 was highly expressed in capillary fraction compared to BCECs and pericytes suggesting that the in vivo condition could influence its expression, as for vLDLR. Moreover, the presence of glial cells significantly down-regulated ABCA1 and up-regulated ABCG1 in BCECs suggesting different regulation mechanisms of their promoters in these cells. Since several years, ABCA1 and ABCG1 have been under intense scrutiny because they are direct target genes of the Liver X activated Receptor (LXR) nuclear receptors (Beaven and Tontonoz, 2006). LXRs act as molecular sensors of the cellular cholesterol concentrations transferring cellular lipids to the ApoE and/or ApoA1 particles. In the brain, LXRs are involved in lipid homeostasis and *LXR–/–* mice present several neurodegenerative disorders (Wang et al., 2002). These very interesting data had lead to the study of these receptors in neurodegenerative disorders and in particular in AD. The synthetic agonist of these nuclear receptors, TO901317, when injected in wild-type or transgenic mice overexpressing

Fig. 3 – ABC transporters expression detected by RT-PCR in bovine BCEC monolayer (E), in BCECs co-cultured with glial cells (Coc), in bovine capillary extracts (BC) and in pericytes (P). As described previously, densitometry values for conditions (E), (Coc) and (P) represent the mean ± SE-fold intensity of four experiments normalized to β-actin intensity. *$p<0.05$ versus BCEC monolayer (E) control (one-way ANOVA, Tukey test).

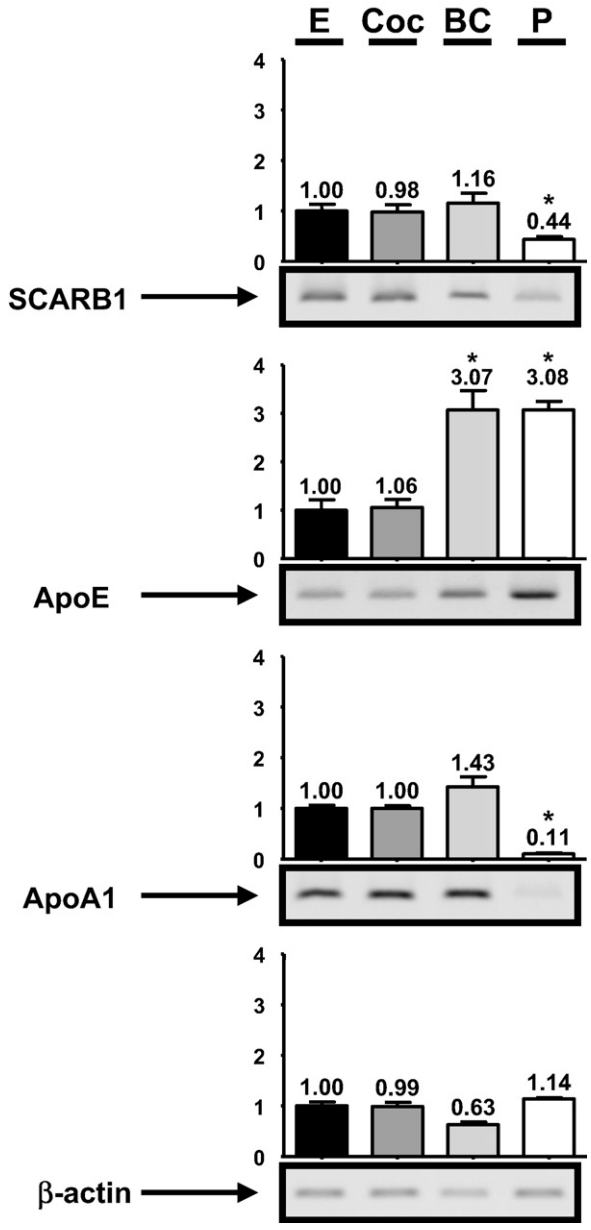


Fig. 4 – SCARB1, ApoE and ApoA1 expression detected by RT-PCR in bovine BCEC monolayer (E), in BCECs co-cultured with glial cells (Coc), in bovine capillary extracts (BC) and in pericytes (P). As Figs. 3 and 4, densitometry values for conditions (E), (Coc) and (P) represent the mean ± SE-fold intensity of four experiments normalized to β-actin intensity. *$p<0.05$ versus BCEC monolayer (E) control (one-way ANOVA, Tukey test).

the precursor of the Aβ peptide, increases the brain transcriptional expression of both ABCG1 and ABCA1 and decreases brain accumulation of Aβ peptide (Burns et al., 2006; Koldamova et al., 2005b; Riddell et al., 2007). In transgenic mice, ABCA1 deficiency promotes amyloidogenesis (Hirsch-Reinshagen et al., 2005; Koldamova et al., 2005a; Wahrle et al., 2005) while ABCA1 overexpression reduces amyloid deposition probably through ApoE expression and lipidation (Wahrle et al., 2008). Whether ABCG1 and ABCA1 transporters are regulated by TO901317 at the BBB or whether they mediate Aβ efflux across the BBB remain unknown.

Determination of the expression pattern of ABCA2, ABCA3 and ABCA7 at the BBB was highlighted by studies reporting that they were induced by cholesterol and that ABCA7 mediates ApoE- and ApoA1-dependent release from cells (Chan et al., 2008; Ikeda et al., 2003). Moreover, ABCA2 overexpression causes a phenotype similar to that of cholesterol-depleted cells sequestering unesterified cholesterol into endolysosomal compartments (Mack et al., 2008). As shown in Fig. 3, they were expressed in bovine brain capillary extracts and in our BBB model. ABCA3 transcriptional expression was not influenced by the presence of glial cells while ABCA2 and ABCA7 mRNA were significantly upregulated, suggesting that glial cells play an important role in modulating the expression of these transporters, for brain cholesterol supply/elimination. ABCA3 and ABCA7 were expressed by pericytes whereas ABCA2 was absent in these cells suggesting that transcription level of ABCA2 detected in capillary fraction is exclusively from endothelial origin. Interestingly, ABCA2 has also been suggested to act as a drug resistance gene because this protein is highly expressed in cell lines found resistant to estramustine and mitoxantrone (Boonstra et al., 2004; Laing et al., 1998). In accordance with previous studies which have already demonstrated that glial cells promotes the expression of the multi-drug resistance associated protein 6 (MRP6) and P-gp, (Berezowski et al., 2004; Fenart et al., 1998) our results suggest that glial cells may exert an important function in regulating drug resistance and cholesterol metabolism genes.

3.3. *SCARB1 and apolipoproteins*

As shown by our results, SCARB1 is expressed in BCECs, in pericytes and in capillaries. It has become generally accepted that HDLs protect against atherosclerosis and possibly against neurodegenerative diseases by modulating sterol flux. SCARB1 acts as the prime receptor for HDLs in vitro and in vivo (Trigatti et al., 2003). In Porcine BCECs, SCARB1 was shown to be localized essentially in apical (plasma) side and involved in cholesterol efflux via ApoA1 (Panzenboeck et al., 2002). Moreover, this receptor seems also involved in HDL and HDL-associated α-tocopherol transcytosis (Balazs et al., 2004; Sovic et al., 2004). Our results suggest that the presence of glial cells did not influence transcriptional expression of SCARB1.

Like ABCA1 and ABCG1, ApoE and SCARB1 expressions are under the control of LXR transcriptional events, and studies suggest tissue-specific regulation (Malerod et al., 2002). In the CNS, ApoE and ApoA1 are the most important proteins for cholesterol transport. We then investigated ApoE and ApoA1 expressions in our BBB model. Both apolipoproteins are expressed in BCECs as previously demonstrated in porcine BCECs (Panzenboeck et al., 2002) and in capillaries. In pericytes, ApoA1 mRNA displayed a very weak signal. These results demonstrate that BCECs are able to synthesize both apolipoproteins and that pericytes contribute for the majority of ApoE signal measured in capillary fraction. These data suggest a determinant role for the BBB in cerebral cholesterol homeostasis. ApoE plays a central role in AD because this apolipoprotein mediates Aβ peptide deposition in senile plaques; indeed, *ApoE−/−* mice did not show any amyloid deposits (Bales et al., 1997). Moreover, in vivo, ApoE can

interact with Aβ peptide to form a complex which is recognized by receptors from the LDLR family for transcytosis across the BBB (Jaeger and Pietrzik, 2008; Shibata et al., 2000). In rabbits and transgenic mice fed with a cholesterol-enriched diet, cerebral ApoE protein levels were up-regulated (Elder et al., 2007; Wu et al., 2003). Therefore, investigating these effects using our in vitro model could spotlight the function of such receptors and transporters at the BBB level.

In conclusion, our study establishes for the first time transcriptional profiles of transporters and receptors involved in cholesterol transport expressed in BCECs. Glial cell population influences the transcriptional expression at the BBB endothelium of several transporters and receptors probably by releasing diffusible factors. Altogether, these results contribute to highlight intercommunication between BCECs and glial cells in the frame of CNS sterol homeostasis and confirm the importance of the BBB in this process. Further protein detection using specific antibodies and transport studies are needed to understand the functional implication of this transporter and receptor expression pattern in BBB physiology.

4. Experimental procedures

4.1. *Cell culture*

The in vitro model of BBB consists of a co-culture of bovine cerebral endothelial cells and rat glial cells, as previously described (Berezowski et al., 2004; Cecchelli et al., 1999, 2007).

Briefly, newborn rat (<3 days) were guillotined in accordance with French guidelines and their brain extracted. Primary cultures of rat glial cells, plated on the bottom of six-well plates were made from cerebral cortex after the meninges has been cleaned off. Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 2 mM glutamine and 50 μg/mL gentamycin. Three weeks after seeding, cultures of glial cells are stabilized and used for co-culture.

Endothelial cells are isolated by mechanical homogenization from the grey matter of bovine brain, microvessels are seeded onto dishes coated with an extracellular matrix secreted by bovine corneal endothelial cells. Endothelial cells migrate out of morphologically identified capillaries and form microcolonies. Cells are amplified before storage in liquid nitrogen. Bovine brains (aged<6 months) were obtained by a French veterinarian authorized by the French Government.

For experiment, endothelial cells are rapidly thawed at 37 °C and seeded onto 60-mm-diameter gelatine-coated dishes in the presence of DMEM supplemented with 10% (v/v) newborn calf serum (CS) and 10% (v/v) horse serum (HS) (Gibco, Carlsbad, CA, USA), 2 mM glutamine, 50 μg/mL gentamycin and 1 ng/mL of basic-fibroblast growth factor (bFGF). At confluence, endothelial cells are harvested for the co-culture.

Filters (Transwell; pore size 0.4 μM) coated with rat-tail collagen prepared by a modification of the method of Bornstein, (1958) are set in six-well plates containing glial cells. Endothelial cells are plated on the upper side of the filters. Under these conditions, co-cultures are left for 12 days in order to obtain properties similar to the ones displayed by the BBB in vivo.

Pericytes were obtained as previously described (Berezowski et al., 2004). For experiments, pericytes were seeded on gelatine-coated 6-well dishes, cultured and used when confluent.

Capillaries were extracted from bovine brain cortex according to the homogenization technique of Meresse et al., (1989).

4.2. *BBB permeability determination*

Tight junctions integrity was checked by paracellular permeability studies using 50 μM of Lucifer Yellow (Sigma, St Louis, MO, USA) per upper chamber. Three inserts were used. The endothelial permeability coefficient (Pe) was calculated in cm/min using method previously described (Berezowski et al., 2004; Cecchelli et al., 1999). Statistical analysis was made using the software Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).

4.3. *RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)*

Twenty-four hours after medium refreshing, BCECs were rinsed twice in sterile cold phosphate buffered saline buffer and lysed using 350 μL of RLT lysis buffer (Qiagen, Valencia, CA, USA) per filter. Three filters were used for each condition.

Extraction of total RNA was performed using RNeasy total RNA extraction kit (Qiagen) following the manufacturer's protocol. Single-strand DNA was synthesized from 1 μg or total RNA by reverse transcription using Moloney-Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Invitrogen, Cergy-Pontoise, France).

DNA amplifications are realized using specific conditions and primers (all custom-synthesized by Invitrogen) listed in Table 1. Depending on primers, 25 to 40 cycles were used to amplify cDNA.

The various sized RT-PCR products were resolved through 1 to 2% agarose gel electrophoresis stained with ethidium bromide (Euromedex, Souffelweyersheim, France) and visualized on a UV transilluminator. Densitometry measurements were performed using Perfect Image Software (ClaraVision, Orsay, France) and statistical analysis was made using the software Prism 5.

A sequencing procedure was carried out to identify all corresponding fragments (Genoscreen, Lille, France).

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