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Apical-to-Basolateral Transport of Amyloid-β Peptides through Blood-Brain Barrier Cells is Mediated by the Receptor for Advanced Glycation End-Products and is Restricted by P-Glycoprotein

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Abstract. Several studies have highlighted the close relationship between Alzheimer's disease (AD) and alterations in the bidirectional transport of amyloid-β (Aβ) peptides across the blood-brain barrier (BBB). The brain capillary endothelial cells (BCECs) that compose the BBB express the receptors and transporters that enable this transport process. There is significant *in vivo* evidence to suggest that P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) restrict Aβ peptides entry into the brain, whereas the receptor for advanced glycation end-products (RAGE) seems to mediate apical-to-basolateral passage across the BBB. However, deciphering the molecular mechanisms underlying these *in vivo* processes requires further *in vitro* characterization. Using an *in vitro* BBB model and specific competition experiments against RAGE, we have observed a significant decrease in apical-to-basolateral (but not basolateral-to-apical) transport of Aβ_{1-40} and Aβ_{1-42} peptides through BCECs. This transport is a caveolae-dependent process and fits with the apical location of RAGE observed in confocal microscopy experiments. Inhibition of P-gp and BCRP using different inhibitors increases transport of Aβ peptides suggesting that these efflux pumps are involved in Aβ peptide transport at the BCECs level. Taken as a whole, these results demonstrate the involvement of the caveolae-dependent transcytosis of Aβ peptides through the BBB in a RAGE-mediated transport process, reinforcing the hypothesis whereby this receptor is a potential drug target in AD.

Keywords: Alzheimer's disease, amyloid-β peptide, BCRP, blood-brain barrier, brain capillary endothelial cells, caveolae, P-gp, RAGE

Supplementary data available online: http://www.j-alz.com/issues/22/vol22-3.html#supplementarydata02

INTRODUCTION

The aggregation and deposition of amyloid-β (Aβ) peptides in the brain as senile plaques are thought to be crucial events in the development of Alzheimer's disease (AD) and subsequent synaptic dysfunction

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and neuron loss [1]. Under normal circumstances, these peptides are cleared through proteinase-mediated degradation processes [2] and efflux across the blood-brain barrier (BBB) into the peripheral circulation [3]. Aβ peptides can also be imported from the blood into the brain across this barrier [4], suggesting that altered bidirectional transport of these peptides across the BBB could contribute to the development and/or severity of the disease process.

The BBB is mainly situated at the brain capillary level and is composed by a three-cell archetype comprising (i) brain capillary endothelial cells (BCECs), (ii) pericytes embedded in the basement membrane and (iii) astrocytic end-feet processes [5]. The BCECs' tight junction form a physical barrier and receptors expressed by these endothelial cells allow receptor-mediated transport (RMT) between the blood and the brain [5]. However, this process remains subject to debate in terms of the BBB and has not been extensively characterized. The receptor for advanced glycation end-products (RAGE, a multiligand receptor expressed in brain capillaries and microvessels) is thought to be involved in Aβ peptides entry into the central nervous system (CNS). Moreover, the up-regulation of this receptor observed in capillaries and microvessels in AD brains and transgenic models of AD suggests that RAGE expression is relevant to the pathophysiology of AD [4,6,7]. However, there is no direct proof of the role of RAGE in BCECs and its cellular location remains unknown. This lack of data on a potential drug target in AD emphasizes the need to use *in vitro* BBB models for deciphering the molecular mechanisms involved in these processes and exploring potential therapeutic applications.

On the other hand, P-glycoprotein (P-gp) expressed at the luminal (blood) surface of BCECs is suspected to restrict Aβ peptides entry into the CNS and mediates its efflux from brain. However, the *in vitro* and *in vivo* studies of the role of P-gp in Aβ peptide transport across the BBB have reported diverging results [8–11]. P-gp and the breast cancer resistance protein (BCRP) have a similar substrate overlap, and BCRP also acts at the BBB level to prevent molecules entry into the CNS.

To clarify the roles of P-gp, BCRP, and RAGE in AD, we investigated the involvement of these transporters/receptor in Aβ peptide transport across the BBB by using an *in vitro* model. This model consists of bovine BCECs co-cultured for 12 days with new-born rat glial cells to induce barrier properties [12]. The BCECs used in this model have been shown to express several receptors and transporters that are characteristic of the BBB *in vivo*, such as P-gp [13–15], LRP1 [15–17], melanotransferrin receptor [18], and multidrug resistance proteins (MRPs) [13]. The BBB model has recently enabled characterization of capillary RMT and transcytosis mechanisms [17–20].

Here, for the first time, we demonstrate that RAGE is exclusively expressed at the apical BCEC membrane and that this receptor is directly involved in apical-to-basolateral transport of Aβ peptides but not in basolateral-to-apical processes. This transport seems to have a caveolae-dependent mechanism, as has been described for other molecules [19,20]. Moreover, we also demonstrate that apical-to-basolateral transport of Aβ peptides are prevented in our BBB model by the efflux pumps P-gp and BCRP.

MATERIALS AND METHODS

Chemicals

[^{3}H]inulin (1.25 Ci/mmol) and [^{14}C]sucrose (630 mCi/mmol) were purchased from PerkinElmer Life And Analytical Sciences (Waltham, MA, USA). Lucifer Yellow (LY), Filipin, Rhodamine 123 (Rho123) and verapamil were purchased from Sigma (Lyon, France). GF120918 was kindly provided by Dr. Stefan Lundquist from AstraZeneca R&D. Fumitremorgin C (FTC) a specific inhibitor of BCRP, was purchased from COGER (Paris, France). Ultra pure forms of Aβ_{1-40} and Aβ_{1-42} peptides were purchased from Invitrogen (Cergy-Pontoise, France). Lyophilized peptides were reconstituted following manufacturer's instructions to avoid peptide aggregation. Briefly, peptides were dissolved in 100% HFIP (1,1,1,3,3,3-hexafluoro-2-propanol from Sigma) and dried. They were then resuspended in 100% DMSO (dimethylsulfoxyde from Sigma) and stored at $-80°$C in phosphate buffered saline buffer (PBS)/Bovine serum albumin (BSA) 5%/Tween 0.03% buffer. Fluorescent Aβ_{1-40} peptide was purchased from rPeptide (Bogart, Georgia, USA). The lyophilized powder was resuspended in 1% NH_4OH, sonicated 30 s and used the same day to avoid aggregation.

Cell culture and capillary fraction

The *in vitro* model of BBB consists of a co-culture of BCECs from bovine origin and rat glial cells, as previously described [12,21]. All animal experimentation was done according to the French Veterinary Council's guide. Briefly, primary cultures of newborn 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) fetal calf serum (FCS) (Integro, Zaandam, The Netherlands), 2 mM glutamine (Sigma) and 50 μg/mL gentamycin (Sigma). Three weeks after seeding, cultures of glial cells were confluent and ready to be used for co-culture.

BCECs were isolated and characterized as previously described [22]. For cell culture, endothelial cells were 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) (Gibco, Carlsbad, CA, USA) and 10% (v/v) horse serum (HS) (Hyclone Laboratories, Logan, UT, USA), 2 mM glutamine, 50 μg/mL gentamycin, and 1 ng/mL of basic-fibroblast growth factor (bFGF). At confluence, endothelial cells were harvested for the co-culture. Filters (Transwell®; pore size 3 μm) coated with rat-tail collagen prepared by a modification of the method of Bornstein were set in six-well plates containing glial cells [23]. Endothelial cells were plated on the upper side of the filters.

BBB permeability studies

For all experiments, tight junctions integrity was checked out by paracellular permeability studies using 50 μM of LY per upper chamber. Three inserts were used for each condition. LY was measured using a fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The endothelial permeability coefficient (Pe) was calculated in cm/min using method previously described [21]. Only experiment values where Pe was $< 1 \times 10^{-3}$ cm/min was taken into account as previously established [24,25].

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

Twenty-four hours after medium refreshing, BCECs solo- or co-cultured with glial cells were washed twice in sterile cold phosphate buffered saline buffer and lysed using RLT lysis buffer (Qiagen, Valencia, CA, USA). Three filters for BCECs 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).

DNA amplifications were realized using specific conditions and primers custom-synthesized by Invitrogen. RAGE primers were designed from bovine sequence (accession number from NCBI database, NM_173982). Forward primer sequence is 5'-CT ggAATggAAACTgAACaC-3' and reverse primer sequence is 5'-CTCggTAgTTAgACTTggTCTC-3', size of the amplified cDNA band is 196 pb. Amplification was carried out for 30 cycles with an annealing temperature of 60°C. β-actin primers and conditions were previously used and described [13,15]. The various sized RT-PCR products were resolved through 1 to 2% agarose gel electrophoresis, revealed with GelRed® staining nucleic acid (Interchim, Montluçon, France) and visualized using Gel Doc^{TM} XR (Biorad, Marnes-la-Coquette, France). Quantifications were performed using Quantity One software (Biorad) and statistical analysis was made using the software Prism 5. A sequencing procedure was carried out to identify all corresponding fragments (Genoscreen, Lille, France).

Preparation of Nε-(carboxymethyl)lysine-human serum albumin (*CML-HSA*)

As CML is the ligand with the highest affinity for the RAGE, we used CML-HSA for inhibition experiments. CML-HSA was prepared as previously described [26]. Briefly, HSA (30 mg/mL), and sodium cyanoborohydride (0.45 M) were dissolved in sodium phosphate buffer (0.2 M; pH 7.8). Glyoxylic acid was then added, and the mixture incubated for 24 h at 37°C. Control protein was prepared under the same conditions, except that glyoxylic acid was omitted. The preparation of CML-modified proteins was characterized by modifications in percentage, assessed via 2,4,6-trinitrobenzenesulfonic acid and by gas chromatography mass spectrometry. CML-HSA and control HSA solutions were then extensively dialyzed for 48 h (1 v/100 v). To avoid any residual sodium cyanoborohydride (62.84 Da) or glyoxylic acid (74.04 Da) presence, the membrane cut-off was 15 000 Da. Endotoxin levels evaluated by chromogenic Limulus assay (ACC, Falmouth, MA, USA) amounted to < 0.002 EU/mg protein in the experimental preparations.

Amyloid-β peptides and inulin transport studies

Prior to the transport experiments, BCECs monolayers were washed once for ten min with prewarmed

Ringer HEPES buffer (RH, pH 7.4; in mM: HEPES, 5; $NaHCO_3$, 6; NaCl, 150; KCl, 5.2; $CaCl_2$, 2.2; $MgCl_2+6H_2O$, 1.2)/BSA 0.5%. For influx studies (apical-to-basolateral transport), filters were transferred to 6-well plates containing 2.5 mL of RH/BSA 0.5% per well. 1.5 mL of RH/BSA 0.5%/LY containing either 12 nM of [^{3}H]inulin or 12 nM of Aβ peptides. Incubations were performed at 37°C or 4° for 2 h with very slight agitation. At the end of the experiment, BBB permeability was evaluated using the LY BBB integrity marker. [^{3}H]inulin was used as a non receptor-mediated transported molecule control and was measured using a liquid scintillation counter (Tricarb 2100TR). Aβ_{1-40} and Aβ_{1-42} peptides were dosed using commercially available kits (Invitrogen) and following manufacturer's instructions. Briefly, A monoclonal antibody specific for the NH_2-terminus of Aβ_{1-40} and Aβ_{1-42} peptides was coated onto the wells. Then, a rabbit antibody specific for the COOH-terminus of the Aβ_{1-40} or Aβ_{1-42} sequences was co-incubated with samples. Mass-balance was calculated to estimate peptides aggregation and degradation and only experiments with mass-balance $>$ 90% were taken into account. To consider influence of filter and collagen coating on molecules transport, percentages of passage were calculated in presence and in absence of BCECs. It consists in dividing [^{3}H]inulin or Aβ peptides amounts measured in the basolateral compartment (brain) by the initial concentration added in apical compartment (blood). Then, final percentages of transport were calculated dividing values obtained with cells by results without cells.

For inhibition studies, transport experiments were performed at 37°C and, apical and basolateral compartments were supplemented with 50 μg/mL of CML-HSA or Ctrl-HSA for 2 h. Final percentages of transport were calculated as described above.

P-glycoprotein and breast cancer resistant protein inhibition experiments

Cells were washed with prewarmed RH/BSA 0.5% for 10 min. Then, filters were transferred into 6-wells plates containing 2.5 mL of RH/BSA 0.5% per well. In the apical compartment was added [^{14}C]sucrose (450 nM) or Rho123 (20 μM) or Aβ_{1-40} or Aβ_{1-42} peptides (120 nM) supplemented or not with 20 mM of verapamil or 0.5 mM of GF120918 or 1 mM of FTC for 30 min. Experiments were also performed without cells. Filters were then placed under slight agitation at 37°C. [^{14}C]sucrose was detected using a liquid scintillation counter (Tricarb 2100TR). Rho123 was measured using a fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Aβ peptides were dosed using commercially available kits cited above and following manufacturer's recommendations. Percentages of passage of [^{14}C]sucrose, Rho 123 and Aβ] peptides across the endothelial cells monolayer were calculated as described above.

Fluorescent experiments and confocal microscopy

For fluorescent Aβ_{1-40} endocytosis studies, cells were first washed 10 min with warm RH/BSA 0.5%. Then, 1 μM of freshly prepared Fluorescent Aβ_{1-40} peptide was placed in the apical compartment during 1 h and cells were placed at 4°C or 37°C with a slight agitation. At the end of the experiment, filters were rinsed five times with cold RH/BSA 0.5% and twice with cold RH before a 10 min fixation step with 4% paraformaldehyde (PFA) at room temperature (RT). After 3 rinses, cells were incubated 2 min with 33258 Hoechst (1 μg/mL) (Sigma) to stain nuclei and preparations were mounted in mounting medium. Fluorescence was observed using a Leica Microsystems microscope (Wetzlar, Germany), and acquisition were done with a Cool-Snap digital camera (Leica Microsystems).

To characterize endocytosis processes and caveolae involvement, the cholesterol-binding agent filipin was used as previously described [20]. Briefly, cells were incubated with or without filipin (3 μg/mL) in the apical compartment for 20 min and washed with warm RH buffer before incubation with fluorescent Aβ_{1-40} peptide. Then samples were observed using a Leica Microsystems microscope. No effect of filipin and fluorescent Aβ_{1-40} peptide on BBB permeability was observed (data not shown). To restore endocytosis process, reversibility experiments were performed. After filipin treatment, BCECs were incubated in DMEM containing 20% HS/CS for 30 min then fluorescent Aβ_{1-40} peptide was added in apical compartment as described above.

For confocal microscopy, BCECs cultured on Transwell® 3 μm inserts were washed 3 times with ice-cold PBS and fixed with 4% PFA for 10 min at RT. Then, filters were cut from the plastic insert and cells were rinsed three times in PBS and permeabilized with triton X-100 0.1% (Sigma) in PBS for 10 min. After three washes, non-specific sites were blocked by 10% normal goat serum in PBS for 30 min at RT. Cells were then incubated with RAGE antibody (1:100) (Abcam, Cambridge, UK) for 1 h at RT and rinsed three times before incubation with the secondary antibodies Alexa

Fluor 568 goat anti-rabbit (1:200) (Molecular Probes, Eugene, OR, USA). The cells were further incubated for 2 min with 33258 Hoechst, washed three times with PBS and the samples were mounted in mounting medium. When cells were incubated in absence of primary antibody, no signal was observed. The cells were examined (magnification 63X) and photographed with Leica DMI 6000 B fluorescent microscope (Leica, Wetzlar, Germany) with excitation at 515–560 nm and 360 ± 40 nm for TRITC and Hoechst respectively. For three-dimensional analysis, the scanning was performed with the Inverted Leica DM IRE2 Confocal and Multiphoton Microscope (Leica) with use of argon and He–Ne lasers, with illumination and emission for TRITC 543 nm and 551–639 nm, respectively. For 33258 Hoechst multiphoton excitation at 360 nm and detection at 400–460 nm was applied. Three-dimensional analysis was performed by Z-axis imaging with optical slices of 0.16 μm. Acquisitions were then analyzed with LAS AF Lite confocal software (Leica).

Statistical analyses

All statistical analyses were performed using Prism software (GraphPad Software Inc., La Jolla, CA, USA); NS no significant, *$p < 0.05$; **$p < 0.005$, ***$p < 0.001$).

RESULTS

The effect of soluble forms of Aβ_{1-40} and Aβ_{1-42} peptides on BBB permeability

The first objective of the present study was to investigate the effect of physiological concentrations of Aβ_{1-40} and Aβ_{1-42} peptides on BBB permeability. By using a technique for permeability evaluation, we examined the impact of a 2-day treatment with soluble forms of Aβ_{1-40} and Aβ_{1-42} peptides in the apical compartment. Given that (i) Aβ peptides are synthesized in the cerebral compartment and (ii) glial cells can induce inflammatory responses that modify the integrity of the BBB [5], we also performed these experiments with Aβ peptides incubated in basolateral chamber. Both Aβ peptides were added initially to the culture medium at four different concentrations (2, 12, 50, and 120 nM). No aggregated forms were detected (by western blotting and ELISA kits) over the 2-day incubation period (data not shown). The results of these permeability studies (using LY as an integrity tracer) demonstrated that the BBB integrity did not change significantly after 2 days of treatment with 2, 12, 50, or 120 nM of the soluble forms of Aβ_{1-40} and Aβ_{1-42} incubated in either the apical (Supplementary Fig. 1A; available online: http://www.j-alz.com/issues/22/vol22-3.html#supplementarydata 02) or the basolateral (Supplementary Fig. 1B) compartments.

The role of the transcellular pathway in Aβ_{1-40} and Aβ_{1-42} peptide transport in our in vitro BBB model

There is *in vivo* evidence to suggest that Aβ peptides can enter the CNS by crossing the BBB [4]. Given that the BCECs' tight junctions prevent the passage of large molecules and allow only transcellular transport [5], we wondered whether Aβ peptides might also be internalized and transcytosed by BCECs. Since all these steps could be attenuated or inhibited at 4°C, we investigated the role of the transcellular pathway in Aβ peptide transport by comparing the results of experiments performed at 37°C and at 4°C. Inulin was included as a control because its apparent molecular weight is very similar to those of the Aβ peptides (almost 4.5 kDa) and it lacks a specific receptor on the BCEC membrane. Whereas apical-to-basolateral transport of inulin remained unchanged at 4°C (relative to 37°C), the transport of Aβ_{1-40} and Aβ_{1-42} peptides fell significantly (by 62% and 42%, respectively). This finding suggested that transcellular transport processes were operating (Fig. 1A). To investigate internalization differences at the BCEC level, we performed fluorescent experiments with FITC-Aβ_{1-40} peptide. As shown in Fig. 1B, less Aβ peptide was internalized at 4°C than in the control experiment at 37°C. The observed decreases in Aβ peptide transport across the BCEC monolayer suggested the presence of transcellular pathway.

It has been reported that caveolae vesicles are involved in this process at the BBB [20]. We used filipin to study the involvement of these vesicles in Aβ peptides endocytosis. This membrane cholesterol-binding agent provokes the disassembly of caveolae vesicles and has already been assessed in our BBB model; no change in permeability was measured after 20 min of filipin treatment (data not shown and [20]). In the present study, pre-incubation of BCECs with 3 μg/mL of filipin prior to the addition of fluorescent Aβ peptide resulted in a decrease in the fluorescent signal (Fig. 2B, compared with the control experiment in Fig. 2A). We also investigated the reversibility of the effect of filipin; after treatment and multiple washing

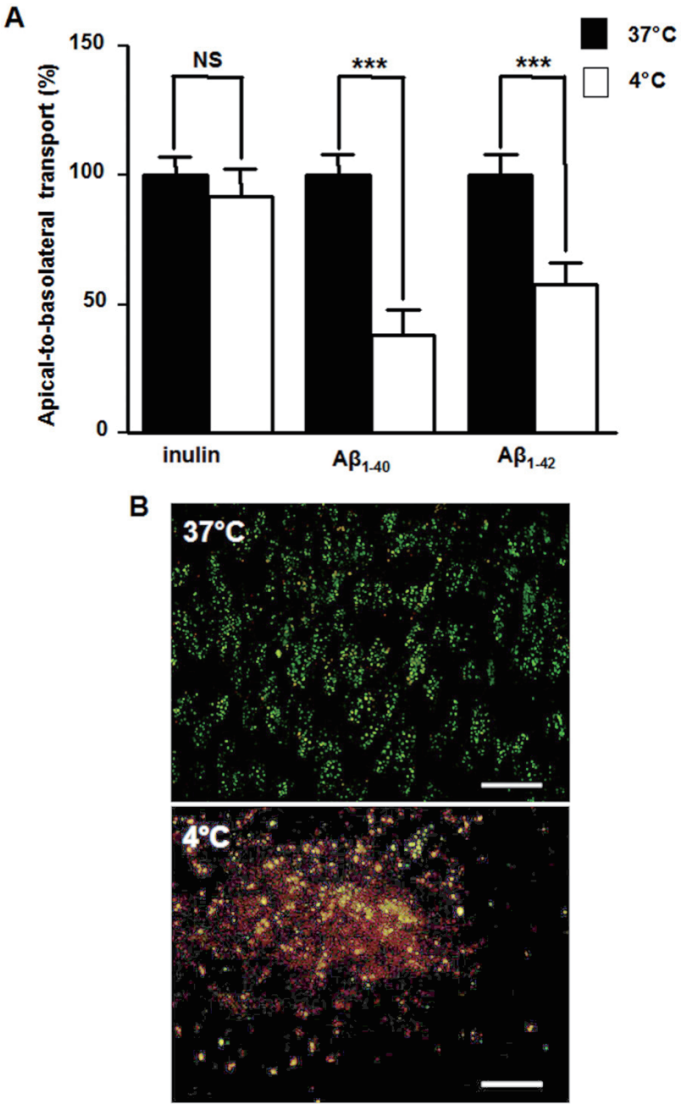

Fig. 1. The effect of temperature on the apical-to-basolateral transport and endocytosis of inulin, Aβ_{1-40} and Aβ_{1-42} peptides. A) Twelve nM of radiolabelled inulin or soluble forms of Aβ peptides were added to the apical compartment. The transport of these molecules across a monolayer of BCECs seeded on 3 μm Transwell® filters was assayed at 37°C or 4°C for 2 h with very slight stirring. The same experiments were performed with collagen coated-filters without cells, in order to take account of their influences on transport. The amounts of [^{3}H]inulin or Aβ peptides measured in the basolateral compartment (brain) were then divided by the initial concentration added to the apical compartment (blood). Final transport percentages were calculated by dividing values obtained in the presence of cells by those obtained in the absence of cells. Monolayer integrity was checked in each case by using LY (data not shown). Data represent means ± SEM; $n = 6$. ***$p < 0.001$ in a paired t-test. B) Fluorescent Aβ_{1-40} peptide was incubated for 1 h in the apical compartment of co-cultures at 4°C or 37°C. After multiple washing and fixation, samples were observed with fluorescent microscopy. The lower signal observed at 4°C suggests a decrease in the internalization of this peptide, compared with the control condition. Scale bar: 25 μm.

steps, BCECs were incubated with fluorescent Aβ peptides. As shown in Fig. 2C, this short reversal treatment restored normal endocytosis by the BCECs and indicated that the cells' fundamental functions were not altered by filipin treatment (as previously demonstrated in [20]). Taken as a whole, these results suggest that a transcellular RMT process is involved in Aβ peptides transfer across the BBB involving caveolae vesicles.

Although RAGE is thought to be involved in Aβ peptides transport from blood to brain at the BBB level, there is no direct evidence of its involvement at the BCEC membrane and its exact cellular location remains

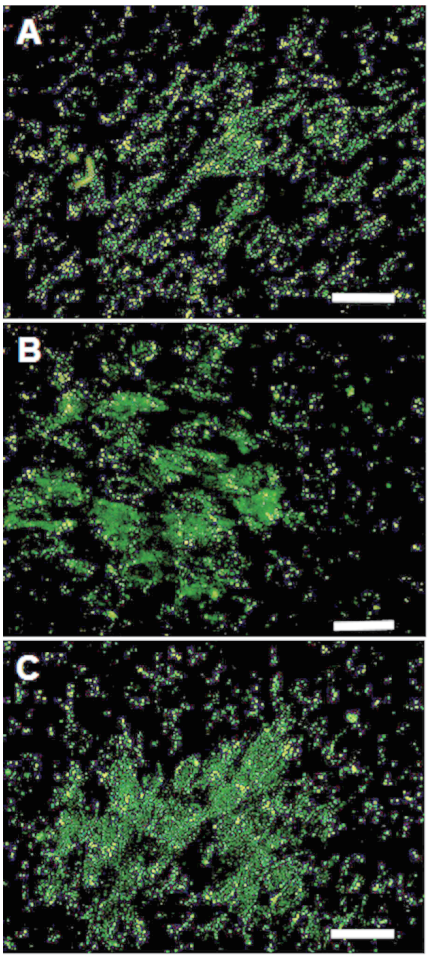

Fig. 2. The effect of filipin on endocytosis of FITC-Aβ peptides by BCECs. A) The cells were incubated in the absence (A) or presence of filipin (3 μg/mL). B) prior to the addition of fluorescent Aβ peptide and endocytosis was assayed as described in the Materials and Methods section. C) Immediately after filipin treatment, the cells were incubated in DMEM containing 20% HS/CS for 30 min, in order to reverse the effects of this cholesterol-binding agent before examining Aβ peptide endocytosis again. Scale bar: 25 μm.

unknown. Hence, we decided to characterize RAGE expression and location in our BBB model.

Expression of RAGE by BCECs

Using our *in vitro* BBB model, we first examined RAGE expression in BCECs cultured alone (E) or in presence of glial cells (Coc). The influence of these cells on the properties of BBB is very well documented. Indeed, the presence of glial cells reinforces the tightness of the endothelial junctions, reduces the permeability of the BBB, and modifies the expression patterns of several receptors and transporters expressed by the BCECs [5,13,15]. In view of our inability to find an efficient bovine anti-RAGE antibody for immunoblotting, we used an RT-PCR technique. As shown in Fig. 3A, a slight but significant increase in RAGE expression (1.61-fold versus the (E) condition) was observed in BCECs cocultured with glial cells for 12 days.

In RMT, BCECs express several specialized receptors at their luminal (blood) and/or abluminal (cerebral) faces. We therefore investigated the location of RAGE in BCECs by using confocal microscopy. As shown in Fig. 3B, this receptor was exclusively expressed at the luminal membrane, which is consistent with its putative role in the apical-to-basolateral transport of Aβ peptides.

The role of RAGE in Aβ_{1-40} and Aβ_{1-42} peptide influx and efflux across the BCEC monolayer

To determine whether RAGE was required for Aβ_{1-40} and Aβ_{1-42} peptide influx across the monolayer of BCECs seeded on Transwell® filters, competitive experiments were carried-out using the CML-HSA ligand, which presents a very high affinity for this receptor [26]. This glycation end-product was synthesized as described in Material and Methods and non-glycated human serum albumin was used as a control (Ctrl-HSA). The transport of inulin and Aβ_{1-40} and Aβ_{1-42} peptides were then studied in presence of 50 μg/mL of CML-HSA or Ctrl-HSA in the brain or the blood compartment. No changes in general BBB permeability were seen under any of these experimental conditions (data not shown). A significant decrease in the apical-to-basolateral transport of Aβ_{1-40} and Aβ_{1-42} peptides was observed in the presence of CML-HSA, relative to Ctrl-HSA (a drop of 39 and 30% for Aβ_{1-40} and Aβ_{1-42}, respectively) (Fig. 4A). This result is consistent with the luminal location of RAGE mentioned above (Fig. 3B). In contrast, the basolateral-to-apical transport of each peptide was not affected by the presence of CML-HSA (Fig. 4B). Neither apical-to-basolateral nor basolateral-to-apical transports of inulin were significantly affected by Ctrl-HSA or CML-HSA treatments.

Roles of P-gp and BCRP in Aβ peptide transport

Studies of P-gp's contribution to Aβ_{1-40} and Aβ_{1-42} peptide transport across the BBB have reported conflicting results and conclusions [8–11,27,28]. In our BBB model, P-gp has been functionally characterized for over ten years and the luminal location of this

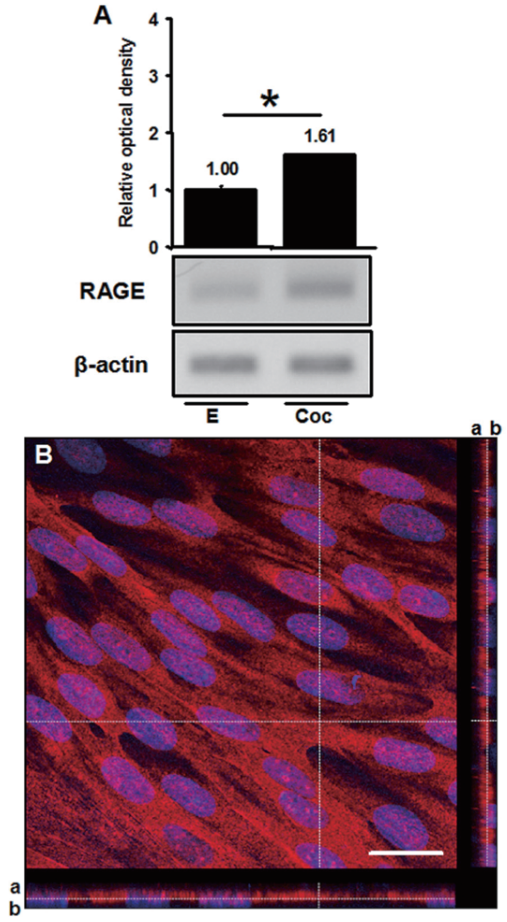

Fig. 3. RAGE expression and cell location in BCECs. A) RAGE expression detected by RT-PCR technique in a bovine BCEC monolayer (E) and in BCECs co-cultured with glial cells (Coc). Analysis was performed using the primers and conditions given in the Materials and Methods section. Expression of β-actin was determined as a simple quantity control. *p $<$ 0.05 in a paired t-test. B) Three-dimensional analysis of the cellular location of RAGE in BCECs. RAGE (red) was detected as described in the Materials and Methods section. No signal was detected when BCECs were incubated in the absence of anti-RAGE primary antibody (not shown). Nuclei were stained with 33258 Hoechst and appear in blue. The three-dimensional analysis (performed always from the top of the filter downwards) was obtained from 0.16 μm-wide optical slices. The results of a Z-axis analysis along the vertical white line (Y1–Y2) are presented as strips to the right of the main panels. The results of the Z-axis analysis along the horizontal white line (X1-X2) are presented as strips below the main panels. The apical side (start of the scan) is indicated by a black letter "a" and the basolateral side is indicated by a black letter "b". Scale bars: 20 μm.

efflux pump was recently confirmed [14,29,30]. BCRP shows similar substrate overlap with P-gp and its involvement in Aβ_{1-40} transport was suggested at the microvessel cells level [27]. Therefore, we investigated the involvement of these efflux pumps in Aβ_{1-40} and

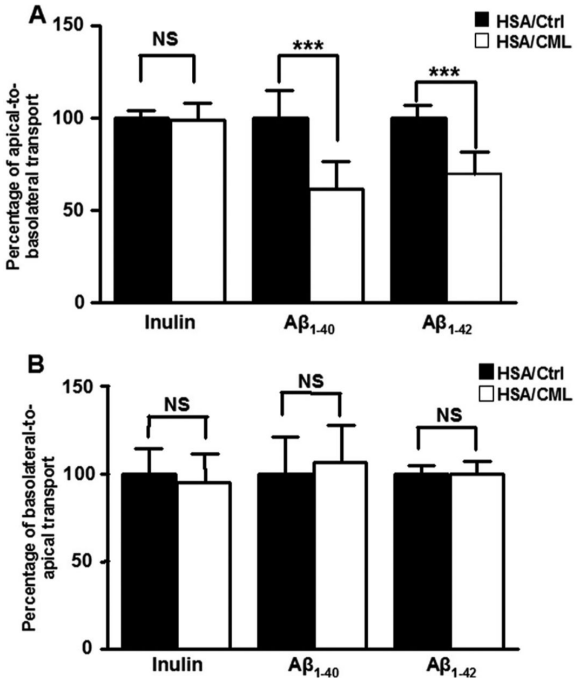

Fig. 4. RAGE inhibition experiments using the CML-HSA ligand. During the transport experiments, native or glycated HSA (CML-HSA) was added at a concentration of 50 μg/mL to the same compartment as inulin or Aβ_{1-40} or Aβ_{1-42} peptides. Involvement of RAGE was determined for apical-to-basolateral (A) and basolateral-to-apical (B) transport of inulin, Aβ_{1-40} and Aβ_{1-42} peptides, as described in the Materials and Methods section. For each monolayer of BCECs seeded on a Transwell®, permeability was measured using the integrity marker LY (data not shown). Data represent the mean $\pm$ SEM; $n = 6$. NS: non-significant; ***$p < 0.001$ when comparing samples treated with HSA alone versus those with HSA/CML in a paired t-test.

Aβ_{1-42} peptide apical-to-basolateral transport by using verapamil and GF120918 to inhibit P-gp and by using Fumitremorgin C (FTC) to inhibit BCRP. Rho123 was used substrate of P-gp [14] and BCRP [31]. As observed in Fig. 5A, Fig. 5B, and Fig. 5C, Rho123 entry's into the cerebral compartment was increased by 175.4% $\pm$ 8.2, 152.9% $\pm$ 18,7, and 147.0% $\pm$ 4.5, after treatment with verapamil, GF120918 or FTC, respectively. Treatments had no significant effect on the transport of sucrose (used as a paracellular diffusing molecule). As shown by the Fig. 5A, no significant modifications in Aβ_{1-40} and Aβ_{1-42} peptide apical-to-basolateral transport were observed in presence of verapamil. On the contrary, increases of Aβ_{1-40} peptide apical-to-basolateral transports by 136.7% $\pm$ 7.1 and 129.5% $\pm$ 2.5 were observed after GF120918 and FTC treatments respectively (Fig. 5B, C). Same observations were reported for Aβ_{1-42} peptide (152.0% $\pm$ 9.6 and 144.1% $\pm$ 15.1: Fig. 5B, C, respectively).

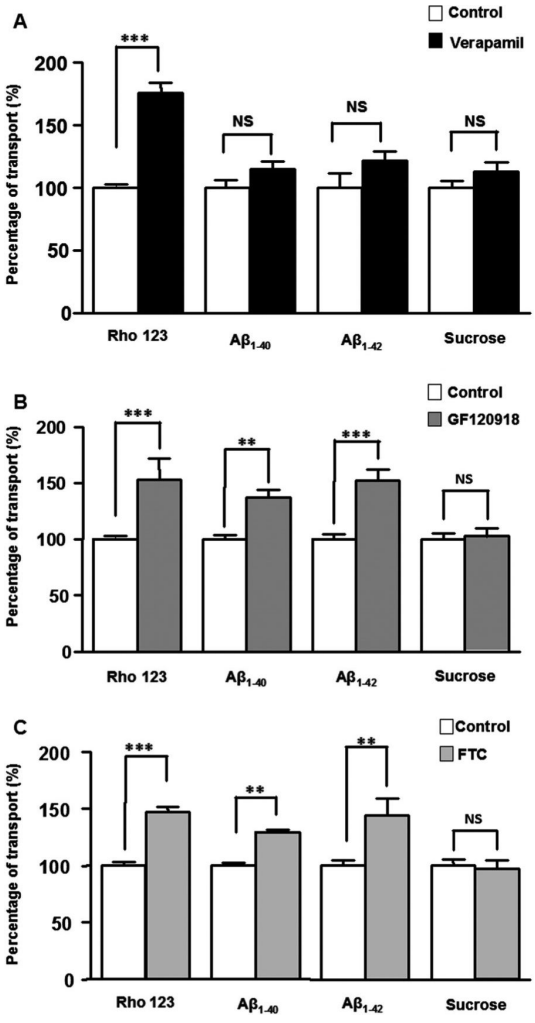

Fig. 5. Determination of the contribution of P-gp (A and B) and BCRP (C) to apical-to-basolateral transport of Aβ_{1-40} and Aβ_{1-42} peptides. Verapamil and GF120918 were used as selective P-gp inhibitors, as previously described whereas FTC was described as a BCRP inhibitor. Aβ_{1-40} and Aβ_{1-42} peptides (120 nM), sucrose (450 nM), and rhodamine 123 (20 μM) were added to the apical compartment in the presence or absence of 20 μM verapamil (A), 0.5 mM GF120918 (B), or 1 mM FTC (C). After 45 min of slight stirring at 37°C, apical and basolateral media were collected and the cells were extensively rinsed. Next, transport was measured as described in the Materials and Methods. For each monolayer of BCECs seeded on a Transwell®, permeability was measured using the integrity marker LY (data not shown). Data represent means ± SEM; $n = 6$. NS: non-significant; ***$p < 0.001$; **$p < 0.01$ when comparing verapamil-treated samples with untreated controls in a paired t-test.

DISCUSSION

Dysregulation of Aβ_{1-40} and Aβ_{1-42} peptide transport across the BBB is thought to play a key role in the development of AD. *In vivo* observations suggest that RAGE is expressed at the BBB and is involved in the apical-to-basolateral transport of Aβ peptides, whereas P-gp prevents this process [4,8]. The involvement of BCECs (which are in direct contact with the plasma) in these processes was suspected but has never been directly demonstrated *in vitro*. Therefore, by using an *in vitro* model of the BBB, we sought to characterize the effects of soluble forms of Aβ_{1-40} and Aβ_{1-42} peptides on BBB permeability and to investigate the mechanisms involved in peripheral Aβ_{1-40} and Aβ_{1-42} peptides entry into the CNS.

In order to focus on molecular transport, we only used soluble forms of these peptides. Most studies of the influence of these forms of Aβ peptides on the BBB integrity have looked at microvessel cells and have shown effects on cell viability [32,33]. However, the brain capillary endothelium represents the largest surface area for blood-brain exchange. Therefore, we first investigated the effect of soluble forms of Aβ_{1-40} and Aβ_{1-42} peptides on BCECs permeability; no changes were observed. Recent evidence suggest that oligomerized forms of Aβ peptides are more aggressive than the soluble forms used in our study and are able to modify endothelial viability and thus BBB integrity [32,33]. The potentially harmful effects of aggregated forms on the capillary/BCECs merit further investigation.

We have previously demonstrated that to reach the brain, large molecules such as low-density lipoproteins and transferrin must first interact with the BCECs' luminal face in a receptor-mediated mechanism and are then incorporated into multivesicular bodies [18–20]. In apical-to-basolateral transport experiments at 4°C, we observed that Aβ peptides transport into the basolateral compartment was lower than at 37°C; this finding suggests that endocytosis and transcytosis processes are involved in this transport. We then explored the mechanisms of endocytosis and showed that caveolae vesicles are involved in this phenomenon. Taken as a whole, our present results strongly suggest that ligand-binding and vesicular mechanisms are involved in apical-to-basolateral Aβ peptide transport.

Although RAGE is known to be expressed in microvessels and capillaries, its involvement in the transport process had not been demonstrated at the BCEC level. In our BBB model, we detected RAGE on the luminal side of the BCECs. Competition by the CML-HSA ligand decreased significantly apical-to-basolateral transport of Aβ peptides. However, the fact that the transport process was not completely abolished suggests that other receptors and mechanisms are probably involved in the CNS entry of Aβ peptides. No

RAGE signal was detected at the basolateral face of BCECs and, in accordance with this observation, no significant inhibition by the CML-HSA ligand (in comparison with HSA alone) was measured in basolateral-to-apical transport experiments.

Lastly, and in line with other studies [8,10,27], our results suggest that P-gp and BCRP significantly prevents Aβ peptides entry into the CNS. The role of P-gp is subject to debate because several studies (using different cell types, different inhibitors, and *in vivo* techniques) have reported its non-involvement in this process [9,11,28]. However, as suggested by our study, the choice of P-gp inhibitor is very important. Indeed, use of verapamil, a competitive inhibitor of P-gp, showed a slight but no significant effect on Aβ peptide transport whereas GF120918, which inhibits the P-gp ATPase activity, clearly increased Aβ peptides entry into the CNS. These discrepancy observations are explained by the inhibition mechanism of these compounds and were already described in others *in vitro* systems [34, 35]. Our observations demonstrated that the choice of P-gp inhibition is crucial to highlight P-gp contribution in cellular process and must be of clinical relevance.

In summary, we have demonstrated for the first time that BCECs are direct involved in RAGE-mediated entry of Aβ peptides into the CNS. Taken as a whole, our findings indicate that the inhibition of the Aβ peptide influx from blood to brain may be a useful strategy for inhibiting Aβ peptide accumulation and preventing the development of pathological changes in AD.

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