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Oxysterols decrease apical-to-basolateral transport of A β peptides via an ABCB1-mediated process in an in vitro blood-brain barrier model constituted of bovine brain capillary endothelial cells

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Abstract:

It is known that activation of the liver X receptors (LXRs) by natural or synthetic agonists decreases the amyloid burden and enhances cognitive function in transgenic murine models of Alzheimer's disease (AD). Recent evidence suggests that LXR activation may affect the transport of amyloid β (A β) peptides across the blood-brain barrier (the BBB, which isolates the brain from the peripheral circulation). By using a well-characterized in vitro BBB model, we demonstrated that LXR agonists (24S-hydroxycholesterol, 27-hydroxycholesterol and T0901317) modulated the expression of target genes involved in cholesterol homeostasis (such as ATP-binding cassette sub-family A member 1 (ABCA1)) and promoted cellular cholesterol efflux to apolipoprotein A-I and high density lipoproteins. Interestingly, we also observed a decrease in A β peptide influx across brain capillary endothelial cells, although ABCA1 did not appear to be directly involved in this process. By focusing on others receptors and transporters that are thought to have major roles in A β peptide entry into the brain, we then demonstrated that LXR stimulation provoked an increase in expression of the ABCB1 transporter (also named P-glycoprotein (P-gp)). Further investigations confirmed ABCB1's involvement in the restriction of A β peptide influx. Taken as a whole, our results not only reinforce the BBB's key role in cerebral cholesterol homeostasis but also demonstrate the importance of the LXR/ABCB1 axis in A β peptide influx - highlighting an attractive new therapeutic approach whereby the brain could be protected from peripheral A β peptide entry.

Abbreviations:

24S-OH-chol: 24S-hydroxycholesterol; 27-OH-chol: 27-hydroxycholesterol; ABCA1: ATP binding cassette sub-family A member 1; ABCB1: ATP binding cassette sub-family B member 1; ABCC1: ATP binding cassette sub-family C member 1; AD: Alzheimer's disease; A β peptides: β -amyloid peptides; Apo: apolipoprotein; BBB: blood-brain barrier; BCECs: brain capillary endothelial cells; BCRP: breast cancer resistance protein; HDL: high density lipoprotein; LXR: liver X receptor;

MRP1: multidrug resistance associated protein 1; RAGE: receptor for advanced glycation end-products; SCARB1: scavenger receptor class B member 1.

1. Introduction

The blood-brain barrier (BBB) is known to be relatively impermeable to plasma circulating molecules, xenobiotics and metabolites. This natural obstacle between blood and brain compartments is essential for maintaining brain homeostasis and protecting the central nervous system (Abbott et al., 2010). The BBB is located within the brain capillaries and its phenotype is displayed by brain capillary endothelial cells (BCECs). The BBB's properties are induced by the cellular environment around the brain capillaries, with astrocyte end-feet and pericytes, the latter sharing a basal membrane with the BCECs (Daneman et al., 2010). Transport of molecules across this barrier can occur through a limited, specific, transcellular pathway that especially involves receptor-mediated transport (Cecchelli et al., 2007).

The best known of the compounds with limited exchange across the BBB is cholesterol, a vital sterol required for cell viability, electric insulation of the axon and membrane dynamics (Saher et al., 2005). This sterol does not cross the BBB (Osono et al., 1995; Spady et al., 1987) or crosses only very slowly (Chobanian and Hollander, 1962; Wilson, 1970) - probably because transport occurs through a limited, lipoprotein-mediated pathway (Candela et al., 2008; Dehouck et al., 1997). In the body, excess cholesterol is metabolized into oxysterols like 24S-hydroxycholesterol (24S-OH-chol) and 27-hydroxycholesterol (27-OH-chol), both of which easily cross the BBB. 24S-OH-chol is exclusively produced by a set of specific neurons (Lutjohann et al., 1996) and is involved in brain cholesterol turnover and elimination (Bjorkhem et al., 1999; Pfrieger, 2003). The clearance of 24S-OH-chol into the circulation (down its natural concentration gradient) has been estimated at 6-7 mg/day (Meaney et al., 2002). 27-OH-chol synthesized by peripheral organs is the major oxysterol in plasma and enters the brain compartment through the BBB at a rate of 4 mg/day (Leoni et al., 2003). 24S-OH-chol and 27-OH-chol have been well characterized as natural agonists of liver X receptor (LXR) nuclear receptors, which are involved in cellular cholesterol homeostasis (Vaya and Schipper, 2007). Activation of LXRs leads to the upregulation of ATP-binding cassette sub-family A member 1 (ABCA1), which mediates reverse cholesterol transport to lipid-poor

apolipoprotein A-I (ApoA-I), ApoE or high density lipoproteins (HDLs) (Kim et al., 2008; Saint-Pol et al., 2012).

The LXR/ABCA1 axis is a very attractive drug target in Alzheimer's disease (AD), a neurodegenerative disorder mainly associated with amyloid- β (A β) peptide accumulation, aggregation and deposition in the brain (Querfurth and LaFerla, 2010). Indeed, stimulation of the LXR pathway by agonists in murine models of AD provoked an ABCA1-dependent decrease in A β burden in the brain, which was correlating with a partial recovery of cognitive function (Burns et al., 2006; Donkin et al., 2010; Fitz et al., 2010; Koldamova et al., 2005b; Lefterov et al., 2007; Riddell et al., 2007; Terwel et al., 2011). Overexpression of ABCA1 leads to the same observations (Wahrle et al., 2008), whereas deletion of ABCA1 is associated with enhanced A β peptide deposition in the brain (Fitz et al., 2012; Hirsch-Reinshagen et al., 2005; Koldamova et al., 2005a; Wahrle et al., 2005). Nevertheless, the cellular and molecular mechanisms that underlie these processes have yet to be described in detail. We and others have suggested that the LXR signaling pathway may affect the transport of A β peptides across the BBB (probably via an ABCA1-dependent process). A β peptides cross the BCECs in both apical-to-basolateral and basolateral-to-apical directions (Zlokovic, 2008). We previously investigated the apical-to-basolateral transport of A β peptides (i.e. influx into the brain) (Candela et al., 2010) using an in vitro BBB model (Cecchelli et al., 2007; Dehouck et al., 1992). We showed that the apical-to-basolateral entry of these peptides is mediated by the receptor for advanced glycation end-products (RAGE) and is restricted by breast cancer resistance protein (BCRP, also known as ABCG2) and P-glycoprotein (P-gp or ABCB1) (Candela et al., 2010). These receptors/transporters are expressed on the luminal side of BCECs (de Vries et al., 2007; Deane et al., 2003; Gosselet et al., 2009; Tai et al., 2009b). More recently, multidrug resistance associated protein 1 (MRP1 or ABCC1) was reported to be involved in the cerebral clearance of A β peptides (Krohn et al., 2011). Modulation of expression and/or activity of these receptors/transporters is considered to be a promising therapeutic strategy in AD, with a view to limiting A β peptide entry into the brain. For example, Deane and collaborators

elegantly used and designed a novel, specific RAGE inhibitor (FPS-ZM1) to produce a marked reduction of A β peptide influx across the BBB and thus a decrease in the brain's A β burden (Deane et al., 2012). In contrast, several studies of ABCB1 or ABCC1 have clearly shown that increasing the transporter's activity or transcription levels leads to a reduction in the brain's amyloid burden (Hartz et al., 2010; Krohn et al., 2011; Qosa et al., 2012).

With these considerations in mind, and given that the BBB is constantly exposed to 24S-OH-chol, 27-OH-chol (Bjorkhem, 2006) and A β peptide fluxes (Zlokovic, 2008), the present study involved the use of an in vitro BBB model to clarify how these two oxysterols can act as LXR agonists on A β peptide influx across BCECs.

2. Results

2.1 24S-OH-chol and 27-OH-chol are not toxic for BCECs in vitro (up to 10 μ M)

We first assessed the toxicity of different concentrations (0-10 μ M) of 24S-OH-chol and 27-OH-chol in BCECs. Oxysterols were added to the compartment in which they are usually generated, i.e. the abluminal compartment (representing the brain) for 24S-OH-chol and the luminal side (representing the blood) for 27-OH-chol. After a 24-hour treatment, the endothelial permeability (Pe) of Lucifer Yellow (a BBB integrity marker) was evaluated, as previously described (Culot et al., 2008; Hallier-Vanuxeem et al., 2009). As shown in Fig. 1, there were no significant changes in Pe values for the oxysterol concentrations tested here (0-10 μ M). The same results were obtained with 0-10 μ M T0901317, a well-known synthetic LXR agonist (Repa et al., 2000). Hence, a concentration of 10 μ M was used in subsequent experiments.

2.2 Expression of ABCA1 is upregulated by LXR agonists

We have previously demonstrated that BCECs express LXRs, ABCA1 and SCARB1 (Gosselet et al., 2009; Saint-Pol et al., 2012). In the present study, we used semi-quantitative RT-PCR to investigate the ability of 24S-OH-chol and 27-OH-chol to stimulate this pathway in BCECs (Fig. 2A). The primers are described in Table 1. The expression levels of LXR- α , LXR- β and SCARB1 were not modified by 24h of treatment with various doses of either 24S-OH-chol or 27-OH-chol. In contrast, expression levels of ABCA1 were modified (Fig. 2A). Using real-time RT-PCR, we then quantified the upregulation of ABCA1 mRNA expression (7.17-fold for 10 μ M 24S-OH-chol and 4.73-fold for 10 μ M 27-OH-chol, relative to control conditions) and confirmed that LXR stimulation did not modify SCARB1 expression (Fig. 2B). Immunoblot assays performed on the BCEC membrane-enriched fraction gave the same results in terms of protein levels (Fig. 2C).

2.3 Oxysterol-mediated ABCA1 expression increases cholesterol release to ApoA-I and HDL particles

To investigate the impact of ABCA1 induction on cellular cholesterol efflux, we then studied ABCA1's ability to release cholesterol to ApoA-I and HDL particles. In the absence of oxysterol treatment, BCECs loaded with radioactive cholesterol were able to efflux a small proportion to the medium (Fig. 3A) ($4.86 \pm 0.7\%$, for a bovine serum albumin (BSA)-only control condition). Addition of lipid-free ApoA-I particles did not significantly increase the extent of release ($4.02 \pm 0.19\%$ versus the control condition), whereas HDL particles increased release by up to 5-fold, compared with control condition ($21.46 \pm 0.73\%$). In the absence of cholesterol acceptors, cholesterol efflux was greater after 24h of treatment with $10 \mu\text{M}$ of 24S-OH-chol or 27-OH-chol (Fig. 3B) ($160.72 \pm 9.17\%$ and $131.17 \pm 13.69\%$, respectively, compared with dimethyl sulfoxide (DMSO) only). Fig. 3C shows that both these oxysterols also increased cellular cholesterol release in the presence of ApoA-I ($204.77 \pm 17.65\%$ for 24S-OH-chol and $209.03 \pm 11.57\%$ for 27-OH-chol, compared with DMSO). Oxysterol stimulation also promoted cellular cholesterol efflux in the presence of HDL particles in the medium ($146.45 \pm 0.68\%$ for 24S-OH-chol and $119.45 \pm 5.02\%$ for 27-OH-chol, compared with the control) (Fig. 3D).

2.4 Apical-to-basolateral transport of soluble A β peptides is decreased by LXR stimulation

We previously used our in vitro BBB model to investigate apical-to-basolateral (i.e. blood-to-brain) transport of A β peptides (Candela et al., 2010). Since 24S-OH-chol and 27-OH-chol are also exchanged across BCECs, we decided to assess the impact of the two oxysterols on A β peptides and inulin influx across BCECs. Inulin is used as a non-specific vesicular transport marker (Brillault et al., 2002; Candela et al., 2010; Saint-Pol et al., 2012). As shown in Fig. 4, both oxysterols decreased significantly the apical-to-basolateral transport of naïve forms of A β_{1-40} (diminution of $14.7 \pm 1.71\%$ for 24S-OH-chol and $15.77 \pm 1.63\%$ for 27-OH-chol, compared with untreated condition) and A β_{1-42} peptides (diminution of $26.16 \pm 4.42\%$ and $24.86 \pm 5.7\%$, respectively) across BCECs. Transport of [^3H]-inulin was not significantly modified after LXR

pathway stimulation with 10 μ M 24S-OH-chol ($103.88 \pm 3.78\%$, compared with DMSO) or 10 μ M 27-OH-chol ($103.76 \pm 2.15\%$).

2.5 ABCA1 is not involved in A β peptide influx across BCECs

Given that 24S-OH-chol and 27-OH-chol were found to induce significant ABCA1 expression, we then investigated the compounds' possible roles in the decrease in A β peptide apical-to-basolateral transport across BCECs reported in Fig. 4. We used probucol (a specific ABCA1 inhibitor (Favari et al., 2004; Saint-Pol et al., 2012)) to clarify ABCA1's involvement in this process. As in previous studies (Do et al., 2011; Saint-Pol et al., 2012; Stefulj et al., 2009), probucol was used at a concentration of 10 μ M and was not toxic for BCECs at this concentration (data not shown). As shown in Fig. 5, inhibition of ABCA1 did not modify the decrease in A β peptide influx mediated by 24S-OH-chol ($67.24 \pm 2.41\%$ and $67.69 \pm 4.29\%$ in the presence and absence of probucol) or 27-OH-chol ($68.12 \pm 4.9\%$ and $72.41 \pm 1.84\%$ in the presence and absence of probucol), suggesting that ABCA1 is not directly involved in A β peptide influx across BCECs. Probucol treatment did not modify [3 H]-inulin influx across BCECs (data not shown).

2.6 ABCB1 expression and activity are increased after treatment with oxysterols

We have previously used the present in vitro BBB model to show that A β peptide influx across BCECs is mediated by RAGE and is restricted by ABCB1 and ABCG2 (Candela et al., 2010). More recently, involvement of ABCC1 in A β peptide transport across the BBB has been suggested as to be much more important than other ABC transporters (Krohn et al., 2011). Given that ABCA1 is not responsible for the decrease in A β peptides observed after oxysterol treatment (Fig. 5), we then studied the expression of these four A β peptide receptors/transporters after 24 hours of treatment with 10 μ M of 24S-OH-chol or 27-OH-chol (Fig. 6A). An RT-qPCR analysis indicated that transcriptional expression of RAGE, ABCC1 and ABCG2 were not modulated by oxysterol treatment, whereas ABCB1 expression was 1.8-fold and 1.7-fold greater (compared with

the control condition) after treatment with 24S-OH-cholesterol and 27-OH-cholesterol, respectively. Therefore, the ABCB1 efflux pump is a potential target gene in the oxysterol-induced reduction of A β peptide influx across BCECs. ABCB1 induction by oxysterols was confirmed by immunoblot experiments in BCEC membrane-enriched fractions, which revealed that ABCB1 was respectively 3-fold, 2.7-fold and 2.3-fold more abundant after treatments with 24S-OH-cholesterol, 27-OH-cholesterol and T0901317 (Fig. 6B). To confirm ABCB1's involvement in this oxysterol-modulated process, we investigated its post-oxysterol activity by using colchicine, a well-known substrate of ABCB1 (Decleves et al., 2006). As shown in Fig. 6C, [3 H]-colchicine influx across BCECs was reduced by treatment for 24h with 10 μ M 24S-OH-cholesterol and 10 μ M 27-OH-cholesterol ($84.55 \pm 3.03\%$ and $83.05 \pm 1.49\%$, respectively, relative to the untreated condition), demonstrating that both oxysterols increase ABCB1 expression and activity and thus that the transporter may be involved in the restriction of A β peptide influx across BCECs.

2.7 ABCB1 is involved in the oxysterol-mediated reduction in A β peptide influx across BCECs

When ABCB1 is active, it limits substrate influx across BCECs and decreases the compounds' accumulation within the cell. Hence, inhibition of this efflux pump leads to an accumulation of its substrates in BCECs and a progressive increase in influx processes (Fenart et al., 1998). To inhibit ABCB1, we used A β peptides as competitive inhibitors (Kuhnke et al., 2007) and measured [3 H]-colchicine accumulation and influx across BCECs. In each condition, competition with A β peptides led to an almost 2-fold increase in [3 H]-colchicine accumulation in BCECs, when compared with the same condition in the absence of A β peptides ($212.20 \pm 1.3\%$, $201.03 \pm 10.68\%$ and $211.03 \pm 21.14\%$ for the DMSO, 24S-OH-cholesterol and 27-OH-cholesterol conditions, respectively) (Fig. 7A). In presence of A β peptides, significant increases in [3 H]-colchicine influx across BCECs ($129.96 \pm 15.61\%$, $140.85 \pm 6.92\%$ and $132.17 \pm 7.01\%$ for the DMSO, 24S-OH-cholesterol and 27-OH-cholesterol conditions, respectively) (Fig. 7B) were observed. When reverse experiments were performed (Fig. 7C), increases of apical-to-basolateral transports of A β peptides were

measured in presence of colchicine ($135.42 \pm 2.23\%$, $142.18 \pm 3.39\%$ and $115.78 \pm 3.95\%$ for the DMSO, 24S-OH-chol and 27-OH-chol conditions, respectively). Altogether, these results confirm that LXR stimulation by oxysterols restricts A β peptide influx across BCECs via what is probably an ABCB1-mediated process.

Discussion

The results of several recent studies suggest that the LXR/ABCA1 axis is an attractive therapeutic approach in AD. Firstly, amyloid plaque load and neuro-inflammation are exacerbated in *lxr* knock-out mice (Zelcer et al., 2007). Furthermore, overexpression of *Abca1* decreases the amyloid burden (Wahrle et al., 2008), whereas *Abca1* deletion promotes the AD phenotype (Fitz et al., 2012; Hirsch-Reinshagen et al., 2005; Koldamova et al., 2005a; Wahrle et al., 2005). Lastly, stimulation of the LXR by natural or synthetic agonists increases ABCA1 expression and decreases A β peptide accumulation, synthesis and clearance (Burns et al., 2006; Donkin et al., 2010; Fitz et al., 2010; Koldamova et al., 2005b; Lefterov et al., 2007; Riddell et al., 2007; Terwel et al., 2011). Although it is not clear how ABCA1 affects A β peptide proteostasis, the latter results have prompted the hypothesis whereby this lipid transporter may be directly involved in bidirectional A β peptide transport across the BBB. Brain capillary endothelial cells (BCECs) and the surrounding brain pericytes express the receptors and ABC transporters directly involved in the exchange of A β peptides between brain and blood compartments (Zlokovic, 2008). Although previous reports state that ABCA1 was not involved in cerebral efflux of naïve form of A β peptides (Akanuma et al., 2008b; Saint-Pol et al., 2012), the putative link between LXR/ABCA1 axis stimulation and A β peptide influx into the brain has never (to the best of our knowledge) been investigated. This influx must not be neglected, since an elegant study by Zlokovic's group demonstrated that reducing A β entry into the brain may be also a valid strategy for preventing amyloid burden and cognitive deficits in AD transgenic mice (Deane et al., 2012).

With these considerations in mind, we designed the present study to characterize the effect of the LXR stimulation by natural and synthetic agonists on cholesterol cellular efflux and A β peptide influx across the BBB. The model used in this study consists of a co-culture of bovine BCECs with glial cells (Cecchelli et al., 2007; Dehouck et al., 1990) and has previously been used to investigate the process of influx of naïve forms of A β peptides into the brain (Candela et al., 2010).

We first assessed the toxicity of natural (24S-OH-cholesterol and 27-OH-cholesterol) and synthetic (T0901317) LXR agonists on BBB permeability. In our culture conditions, no toxicity was observed following 24 hours of treatment with a range of concentrations (0-10 μ M). This finding agrees with previous studies in porcine BCECs (Panzenboeck et al., 2006), brain pericytes (Saint-Pol et al., 2012) and other cells, in which a toxic effect beyond a concentration of 25 μ M was evidenced (Kolsch et al., 2001; Riendeau and Garenc, 2009).

Our results also showed an increase in ABCA1 expression in bovine BCECs after oxysterol treatment. ABCA1 is the major LXR target gene (Whitney et al., 2002) and is known to be a key ABC transporter involved in brain cholesterol homeostasis (Hirsch-Reinshagen et al., 2005; Wong et al., 2006). This ABC transporter is widely expressed by central nervous system cells (Abildayeva et al., 2006), choroid plexus epithelial cells (Fujiyoshi et al., 2007) and BBB cells such as brain pericytes (Saint-Pol et al., 2012) and BCECs (Akanuma et al., 2008a; Gosselet et al., 2009; Panzenboeck et al., 2002). In line with previous studies, our data show that oxysterol-mediated upregulation of ABCA1 in BCECs is correlated with an increase in cholesterol efflux to ApoA-I and HDL particles (Do et al., 2011; Panzenboeck et al., 2002; Panzenboeck et al., 2006). Furthermore, our previous results on brain pericytes demonstrated that 24S-OH-cholesterol-mediated ABCA1 expression was also associated with an increase in cholesterol efflux from pericytes to ApoA-I and HDL particles (Saint-Pol et al., 2012). Taken as a whole, these data strongly reinforce the hypothesis in which the BBB plays a major role in brain and peripheral cholesterol homeostasis (Karasinska et al., 2009).

Furthermore, our results showed that treatment with oxysterols provoked a decrease in the influx of soluble forms of A β peptides across BCECs (in the apical-to-basolateral direction) and thus could reduce A β entry into the brain and limit its further accumulation in this compartment. Importantly, this effect was observed using the naïve A β peptides that are very frequently used in transport studies. It is noteworthy that A β peptides reportedly interact with several apolipoproteins (apoE, apoA-I) and other molecules such as α -macroglobulin 2. It remains to be seen whether influx

of these complexes is also decreased. Although ABCA1 is upregulated after stimulation of the oxysterol-mediated LXR pathway, we demonstrated that this transporter was not directly involved in the decrease in A β peptide influx across BCECs. In line with two previous reports focusing on the efflux of naïve forms of A β peptides across the BBB, these data suggest that ABCA1 is not directly involved in A β peptide uptake or influx. Firstly, a study of *Abca1*^{-/-} mice found that the lack of ABCA1 did not affect the efflux of a naïve form of A β peptides across the BBB (Akanuma et al., 2008b). Secondly, our previous study demonstrated that brain pericytes (the first cell type encountered by A β peptides prior to their elimination from the brain across the BBB) also express ABCA1 but that transporter is not involved in A β uptake or cellular efflux (Saint-Pol et al., 2012).

To understand how LXR stimulation by oxysterols decreases A β peptide influx into BCECs, we then observed the oxysterols' effects on RAGE, ABCB1, ABCC1 and ABCG2 expressed by these cells. It is now accepted that RAGE mediates apical-to-basolateral transport of naïve A β peptide across BCECs (Candela et al., 2010; Deane et al., 2003), whereas this transport is restricted by ABCB1, ABCG2 and probably ABCC1 (Candela et al., 2010; Krohn et al., 2011; Tai et al., 2009a). We found that LXR stimulation increased transcriptional and proteins levels of ABCB1 but not RAGE, ABCC1 and ABCG2. Furthermore, activity assays using the ABCB1 substrate colchicine (Balayssac et al., 2005) demonstrated that (as had been observed for A β peptides) the influx of this molecule across BCECs was reduced by oxysterol treatment. Competition between colchicine and A β peptides led to an accumulation of colchicine in BCECs and an increase in apical-to-basolateral transport of this molecule across BCECs, confirming that oxysterol-induced expression of ABCB1 is correlated with an increase in its activity in BCECs. Reverse experiments give the same conclusion. These results demonstrate that the ABCB1 efflux pump is involved in oxysterol-mediated restriction of naïve forms of A β peptides across BCECs.

As mentioned above, limiting the entry of A β peptides into the brain compartment is a pertinent therapeutic approach in AD (Deane et al., 2012). ABCB1 is a key ABC transporter involved in the restriction of A β peptide influx across the BBB and postmortem investigations of

brain tissue have shown that ABCB1 expression levels in vessels surrounding A β plaques (Vogelgesang et al., 2002) and hippocampal blood vessels (Wijesuriya et al., 2010) are lower in AD patients than in age-matched patients. Moreover, ABCB1 is less active in AD patients than in non-AD patients (van Assema et al., 2012). Thus, targeting ABCB1 expression and/or its activity appears to be a potentially valid way of limiting A β peptide accumulation in the brain. Moreover, previous research has demonstrated that increasing the ABCB1 expression at the BBB level leads to a further decrease in the brain A β burden (Hartz et al., 2010; Qosa et al., 2012). Our present data showed that the decrease in amyloid burden after LXR stimulation by oxysterols is probably due to upregulation of ABCB1 expression/activity in BCECs. Although it is not known how LXR stimulation regulates ABCB1, this process appears to be specific to the BCECs because our previous research did not observe these effects in brain pericytes (Saint-Pol et al., 2012). Upregulation of ABCB1 by LXR agonists has been observed in brain capillaries and blood monocyte (Elali and Hermann, 2012; Langmann et al., 2006) but it is not yet clear understand how LXRs contribute to this process in molecular terms. It is likely that other nuclear receptors expressed by BCECs (Akanuma et al., 2008a; Panzenboeck et al., 2006) (such as the pregnane and farnesoid X receptors) are involved in this process. Thus, LXR pathway stimulation by agonists such as oxysterols may not only be beneficial in the context AD but also represents a new potential therapeutic approach for restoring and/or optimizing the restriction of ABCB1 substrate entry into the brain.

In conclusion, the present study demonstrated the impact of LXR pathway stimulation by 24S-OH-chol and 27-OH-chol in BCECs on (i) ABCA1-mediated cholesterol homeostasis and (ii) the restriction of A β peptide influx from the blood to brain by an ABCB1-mediated process and thus confirmed the strong relationship between LXR nuclear receptors and AD. Taken as a whole, these data reinforce previous suggestions that stimulation of LXR receptors represents a new therapeutic strategy for protecting the brain against the characteristic amyloid burden in AD.

4. Experimental Procedure

4.1. Chemicals

24S-hydroxycholesterol (24S-OH-chol), 27-hydroxycholesterol (27-OH-chol) and T0901317 were respectively purchased from Enzo Life Science (Villeurbanne, France), Avanti Polar Lipids (Alabaster, AL, USA) and Sigma (St Louis, MO, USA). The compounds were dissolved in DMSO (Sigma) at concentrations of 24.8 mM, 12.4 mM and 10.4 mM, respectively. Apolipoprotein A-I (ApoA-I) and high density lipoproteins (HDLs) were purchased from VWR (Fontenay-sous-Bois, France). Bovine serum albumin (BSA), Lucifer Yellow (LY) and colchicine were also purchased from Sigma. [³H]-cholesterol (43 Ci/mmol), [³H]-colchicine (85.3 Ci/mmol), [¹⁴C]-sucrose (630 mCi/mmol) and [³H]-inulin (1.25 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA, USA). Ultrapure forms of A β ₁₋₄₀ and A β ₁₋₄₂ peptides were purchased from Life Technologies SAS (Saint Aubin, France) and resuspended as previously described (Candela et al., 2010). A β ₁₋₄₀ and A β ₁₋₄₂ ELISA kits were also purchased from Life Technologies SAS. Fluorescein isothiocyanate (FITC)-labeled A β ₁₋₄₀ peptide was purchased from rPeptide (Bogart, GA, USA) and was used freshly resuspended in 1% NH₄OH according to the manufacturer's instructions (Candela et al., 2010).

4.2. Cell culture

The in vitro BBB model used in the present study was based on a co-culture of bovine brain capillary endothelial cells (BCECs) and rat glial cells that induce BBB properties (Cecchelli et al., 2007). All animal experiments were performed in compliance with the French Veterinary Council's guidelines.

Briefly, glial cells were extracted from parietal cortexes of newborn rats and seeded (after the removal of meninges and mechanical dissociation) on the bottom of uncoated, six-well plates containing Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FCS (fetal calf serum, Life Technologies), 2 mM L-glutamine (Sigma) and 50 μ g/mL gentamicin

(Biochrom A.G., Berlin, Germany). Three weeks after seeding, the cells were confluent and were used for co-culture.

Next, BCECs were extracted and isolated from bovine brain capillaries and characterized as previously described (Candela et al., 2010; Dehouck et al., 1990; Dehouck et al., 1992). For cell culture, BCECs were seeded in 60 mm gelatin-coated dishes containing DMEM supplemented with 10% (v/v) horse serum (HS, Life Technologies), 10% (v/v) calf serum (CS, Integro b.v., Zaandam, The Netherlands), 2 mM L-glutamine and 50 µg/mL gentamicin and 1 ng/mL of basic fibroblast growth factor (HS/CS). At confluence, BCECs were ready to be used for co-culture. Filters (Costar Transwell®, pore size 0.4 or 3 µm, Corning, Avon, France) were coated with rat-tail collagen and set in six-well plates containing the glial cells. Endothelial cells were seeded on the upper side of filters. The medium was changed every two days. The endothelial cells showed BBB properties after 12 days of co-culture (Cecchelli et al., 2007).

4.3. Evaluation of BCEC permeability after treatment with compounds

The integrity of tight junctions after 24h of treatment with different doses (0-10 µM) of either 24S-OH-chol (in the abluminal compartment), 27-OH-chol, T0901317 or probucol (in the luminal compartment) was checked by calculating the endothelial permeability coefficient (P_e) of LY (50 µM per filter), as previously described (Cecchelli et al., 1999). Three filters were used per condition and LY concentrations were measured with a spectrophotometer (Biotek, Colmar, France).

4.4. mRNA extraction and PCR analysis

After 12 days of co-culture, BCECs were treated for 24h with 24S-OH-chol or 27-OH-chol (0-10 µM). Next, cells were rinsed twice with cold calcium- and magnesium free phosphate buffered saline (PBS-CMF: 8 g/L NaCl, 0.2 g/L KCl, 0.2 g/L KH₂PO₄, 2.87 g/L NA₂HPO₄(12H₂O), pH 7.4) and lysed with RNeasy lysis buffer (Qiagen, Valencia, CA, USA). Lysates were frozen at -

20°C prior to thawing for total RNA extraction. mRNAs were extracted according to the Qiagen RNeasy Mini Kit protocol and were assayed by measuring absorbance at 260, 280 and 320 nm with a Tek3 microplate reader protocol (Synergy™ H1, Biotek). cDNAs were obtained from 0.5 µg of mRNA using iScript™ Reverse Transcription Supermix (BioRad, Marnes-la-Coquette, France), according to the manufacturer's instructions. Semi-quantitative cDNA amplification analysis was performed by RT-PCR using custom-designed primers (reported in Table 1) and as previously described (Saint-Pol et al., 2012). Amplification (number of cycles/annealing temperature) was carried out as follows for each gene : ABCA1 (32/55°C), SCARB1 (30/55°C), LXR- α (30/55°C), LXR- β (30/50°C) and β -actin (25/55°C). All cDNA amplification products were then sequenced using the Sanger method (GenoScreen, Lille, France). Quantitative amplification (qPCR) of cDNA was performed using Sso Fast EvaGreen Master Mix (BioRad) and custom-designed primers (Table 1). Amplification was carried out for 40 cycles with an annealing temperature of 60°C using a CFX96 thermocycler (BioRad). The efficiency was determined for each primer pair and used in the calculation method (CFX Manager, BioRad). Melting curve analysis was performed after the amplification cycles, in order to check the specificity/purity of each amplification. Gene expression levels were evaluated according to the $\Delta\Delta C_t$ method and were normalized against β -actin expression.

4.5. Immunoblots

Brain capillary endothelial cells were seeded on Transwell® filters coated with rat tail collagen. The filters were then set into dishes with glial cells. After 12 days of co-culture, BCECs were treated for 24h with DMSO vehicle (as a control) or 10 µM of either T0901317, 24S-OH-chol or 27-OH-chol. Cells were then rinsed twice with warm PBS at 37°C and then incubated for 5 min with 1 mg/mL of collagenase. Cells were centrifuged at 2500 rpm at 4°C for 8 min. Pellets were resuspended with a pestle in 20 mM tricine/250 mM sucrose/1 mM EDTA buffer. The plasma membrane fraction was isolated as previously described (Saint-Pol et al., 2012). Protein extracts

were assayed using the Bradford method; 8 µg of extract were then electrophoresed on 7.5% sodium dodecyl sulfate polyacrylamide gel (BioRad) and electrotransferred onto nitrocellulose membranes (GE Healthcare, Saclay, France). After a 90-min incubation in blocking buffer (25 mM Tris-HCl (pH 8.0), 125 mM NaCl, 0.1% Tween 20, and 5% skimmed milk), the membranes were incubated with primary antibody (anti-ABCA1 (2 µg/mL, Abcam, Cambridge, UK), anti-SCARB1 (0.4 µg/mL, Abcam) or anti-Pgp (5 µg/mL, GeneTex, Paris, France)) overnight at 4°C, rinsed three times with blocking buffer and incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Dako) for 1h at room temperature. The HRP was assayed with an enhanced chemiluminescence kit (GE Healthcare) and revealed on chemiluminescence-sensitive films (GE Healthcare). The bands' optical densities were measured using TotalLab TL 100 1D Gel Analysis software (Nonlinear Dynamics, Newcastle, UK).

4.6. Apical-to-basolateral transport of Aβ peptides

After a 24h-treatment with either DMSO (the control condition, 1/400) or 10 µM 24S-OH-cholesterol or 27-OH-cholesterol, Aβ peptide influx (apical-to-basolateral transport) experiments were performed as previously described (Candela et al., 2010). Firstly, filters bearing BCECs were rinsed for 10 minutes in pH 7.4 Ringer HEPES buffer (RH; HEPES 5 mM; NaHCO₃ 6 mM; NaCl 150 mM; KCl 5.2 mM; CaCl₂ 2.2 mM; MgCl₂ + 6H₂O 1.2 mM) supplemented with 0.5% BSA, and transferred into six-well plates containing 2.5 mL RH/0.5% BSA in each well. Next, 1.5 mL RH/0.5% BSA/LY with 12 nM of either [³H]-inulin or ultrapure Aβ peptides were placed on the filters. During the 2-hour transport assay, cells were incubated at 37°C with very slight agitation. The BBB's permeability and non-receptor-mediated transport were respectively evaluated by measuring LY permeability (see below) and [³H]-inulin transport with a liquid scintillation counter (TriCarb 2100TR, PerkinElmer). Radioactivity was measured as disintegrations per minute (DPM). Each form of Aβ peptides (Aβ₁₋₄₀ and Aβ₁₋₄₂) was assayed with appropriate ELISA kits (Life Technologies), according to the manufacturer's instructions. Briefly, a monoclonal antibody

specific for the NH₂-terminus of A β ₁₋₄₀ and A β ₁₋₄₂ peptides was coated onto the wells. Next, a rabbit antibody specific for the COOH-terminus of the A β ₁₋₄₀ or A β ₁₋₄₂ sequences was co-incubated with samples. The mass balance was calculated in order to estimate peptide aggregation and degradation. Experiments with at least 90% of A β peptide mass balance were taken into consideration. The percentage passage calculation consisted in dividing the amounts of [³H]-inulin or A β peptides in the basolateral compartment (modeling the brain) by the initial concentration added to the apical compartment (modeling the blood). To consider the influence of the filter and the collagen coating on molecular transport, the percentage passage was calculated in the presence and absence of BCECs. The final transport percentage was thus calculated by dividing the value obtained in the presence of cells by the value obtained in the absence of cells.

Since LY is the most commonly used control molecule for both [³H]-inulin and A β peptide conditions, the percentage of test compound across BCECs was evaluated according to the following equation:

$$\% \text{ "normalized" compound transport} = 100 \times (\% \text{ transport of a test compound across the filter}) / (\% \text{ LY transport across the filter}).$$

Each assay was performed using three filters and each experiment was performed three times.

For inhibition studies, A β ₁₋₄₀ peptide influx was assayed for 2h, as described below. RH/0.5% BSA/[¹⁴C]-sucrose (as BBB integrity marker) was supplemented with 12 nM FITC-labeled A β ₁₋₄₀ or [³H]-inulin in the presence or absence of 10 μ M probucol. As influx studies using ultrapure forms of A β peptides:

$$\% \text{ "normalized" FITC-A}\beta_{1-40} \text{ peptide or } [^3\text{H}]\text{-inulin transport} = 100 \times (\% \text{ FITC-A}\beta_{1-40} \text{ transport or } [^3\text{H}]\text{-inulin transport across the filter in the presence or absence of probucol}) / (\% [^{14}\text{C}]\text{-sucrose transport for the corresponding filter}).$$

Each assay was performed using three filters and each experiment was performed at least twice.

4.7. [³H]-Cholesterol efflux studies

Radioactive HS/CS medium was obtained by incorporating [³H]-cholesterol (0.5 μCi/mL) with HS and CS for 6 h at 37°C. Cells were incubated for 36 h with this medium to allow incorporation of the labeled cholesterol. Cells were then rinsed twice with pre-warmed PBS-CMF buffer and once with pre-warmed DMEM/0.1% BSA. Next, the cells were equilibrated in DMEM/0.1% BSA for 24 h. During this step, 10 μM of 24S-OH-chol or 27-OH-chol were added in the compartment in which they are usually generated in vivo (ie. abluminal compartment (brain) for 24S-OH-chol and luminal compartment (blood) for 27-OH-chol). Cells were rinsed once with pre-warmed DMEM/0.1% BSA. ApoA-I (20 μg/mL) or HDLs (50 μg/mL) were then added for 8 h. After this period, apical and basolateral media were collected and cell debris was removed by centrifugation at 4000 rpm for 4 min at 4°C. The cells were washed four times with cold RH buffer and lysed with RH/1% Triton X-100. Aliquots of cell lysates and both apical and basolateral media were analyzed in a liquid scintillation counter (Tri-Carb 2100TR). Cellular cholesterol efflux was calculated according to the following equation:

$$\% \text{ total efflux} = (\text{DPM}_{\text{apical}} + \text{DPM}_{\text{basolateral}}) \times 100 / (\text{DPM}_{\text{apical}} + \text{DPM}_{\text{basolateral}} + \text{DPM}_{\text{cell lysate}}).$$

Each assay was performed using three filters and each experiment was run at least three times.

4.8. ABCB1 activity assay

Brain capillary endothelial cells were first treated for 24h with either DMSO (the control condition) or 10 μM 24S-OH-chol or 27-OH-chol. [³H]-colchicine was used as the ABCB1 substrate (Balayssac et al., 2005). Before starting apical-to-basolateral transport experiments, filters containing BCECs were rinsed for 10 minutes in RH buffer supplemented with 0.5% BSA and then transferred into six-well plates containing 2.5 mL RH/0.5% BSA per well. Experiments started when 1.5 mL RH/0.5% BSA/LY with 50 nM [³H]-colchicine was added to the luminal compartment. During the 2-hour transport assay, cells were incubated at 37°C with very slight agitation. Apical and basolateral media were collected and measured for LY and [³H]-colchicine amounts. Cells were rinsed four times in cold RH/0.5% BSA and lysed with RH/0.5% BSA/1%

Triton X-100. The BBB's permeability was evaluated by measuring LY permeability. The [³H]-colchicine radioactivity was measured in DPM. As mentioned for Aβ peptide influx experiments, we considered the influence of the filter and the collagen coating on molecular transport and so calculated the percentage passage in the presence and absence of BCECs. The percentage of [³H]-colchicine influx across BCECs was evaluated according to the following equation:

$$\% \text{ "normalized" } [^3\text{H}]\text{-colchicine transport} = 100 \times (\% [^3\text{H}]\text{-colchicine transport across the filter}) / (\% \text{ LY transport across the filter}).$$

Each assay was performed using three filters and each experiment was performed at least twice. For competition assays, 60 nM of ultrapure Aβ₁₋₄₀ competed with 50 nM [³H]-colchicine as a ABCB1 substrate. Transport was evaluated as previously described:

$$\% \text{ "normalized" } [^3\text{H}]\text{-colchicine transport} = 100 \times (\% [^3\text{H}]\text{-colchicine transport across the filter in the presence or absence of ultrapure A}\beta_{1-40}) / (\% \text{ LY transport across the corresponding filter}).$$

In reverse experiments, apical-to-basolateral transport of 12 nM of ultrapure Aβ₁₋₄₀ was measured in presence of colchicine (240 nM). Experiments and calculation method were similar to what is described above.

Each assay was performed using three filters, and each experiment was performed at least twice.

4.9. Statistical analysis

All statistical analyses were performed with Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA), using a one-way analysis of variance (ANOVA) and then appropriate post hoc tests. Data are reported as the mean ± SD or (where specified) the mean ± SEM.

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Figure legends:

Table 1: List of the custom primers used for PCR analysis

From left to right: cDNA targeted for PCR amplification, species used for primer design, size (in bp) of the amplification products, forward (F) and reverse (R) primer sequences, cDNA accession numbers from the NCBI database.

Fig. 1: Brain capillary endothelial cell integrity after 24S-OH-cholesterol, 27-OH-cholesterol or T0901317 treatment. The cells were pre-treated for 24h with 0 μ M, 0.01 μ M, 0.1 μ M, 1 μ M or 10 μ M of 24S-OH-cholesterol, 27-OH-cholesterol or T0901317. Cells were incubated for 1h (with a measurement every 20 min) with LY (an integrity marker for BCECs). Each point represents Pe LY, relative to the control (vehicle-treated) condition. n=6.

Fig. 2: 24S-OH-cholesterol and 27-OH-cholesterol induce ABCA1 expression in BCECs. Cells were pre-treated for 24h with DMSO (control) or different concentrations of 24S-OH-cholesterol (abluminal treatment) and 27-OH-cholesterol (luminal treatment). The LXR, ABCA1 and SCARB1 mRNAs (A) were analyzed using RT-PCR with the primers listed in Table 1. The ABCA1 and SCARB1 mRNAs were then quantified (B) using RT-qPCR. Each bar represents mRNA expression normalized against the housekeeping gene β -actin. The results correspond to the mean \pm SEM of two experiments pooled from three filters. Statistical analysis was performed using regular two-way ANOVA with Tukey's multiple comparisons tests. Protein expression levels for ABCA1 and SCARB1 (C) were assessed by immunoblotting of a membrane-enriched BCEC fraction, as described in the Experimental Procedure. T0901317 (10 μ M) was used as a positive control for LXR pathway stimulation. SCARB1 was used as a membrane control, since its expression was not regulated by either of the oxysterols.

Fig. 3: LXR pathway activation by natural ligands leads to an increase in BCEC cholesterol release to ApoA-I or HDL particles. The BCECs were first labeled with [³H]-cholesterol (0,5 μCi/mL) for 36h at 37°C and then equilibrated for 24h in DMEM/0.1% BSA at 37°C in either the absence of oxysterol (DMSO, control) or with 10 μM 24S-OH-chol or 10 μM 27-OH-chol. Cholesterol release was measured after 8h at 37°C in absence of any particles (BSA only) or in the presence of either ApoA-I (20 μg/mL) or HDL (50 μg/mL) particles. A represents cholesterol release from BCECs without oxysterol treatment (the control condition). The effect of oxysterol treatment is observed for the BSA-only condition (B), the ApoA-I condition (C) and the HDL condition (D). Each bar represents the mean ± SEM; n=12-18. NS: non-significant; *p<0.05; ***p<0.001 (in a one-way ANOVA, followed by Bonferroni's correction for multiple comparisons).

Fig. 4: Apical-to-basolateral transport (influx) of Aβ peptides across BCECs is decreased by both oxysterols. The BCECs were pre-treated at 37°C for 24h with either DMSO (the control condition) or 10 μM 24S-OH-chol or 27-OH-chol. Cells were then incubated for 2h with Aβ₁₋₄₀ or Aβ₁₋₄₂ peptides or with [³H]-inulin during gentle agitation at 37°C. Aβ peptides were assayed with ELISAs and [³H]-inulin was measured (in dpm) in a scintillation counter. Influx of Aβ peptides or [³H]-inulin was calculated first by dividing the amount measured in the basolateral (brain) compartment by the amount in the luminal (blood) compartment. These values were then divided by those obtained in the absence of cells. The final influx percentage was obtained by dividing each value by the corresponding Pe LY, in order to take account of any slight, post-treatment changes in monolayer integrity. Each bar represents the mean ± SEM; n=9-12. The percentages of apical-to-basolateral transport for Aβ₁₋₄₀ and Aβ₁₋₄₂ peptides were respectively 13.06 ± 0.85% and 12.98 ± 0.90%. NS: non-significant; **p<0.01; ***p<0.001 (in a one-way ANOVA, followed by Bonferroni's correction for multiple comparisons).

Fig. 5: ABCA1 is not involved in A β ₁₋₄₀ influx across BCECs. Cells were pre-treated with either DMSO (control), 24S-OH-cholesterol (10 μ M) or 27-OH-cholesterol (10 μ M) for 24h at 37°C. The cells were then incubated for 2h with FITC-A β ₁₋₄₀ or [³H]-inulin during gentle agitation at 37°C in the presence or absence of 10 μ M probucol. A β ₁₋₄₀ peptide or [³H]-inulin influx was calculated by dividing the amount measured in the basolateral (brain) compartment by the amount in the luminal (blood) compartment. These values were then divided by those obtained in the absence of cells. The final influx percentage was obtained by dividing each value by the corresponding Pe [¹⁴C]-sucrose, to take into account any slight post-treatment changes in monolayer integrity. Each bar represents the mean \pm SEM; n=6. NS: non-significant; *p<0.05 (in a one-way ANOVA, followed by Bonferroni's correction for multiple comparisons).

Fig. 6: Effect of 24S-OH-cholesterol and 27-OH-cholesterol on transporters involved in A β peptide influx across BCECs. Cells were pre-treated for 24h with DMSO (control), 24S-OH-cholesterol (10 μ M) or 27-OH-cholesterol (10 μ M). ABCB1, RAGE and ABCC1 and ABCG2 mRNA (A) were analyzed using RT-qPCR with the primers listed in Table 1. Each bar corresponds to the mean \pm SEM of mRNA expression normalized against β -actin. Statistical analysis was performed using regular two-way ANOVA with Tukey's multiple comparisons test. Protein expression of ABCB1 (B) were assessed by immunoblotting the membrane-enriched fraction of BCECs in the absence of treatment or treated for 24h with either 24S-OH-cholesterol (10 μ M), 27-OH-cholesterol (10 μ M) or T0901317 (10 μ M). SCARB1 was used as a loading control. (C) ABCB1 activity after treatment with 10 μ M 24S-OH-cholesterol or 27-OH-cholesterol was assessed by measuring the apical-to-basolateral transport of 50 nM [³H]-colchicine for 2h. Each bar corresponds to the mean \pm SEM; n=6. NS: non-significant; *p<0.05 (in a one-way ANOVA, followed by Bonferroni's correction for multiple comparisons).

Fig. 7: ABCB1 is involved in the oxysterol-mediated reduction of A β peptide influx. (A) Accumulation of [³H]-colchicine in BCECs and its influx (B) were measured after competition

experiments for 2h, with 60 nM ultrapure A β ₁₋₄₀ used as a competitive ABCB1 substrate. In (C), A β peptide (12 nM) apical-to-basolateral transport was also investigated in presence of colchicine (240 nM) as a competitive inhibitor. Each bar corresponds to the mean \pm SEM; n=6. *p<0.05; **p<0.01; ***p<0.001 (in a paired t-test).