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Effect of amyloid beta on ATP-Binding Cassette transporter expression and activity in porcine brain microvascular endothelial cells

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Abstract

Background: Deposition of amyloid- β peptide ($A\beta_{(1-42)}$) within the brain is characteristic of Alzheimer's disease. Little is known of the effects of $A\beta_{(1-42)}$ on blood-brain barrier (BBB) ATP-binding Cassette (ABC) efflux transporters which influence BBB permeability. The effects of $A\beta_{(1-42)}$ on ABCB1, ABCC5 and ABCG2 activity and expression and pregnane X receptor (PXR) and constitutive androstane receptor (CAR) transcription factors expression were determined in primary porcine brain endothelial cells (PBECs).

Methods: The effect of $A\beta_{(1-42)}$ on transporter activity was determined by measurement of intracellular accumulation of the fluorescent probes calcein (ABCB1), GS-MF (ABCC5) and Hoechst 33342 (ABCG2). Expression of transporters and transcription factors was assessed by Western blotting.

Results: Treatment of PBECs with $A\beta_{(1-42)}$ significantly decreased activity of ABCB1 ($A\beta_{(1-42)}$ at 10 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$), ABCC5 ($A\beta_{(1-42)}$ at 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$) and ABCG2 ($A\beta_{(1-42)}$ at 10 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$). $A\beta_{(1-42)}$ also significantly decreased expression of ABCB1 ($p < 0.05$ at 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$), ABCG2 ($p < 0.05$ at 25 $\mu\text{g/ml}$ and $p \leq 0.001$ at 50 $\mu\text{g/ml}$), ABCC5 ($p < 0.05$ at 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$), PXR ($p < 0.05$ at 10 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ $A\beta_{(1-42)}$) and CAR ($p < 0.05$ at 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ $A\beta_{(1-42)}$).

Conclusion: $A\beta_{(1-42)}$ inhibits multiple ABC transporters and PXR and CAR in PBECs.

General Significance: $A\beta_{(1-42)}$ reduces ABC transporter activity and expression in BBB endothelial cells and has the potential to influence BBB permeability characteristics.

Keys words: ABCB1, ABCG2, ABCC5, nuclear receptors, Amyloid- β , Alzheimer's disease, porcine brain endothelial cells.

1. Introduction

The blood-brain barrier (BBB) forms the highly selective interface between the central nervous system (CNS) and the systemic circulation, regulating molecular transport and maintaining an optimal environment for neuronal and glial function [1,2]. The polyspecific ATP-binding cassette (ABC) efflux transporters ABCB1 (MDR1, P-glycoprotein), ABCC5 (multidrug resistance-related protein 5) and ABCG2 (breast cancer resistance protein) are highly expressed in BBB endothelial cells, and actively transport an array of xenobiotics, including many therapeutic drugs, resulting in reduced CNS penetration [3,4,5]. ABC efflux transporters also transport a variety of endogenous compounds including glucocorticoid, cytokines, bilirubin, cholesterol and lipid [6,7] and, notably, ABCB1 has been reported to transport the amyloid- β ($A\beta_{(1-42)}$) peptide [8].

Alzheimer's disease is a progressive fatal neurodegenerative disorder and the most common form of dementia. One of the key pathological characteristics of Alzheimer's disease is aggregation of the $A\beta_{(1-42)}$ peptide within the brain, leading to production of cytotoxic oligomers [9], plaque formation [10] and subsequent neurological deficit [11]. Amyloid- β is transported across the BBB, both from blood to brain and brain to blood, with influx into the brain mediated by the receptor for advanced glycation end products (RAGE) and clearance via the low density lipoprotein-related protein (LRP) receptor 1 (LRP1) [12], such that the peptide is in a dynamic equilibrium [13]. Furthermore, it has been postulated that reduced $A\beta_{(1-42)}$ clearance from the brain, i.e. reduced efflux transport of $A\beta_{(1-42)}$, may be associated with accumulation of $A\beta_{(1-42)}$ in Alzheimer's disease [14].

Whilst it has been demonstrated $A\beta_{(1-42)}$ reduces ABCB1 expression and activity in brain endothelial cells [8,15,16], and $A\beta_{(1-42)}$ either has no effect [16] or up-regulates [17] ABCG2 expression, no studies yet report the effects of $A\beta_{(1-42)}$ on the activity or expression of BBB ABCC5. The pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) are widely expressed in BBB brain endothelial cells [18,19,20] and are key regulators of ABC transporter expression. ABC efflux transporter substrates, and inhibitors, act as PXR and CAR ligands [21,22] however to date, there are no reports of the effects of $A\beta_{(1-42)}$ on PXR and CAR expression.

In the present study the effects of $A\beta_{(1-42)}$ on the activities of ABCB1, ABCG2 and ABCC5, and on the expression of ABCB1, ABCG2, PXR and CAR were investigated in a well-characterised BBB model comprised of primary porcine brain endothelial cells

[23,24,25] to obtain a better understanding on the potential effect of A β ₍₁₋₄₂₎ on BBB function.

2. Materials and methods

2.1. Chemicals

A β ₍₁₋₄₂₎ peptide was purchased from Cellmano Biotech Limited (China). Dulbecco's phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), Neutral Red powder, Thioflavin T, heparin sodium salt from porcine intestinal mucosa, trypsin-EDTA solution, verapamil, Ko143, penicillin/streptomycin and puromycin dihydrochloride were purchased from Sigma-Aldrich (UK). Calcein and HEPES were purchased from Invitrogen (Paisley, Scotland). C219 anti-ABCB1 monoclonal antibody was purchased from MERCK (Darmstadt Germany). BXP-53 anti-ABCG2 monoclonal antibody, anti-MRP5 antibody (ab115327), anti-PXR (ab192579), anti-constitutive androstane receptor antibody (ab186869) and anti-vinculin (ab129002) were purchased from Abcam (Cambridge, UK). Mouse IgG anti- β -actin antibody, Collagen type I (rat tail) and fibronectin (human) were purchased from SLS Ltd Nottingham, UK). DMEM (low glucose, no pyruvate, no glutamine, no phenol red) and DMEM (high glucose, no pyruvate), were purchased from Life Technologies Ltd (Paisley, UK). Cell culture plastic ware was obtained from Greiner Bio-one (Stonehouse, UK) and plasma-derived serum from First Link (Birmingham, UK). Bradford protein assay reagent and Western blotting materials were obtained from Bio-Rad (Hemel Hempstead, UK).

2.2. Isolation and culture of PBECs

Isolation of porcine brain microvessels was based on the method described by Skinner *et al.*; [23,26]. Microvessels were either immediately maintained in culture or stored at liquid nitrogen. Microvessels were seeded into 6-well plates pre-coated with 100 μ g/ml type I rat tail collagen and 7.5 μ g/ml human fibronectin and maintained in growth medium composed of DMEM (low glucose without phenol red) supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, 2 mM glutamine, 10 % (v/v) plasma derived serum and 125 μ g/ml heparin in a humidified atmosphere at 37 °C with 5 % CO₂ for 24 h. Microvessels were treated for 48 h with puromycin dihydrochloride, 4 μ g/ml, and maintained in growth medium

and astrocyte- conditioned medium (1:1) for 6 days, to allow proliferation of PBECs. PBECs were then used for Western blotting studies or subcultured into 96-well plates.

2.3. Culture of astrocytes

The CTX-TNA2 rat astrocyte cell line (passages 8–20) was maintained in DMEM supplemented with 10 % (v/v) foetal bovine serum with 100 U/ml penicillin and 100 mg/ml streptomycin at a seeding density of 1×10^6 cells per 75 cm² flask. When cells reached confluency, astrocyte conditioned medium (ACM) was collected and stored at -20 °C until use.

2.4. Measurement of ABCB1, ABCG2 and ABCC5 functional activity

Functional activity of ABCB1, ABCG2 and ABCC5 was measured as described previously [24,27]. PBECs in 96-well plates were treated with either 10 µg/ml, 25 µg/ml or 50 µg/ml A β ₍₁₋₄₂₎ in treatment medium (DMEM low glucose without phenol red supplemented with 1 % (v/v) FBS and 2 mM L-glutamine) for 48 h at 37 °C in 5% CO₂. Cells were initially incubated with the above medium containing either 10 µM verapamil (ABCB1 inhibitor) [28], 0.5 µM Ko143 (ABCG2 inhibitor) [29] or 25 µM MK571 (ABCC5 inhibitor) [30] for 30 min at 37 °C. The above media were then replaced with media plus inhibitors containing either calcein-AM, 0.5 µM (to measure ABCB1 activity), Hoechst 33342, 1 µM, (to measure ABCG2 activity) and CMFDA, 4 µM, (to measure ABCC5 activity). Following a 30 min incubation at 37 °C PBECs were washed twice with warm PBS and intracellular fluorescence of calcein (excitation 484 nm; emission 530 nm), Hoechst 33342 (excitation 370 nm; emission 450 nm) and GS-MF (excitation 492 nm; emission 516 nm) measured using a Safire multiplate reader (Tecan, Germany). Relative fluorescence units were normalised for the protein content of each individual well and functional activity is represented as % RFU/ug protein compared to non-treated control.

2.5. Cell viability

The effect of A β ₍₁₋₄₂₎ on PBECs viability was determined using the Neutral Red assay [31]. PBECs in 96-well plates were treated with either 10 µg/ml, 25 µg/ml or 50 µg/ml A β ₍₁₋

42) in treatment medium for 48 h at 37 °C in 5% CO₂. Following treatment, the medium was removed and replaced with DMEM (low glucose without phenol red supplemented with 2 mM L-glutamine) containing Neutral Red, 40 µg/ml. Cells were incubated for 2 h at 37 °C in a humidified atmosphere with 5% CO₂, washed twice with PBS, 150 µl of destain solution (1 % (v/v) acetic acid in 50 % (v/v) ethanol) added to each well and plates agitated rapidly for 15 minutes on a rocking platform. Fluorescence intensity was measured with a Safire multiplate reader (Tecan, Germany), with excitation and emission wavelengths of 530 and 645 nm, respectively.

2.6. Measurement of Aβ₍₁₋₄₂₎ peptide monomer aggregation using the Thioflavin T assay

Aggregation of Aβ₍₁₋₄₂₎ peptide was monitored using the Thioflavin T assay as described by Kokkoni et al. with modifications [32]. Briefly, Aβ₍₁₋₄₂₎ monomer peptide was dissolved in treatment medium to concentrations of 10 µg/ml, 25 µg/ml and 50 µg/ml and the peptide incubated at 37 °C (Galaxy 14S Incubator, New Brunswick, UK). At 0, 24, 48, 72 and 96 h, a 10 µl sample of Aβ₍₁₋₄₂₎ peptide solution was transferred to 96-well plates, 90 µl of 4 µM Thioflavin T solution added and fluorescence intensity was measured with a Safire multiplate reader (Tecan, Germany) with a 10 s shake prior to each fluorescence reading, and excitation and emission wavelengths of 440 nm and 495 nm, respectively. During data analysis the fluorescent reading of the vehicle control (DMEM with Thioflavin T but no Aβ₍₁₋₄₂₎ peptide) was subtracted from the test Aβ₍₁₋₄₂₎ and Thioflavin T fluorescent reading.

2.7. Effect of Aβ₍₁₋₄₂₎ on ABCB1, ABCG2 and ABCC5 functional activity

Porcine brain endothelial cells were treated with 10 µg/ml, 25 µg/ml and 50 µg/ml Aβ₍₁₋₄₂₎ for 1 h, 24 h and 48 h at 37 °C in 5 % CO₂. The effect of Aβ₍₁₋₄₂₎ on ABCB1, ABCG2 and ABCC5 activity was measured spectrofluorometrically as described in section 2.4.

2.8. Western blotting

Porcine brain endothelial cell lysates were prepared using Cellytic M lysis buffer (Sigma-Aldrich, UK) with protease inhibitor cocktail (Sigma-Aldrich, UK), 1 µl inhibitor cocktail per ml of lysis buffer. Cell lysates were centrifuged at 15,000 g for 15 min at 4 °C and the protein

content of the supernatant determined by the Bradford method using a standard curve of bovine serum albumin [33]. Equal quantities of lysate protein, 15 µg, were separated using 6 %, 8 % and 10 % SDS-polyacrylamide gels for the detection of ABCB1, ABCG2 and both PXR and CAR% respectively, 50 µg protein was separated using 6% SDS-polyacrylamide gels for the detection of ABCC5 and proteins transferred onto polyvinylidene difluoride membranes in 10 mM CAPS buffer, pH 11. Membranes were blocked with 5 % (w/v) low fat milk and incubated overnight at 4 °C with either C219 mouse monoclonal antibody for detection of ABCB1 (1:50), BXP-53 monoclonal antibody for detection of ABCG2 (1:100), anti-MRP polyclonal antibody detection of ABCC5 (1:500) and mouse anti-β-actin-N-terminal monoclonal antibody (1:25,000) for detection of β-actin. PXR was detected with anti PXR polyclonal antibody (1:1000) and CAR with anti-constitutive androstane receptor polyclonal antibody (1:1000). Mouse anti-rabbit anti vinculin antibody, at a dilution of 1:10,000, were used to detect vinculin. Membranes were subsequently incubated with HRP-conjugated secondary antibody for protein detection. Western blots were developed using Enhanced Chemiluminescence reagent (Biorad, UK), images obtained using a ChemiDoc imaging system (Bio-Rad, UK) and analysed with the ImageJ software (NIH, USA).

2.9. Statistical analysis

All experiments were carried out at least four times, unless stated. Data are expressed as mean ± standard deviation and SPSS v20 (IBM) was used to carry out unpaired t-tests. A P-value of less than 0.05 is considered significant.

2. Results

3.1. Determination of ABCB1, ABCG2 and ABCC5 functional activity in porcine brain endothelial cells

Initial fluorimetric studies were carried out to determine ABCB1, ABCG2 and ABCC5 functional activity. Treatment of cells with the ABCB1 inhibitor verapamil significantly increased intracellular accumulation of the fluorescent probe calcein by 7-fold, figure 1. Similarly, treatment with the ABCG2 inhibitor Ko143 increased intracellular accumulation of

the ABCG2 substrate Hoechst 33342 by 2.1-fold, whilst the ABCC5 inhibitor MK571 significantly increased intracellular levels of the ABCC5 substrate GS-MF by 2.4 fold, figure 1.

Fig. 1. Determination of ABCB1, ABCG2 and ABCC5 functional activity in PBECs. Intracellular accumulation of calcein, Hoechst 33342 and GS-MF in control cells and cells pre-treated with ABCB1 inhibitor (verapamil), ABCG2 inhibitor (Ko143) and ABCC5 inhibitor (MK571). Data were analysed using unpaired Student's t-test and are presented as the mean $\pm$ SD of 8 independent experiments, with 4 replicates in each experiment, ** $p \leq 0.001$

3.2. Effect of $A\beta_{(1-42)}$ on the viability of porcine brain endothelial cells

The viability of porcine brain endothelial cells exposed to $A\beta_{(1-42)}$ was determined using the Neutral Red assay. No significant toxicity was observed when cells were exposed to 10 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ $A\beta_{(1-42)}$ over a 48 h period, figure 2.

Fig. 2. The effect of Aβ₍₁₋₄₂₎ on porcine brain endothelial cell viability. The fluorescence intensity of Neutral Red was measured in control cells and cells pre-treated for 48 h with 10 µg/ml, 25 µg/ml and 50 µg/ml Aβ₍₁₋₄₂₎. Data were analysed using unpaired Student's t-test and are presented as the mean ± SD of 4 independent experiments, with 4 replicates in each experiment

3.3. Aβ₍₁₋₄₂₎ aggregation

Initial studies were carried out to establish if Aβ₍₁₋₄₂₎ underwent aggregation in the cell culture conditions employed in this study. There was a significant increase in fluorescent intensity of Thioflavin T over time when Aβ₍₁₋₄₂₎ at 25 µg/ml and 50 µg/ml was incubated in PBEC treatment medium, indicating a time-dependent increase in Aβ₍₁₋₄₂₎ aggregation. However at 10 µg/ml Aβ₍₁₋₄₂₎ the fluorescent intensity was very low and no significant changes in fluorescence were observed over time, figure 3.

Fig. 3. Aggregation Aβ₍₁₋₄₂₎ in PBEC treatment medium. Aβ₍₁₋₄₂₎ peptide solution was freshly prepared at concentrations of 10 μg/ml, 25 μg/ml and 50 μg/ml and incubated for 0, 24, 48, 72 and 96 h in PBEC treatment medium, and a 10 μl sample of Aβ₍₁₋₄₂₎ peptide mixed with 90 μl of 4 μM Thioflavin T. Data are expressed as relative fluorescence units (RFU) and are the mean ± SD of 3 independent experiments with 4 replicates in each experiment. Data were analysed using unpaired Student's t-test, *p<0.05, ** p≤0.001.

3.4. Effect of Aβ₍₁₋₄₂₎ on ABCB1 functional activity

Studies were carried out to determine the effect of Aβ₍₁₋₄₂₎ exposure on the functional activity of the ABCB1 efflux transporter. Treatment with 10 μg/ml, 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎ for 1 h and 24 h significantly increased calcein accumulation in porcine brain endothelial cells, indicating reduced ABCB1 activity, figure 4. At 48 h exposure, there was no significant difference in calcein accumulation in cells pre-treated with 10 μg/ml Aβ₍₁₋₄₂₎ (109 ± 5 % RFU/μg protein) compared to control (100 % RFU/μg protein). However, treatment with 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎ significantly increased calcein accumulation to 132 ± 9 % RFU/μg protein and 144 ± 5 % RFU/μg protein respectively, compared with control (100 % RFU/μg protein) (Figure 4).

Fig. 4. Effects of Aβ₍₁₋₄₂₎ on ABCB1 functional activity. Intracellular accumulation of calcein in control cells and cells pre-treated for 1 h (solid bars), 24 h (striped bars) and 48 h (dotted bars) with 10 μg/ml, 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎. Data were analysed using unpaired Student's t-test and are presented as the mean ± SD of 4 independent experiments, with 4 replicates in each experiment, *p<0.05, **p≤0.001.

3.5. Effect of Aβ₍₁₋₄₂₎ on ABCB1 expression

Western blotting studies were carried out to determine the effect of Aβ₍₁₋₄₂₎ on ABCB1 expression in PBECs. Exposure to Aβ₍₁₋₄₂₎ at 10 μg/ml, 25 μg/ml and 50 μg/ml for 1 h did not significantly affect ABCB1 expression compared to non-treated control cells, figure 4. Whilst treatment with Aβ₍₁₋₄₂₎ for 24 h at 10 μg/ml did not significantly alter ABCB1 expression, expression of the transporter in cells treated with 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎ for 24 h was significantly lower, 72.2 % ± 10 % and 76.2 % ± 12 % respectively, compared to control cells, figure 5. Similar observations were observed when PBECs were treated with Aβ₍₁₋₄₂₎ for 48 h, with Aβ₍₁₋₄₂₎ at 10 μg/ml having no significant effect on ABCB1 expression whilst 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎ significantly reduced ABCB1 expression levels, figure 5.

(A)

(B)

Fig. 5. Effect of Aβ₍₁₋₄₂₎ on ABCB1 expression in PBECS. PBECS were treated for 1 h, 24 h and 48 h with 10 μg/ml, 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎. Panel (A) is a representative Western blot image and panel (B) densitometric data are normalised to β-actin and are presented as a percentage of untreated control cells. Data were analysed using unpaired Student's t-test and are presented as the mean ± SD of 4 independent experiments, *p<0.05.

3.6. Effect of Aβ₍₁₋₄₂₎ on ABCG2 functional activity

Studies were carried out to determine the effect of Aβ₍₁₋₄₂₎ exposure on the functional activity of the ABCG2 efflux transporter. Treatment with 10 μg/ml, 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎ for 1 h and 24 h significantly increased Hoechst 33342 accumulation in porcine brain endothelial cells, indicating reduced ABCG2 activity, figure 6. At 48 h exposure, there was no significant difference in Hoechst 33342 accumulation in cells pre-treated with 10 μg/ml Aβ₍₁₋₄₂₎ (119 % ± 9 % RFU/μg protein) compared to control (100 % RFU/μg protein). However, treatment with 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎ significantly increased Hoechst 33342 accumulation to 130.4 % ± 5% RFU/μg protein and 160 % ± 3 % RFU/μg protein respectively, compared with control (100 RFU/μg protein) (Figure 6).

Fig. 6. Effects of Aβ₍₁₋₄₂₎ on ABCG2 functional activity. Intracellular accumulation of Hoechst 33342 in control cells and cells pre-treated for 1 h (solid bars), 24 h (striped bars) and 48 h (dotted bars) with 10 μg/ml, 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎. Data were analysed using unpaired Student's t-test and are presented as the mean ± SD of 4 independent experiments, with 4 replicates in each experiment, *p<0.05, **p≤0.001.

3.7. Effect of Aβ₍₁₋₄₂₎ on ABCG2 expression

Western blotting studies were carried out to determine the effect of Aβ₍₁₋₄₂₎ on ABCG2 expression in PBECs. Exposure to Aβ₍₁₋₄₂₎ at 10 μg/ml, 25 μg/ml and 50 μg/ml for 1 h did not significantly affect ABCG2 expression compared to non-treated control cells, figure 7. Whilst treatment with 10 μg/ml Aβ₍₁₋₄₂₎ for 24 h did not significantly alter ABCG2 expression, expression levels of the transporter in cells treated with 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎ for 24 h were significantly lower, 81 % ± 1.5 % and 65.2 % ± 16 % respectively, compared to control cells, figure 7. Similar findings were observed when PBECs were treated with Aβ₍₁₋₄₂₎ for 48 h, with Aβ₍₁₋₄₂₎ at 10 μg/ml having no significant effect on ABCG2 expression whilst 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎ significantly reduced ABCG2 expression levels, figure 7.

(A)

(B)

Fig. 7. Effect of Aβ₍₁₋₄₂₎ on ABCG2 expression in PBECs. PBECs were treated for 1 h, 24 h and 48 h with 10 μg/ml, 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎. Panel (A) is a representative Western blot image and panel (B) densitometric data are normalised to β-actin and are presented as a percentage of untreated control cells. Data were analysed using unpaired Student's t-test and are presented as the mean ± SD of 4 independent experiments, *p<0.05, **p≤0.001.

3.8. Effect of Aβ₍₁₋₄₂₎ on ABCC5 functional activity

Studies were carried out to determine the effect of Aβ₍₁₋₄₂₎ exposure on the functional activity of the ABCC5 efflux transporter. Treatment with 10 μg/ml Aβ₍₁₋₄₂₎ for 1 h, 24 h and 48 h did not significantly affect GS-MF accumulation in PBECs, figure 8. However, treatment with 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎ for 1 h, 24 h and 48 h significantly increased GS-MF accumulation (144 % ± 9 % and 141 % ± 7 % RFU/μg protein for 1 h, 142 % ± 10 % and 152 % ± 17 % RFU/μg protein for 24 h and 144 % ± 12 % and 143 % ± 6 % RFU/μg protein for 48 h), compared with control (100 % RFU/μg protein), figure 8.

Fig. 8. Effects of Aβ₍₁₋₄₂₎ on ABCC5 functional activity. Intracellular accumulation of GS-MF in control cells and cells pre-treated for 1 h (solid bars), 24 h (striped bars) and 48 h (dotted bars) with 10 μg/ml, 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎. Data were analysed using Student's unpaired t-test and are presented as the mean ± SD of 4 independent experiments, with 4 replicates in each experiment. *p<0.05.

3.9. Effect of Aβ₍₁₋₄₂₎ on ABCC5 expression

The effect of Aβ₍₁₋₄₂₎ on ABCC5 expression in PBECs was determined by Western blotting. Exposure to Aβ₍₁₋₄₂₎ at 10 μg/ml, 25 μg/ml and 50 μg/ml for 1 h did not significantly affect ABCC5 expression compared to non-treated control cells, figure 9. Whilst treatment with 10 μg/ml Aβ₍₁₋₄₂₎ for 24 h did not significantly alter ABCC5 expression, expression levels of the transporter in cells treated with 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎ for 24 h were significantly lower, 55 % ± 10 % and 65 % ± 9 % respectively, compared to control cells, figure 9. Similar findings were observed when PBECs were treated with Aβ₍₁₋₄₂₎ for 48 h, with Aβ₍₁₋₄₂₎ at 10 μg/ml having no significant effect on ABCC5 expression whilst 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎ significantly reduced ABCC5 expression levels, figure 9.

(A)

(B)

Fig. 9. Effect of $A\beta_{(1-42)}$ on ABCC5 expression in PBECs. PBECs were treated for 1 h, 24 h and 48 h with 10 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ $A\beta_{(1-42)}$. Panel (A) is a representative Western blot image and panel (B) densitometric data are normalised to β -actin and are presented as a percentage of untreated control cells. Data were analysed using unpaired Student's t-test and are presented as the mean $\pm$ SD of 4 independent experiments, * $p < 0.05$.

3.10. The effect of $A\beta_{(1-42)}$ on PXR and CAR expression

This study demonstrates $A\beta_{(1-42)}$ significantly reduces ABCB1, ABCG2 and ABCC5 expression in PBECs. The pregnane X receptor (PXR) and constitutive androstane receptor (CAR) regulate ABCB1, ABCG2 and ABCC2 expression, although no reports document PXR- and CAR-mediated regulation of ABCC5. Therefore studies were carried out to determine the effect of $A\beta_{(1-42)}$ on PXR and CAR expression. In initial control studies, treatment of PBECs with the PXR ligand rifampicin significantly increased both ABCB1 and ABCG2 expression and function, indicating functional PXR in PBECs (data not shown). Exposure of PBECs to $A\beta_{(1-42)}$ at 10 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ for 48 h significantly reduced PXR expression compared to non-treated control cells, figure 10 (A) and (B). Whilst treatment with $A\beta_{(1-42)}$ for 48 h at 10 $\mu\text{g/ml}$ did not significantly alter CAR expression, expression levels of CAR in PBECs treated with 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ $A\beta_{(1-42)}$ were significantly lower, 70 % $\pm$ 12 % and 83 % $\pm$ 7 % respectively, compared to control cells, figure 10 (C) and (D).

(A)

(B)

(C)

(D)

Fig. 10. Effects of Aβ₍₁₋₄₂₎ on PXR and CAR expression in PBECs. PBECs were treated for 48 h with 10 μg/ml, 25 μg/ml and 50 μg/ml Aβ₍₁₋₄₂₎. Panels (A) and (C) are representative Western blot images of PXR and CAR respectively. Panel (B), PXR densitometric data, and panel (D), CAR densitometric data, are normalised to vinculin and are presented as a percentage of untreated control cells. Data were analysed using unpaired Student's t-test and are presented as the mean ± SD of 4 independent experiments, *p<0.05.

4. Discussion

One of the pathological hallmarks of Alzheimer's disease is deposition of amyloid-β peptide (Aβ₍₁₋₄₂₎) into plaques, which accumulate within the brain, leading to neurological dysfunction [34,35]. Clearance of Aβ₍₁₋₄₂₎, from brain, across BBB endothelial cells, into the systemic circulation therefore represents a potential mechanism of reducing Aβ₍₁₋₄₂₎ levels within the CNS. The BBB functions to reduce brain exposure to endogenous and exogenous compounds, and polyspecific ABC efflux transporters, particularly ABCB1, ABCG2 and ABCC5, play a key role in this protective function. In this study we employed an in vitro model of the BBB, comprised of primary PBECs [23,24,25], to investigate the effects of Aβ₍₁₋₄₂₎ on the expression and function of ABC transporters and regulatory receptors in BBB endothelial cells.

Our previous studies employing this PBEC-based BBB model have reported expression and function of ABCB1 and ABCG2 [24], which are known to be highly expressed in the BBB [36,37]. The studies of Warren et al. report relatively high levels of ABCC5 mRNA in

porcine brain endothelial cells [38] but did not investigate ABCC5 expression at the protein level. In addition, the study of Zhang et al. report that ABCC5 mRNA is more highly expressed in primary bovine brain microvessel endothelial cells than other members of the ABCC subfamily [39]. In human brain, ABCC5 has been reported to be the most highly expressed ABCC subfamily member in all human brain regions at the RNA level [40].

In the current study we successfully demonstrate expression of ABCC5 protein in PBECs, which is consistent with the study of Kubo et al. which reports ABCC5 expression by quantitative targeted proteomics in porcine brain capillary [41].

Initial studies were carried out to confirm functional activities of ABCB1, ABCG2 and ABCC5. Intracellular accumulation of calcein in PBECs pre-treated with the ABCB1 inhibitor verapamil was 7.8-fold higher than in control cells, indicating expression of active ABCB1. This finding is similar to those of Smith et al. who observed a 7.5-fold increase in rhodamine-123 intracellular accumulation in cells pre-treated with verapamil [42], whilst a 6-fold increase in intracellular accumulation of calcein in PBECs has also been reported [24,43].

ABCG2 function was assessed by measuring intracellular accumulation of Hoechst 33342 in PBECs pre-treated with the ABCG2 inhibitor Ko143. Ko143 treatment significantly increased intracellular Hoechst 33342 accumulation by 2-fold, which is in keeping with the 2-fold increase reported by Cantrill et al. 2012 [24], and with the 1.6-fold increase in intracellular accumulation of the ABCG2 substrate mitoxantrone reported by Mahringer et al. [44].

The ABCC5 inhibitor MK571 significantly increased intracellular levels of the ABCC5 substrate GS-MF by 2.4-fold. This finding is in agreement with the study of Gutmann et al. which demonstrated a 3-fold increase in GS-MF levels in PBECs treated with MK571[27], and with studies which report MK571 increased intracellular accumulation of GS-MF by approximately 2-fold in the immortalized hBCEC cell line [45]. In initial studies, the specificity of the transporters for probe substrates was analysed. These studies found no significant change in intracellular accumulation of the ABCB1 substrate calcein-AM following treatment with ABCG2 and ABCC5 inhibitors. Similar findings were observed with ABCG2 and ABCC5 substrates, verifying a lack of overlap in substrate specificity (data not shown). Taken together, these findings demonstrate the PBECs employed in the current studies express key BBB-associated ABC efflux transporters.

Prior to investigating the effect of $A\beta_{(1-42)}$ on ABCB1-, ABCG2- and ABCC5-mediated transport, cell viability studies were carried out. $A\beta_{(1-42)}$, at concentrations up to 50 $\mu\text{g/ml}$, had no effect on PBECs viability. This is in agreement with studies reporting $A\beta_{(1-42)}$ at 50 $\mu\text{g/ml}$ had no significant effect, after 48 h treatment, on the viability of hCMEC/D3 brain endothelial cells [16], and is also consistent with the lack of effect of up to 100 $\mu\text{g/ml}$ $A\beta_{(1-42)}$ on the viability of rat brain endothelial cells [46].

The current study presents the first experimental evidence that $A\beta_{(1-42)}$ significantly reduces ABCB1 activity in PBECs by direct inhibition (without an associated reduction in ABCB1 expression) in cells treated with $A\beta_{(1-42)}$ for 1 h.

Reduced ABCB1 activity at 24 h and 48 h is likely due to a combination of inhibition of ABCB1 by $A\beta_{(1-42)}$ and by an $A\beta_{(1-42)}$ -mediated reduction in ABCB1 expression. The effects of $A\beta_{(1-42)}$ on ABCB1 activity are consistent with the studies of Kania et al. that demonstrated a 48 h treatment with 25 $\mu\text{g/ml}$ $A\beta_{(1-42)}$ reduced rhodamine efflux in the hCMEC/D3 brain endothelial cell line [16]. The findings of the present study are also consistent with those of Kuhnke et al. who reported $A\beta_{(1-42)}$, at 25 $\mu\text{g/ml}$, reduced rhodamine efflux in membrane vesicles isolated from LLC-MDR1 cells [8], and with those of Lam et al. 2001, who reported $A\beta_{(1-42)}$, at 100 $\mu\text{g/ml}$, significantly inhibited uptake of [^3H]colchicine by ABCB1 in inverted membrane vesicles isolated from *mdr1* overexpressing CH^RB30 Chinese hamster ovary cells [15].

In the current study, similar reductions in ABCB1 activity were observed following 1 h and 24 h exposure to $A\beta_{(1-42)}$ at 10 $\mu\text{g/ml}$, 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$. Whilst significant ABCB1 inhibition was also observed following 48 h exposure to 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ $A\beta_{(1-42)}$, exposure to $A\beta_{(1-42)}$ at 10 $\mu\text{g/ml}$ did not result in significant inhibition of ABCB1. This lack of inhibitory effect of $A\beta_{(1-42)}$ at 10 $\mu\text{g/ml}$ could be explained by the fact that at 48 h, aggregation of $A\beta_{(1-42)}$ at this lower concentration reduced the amount of $A\beta_{(1-42)}$ monomer available to directly interact with ABCB1. This finding is supported by the studies of Kuhnke et al. that showed $A\beta_{(1-42)}$ in the soluble form, and not the aggregated form, is transported by ABCB1 [8]. To confirm $A\beta_{(1-42)}$ peptide underwent aggregation in the cell culture conditions at the peptide concentrations employed in this study, aggregation was monitored using the Thioflavin T assay. In these conditions, $A\beta_{(1-42)}$ peptide aggregation was observed at 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ however, with 10 $\mu\text{g/ml}$ $A\beta_{(1-42)}$ no time-dependent increase in fluorescence was observed, suggesting the levels of fluorescence obtained were at or near the

sensitivity threshold of the Thioflavin T assay. These findings are consistent with those of Shen and Murphy 1995 who reported a significant increase in Thioflavin T fluorescence intensity when A β ₍₁₋₄₂₎ is incubated over 8 days [47] and with the study of Cukalevski et al. which demonstrated A β ₍₁₋₄₂₎ aggregation increased over 24 h [48]. Furthermore, the latter study reported the fluorescent intensity of Thioflavin T increased as the peptide concentration increased, which is consistent with the current findings that show the fluorescent intensity of Thioflavin T at each time point monitored was higher for 50 μ g/ml A β ₍₁₋₄₂₎ than 25 μ g/ml A β ₍₁₋₄₂₎.

Here we report treatment of PBECs with A β ₍₁₋₄₂₎ at 25 μ g/ml and 50 μ g/ml for 24 h and 48 h significantly reduced ABCB1 expression levels. These findings are in partial agreement with those of Kania et al. who report down regulation of ABCB1 expression in hCMEC/D3 cells after 48 h treatment with 25 μ g/ml A β ₍₁₋₄₂₎, although no significant effect on ABCB1 expression was observed at 24 h exposure [16]. The effects of A β ₍₁₋₄₂₎ on ABCB1 expression in PBECs in vitro are supported by findings in vivo which demonstrate peripherally administration of A β ₍₁₋₄₂₎ leads to a reduction of ABCB1 expression at the mRNA level in the mouse blood-brain barrier [49]. Furthermore, ABCB1 expression has also been reported to be reduced in the BBB of transgenic mice overexpressing human APP, compared with wild type animals [50].

Subsequently, we investigated the effects of A β ₍₁₋₄₂₎ on ABCG2 activity and expression in PBECs. Here we demonstrate A β ₍₁₋₄₂₎ significantly inhibits ABCG2 activity by direct inhibition (without an associated reduction in ABCG2 expression) since, in cells treated with A β ₍₁₋₄₂₎ for 1 h, ABCG2 activity was significantly reduced. We believe this is the first study to report such a finding. Although A β ₍₁₋₄₂₎-mediated inhibition of ABCG2 has not been previously reported, this finding can be rationalised by the fact that ABCG2 possesses broad substrate specificity which overlaps with ABCB1 and that ABCG2 and ABCB1 share common inhibitors. The fact that A β ₍₁₋₄₂₎ is an ABCB1 substrate and inhibitor, supports the finding that the peptide is an ABCG2 inhibitor. Whilst no studies to date have reported A β ₍₁₋₄₂₎-mediated inhibition of ABCG2, there is evidence that A β ₍₁₋₄₂₎ is an ABCG2 substrate. Tai et al. demonstrated increased intracellular accumulation of [¹²⁵I]A β ₍₁₋₄₀₎ in hCMEC/D3 cells treated with the ABCG2 inhibitor fumitrimorgin C [51], Xiong et al. have reported higher accumulation of intravenously administered Cy5.5-labeled A β ₍₁₋₄₀₎ in Abcg2 knockout mice compared to wild type control [17], whilst Do et al demonstrated ABCG2-mediated efflux of [³H]A β ₍₁₋₄₀₎ in HEK293 cells stably transfected with human *ABCG2* cDNA [52].

As with ABCB1, the significant decrease in ABCG2 activity following 24 h and 48 h exposure to $A\beta_{(1-42)}$ is expected to be the result of a combination of the inhibition of ABCG2 activity and reduction of ABCG2 expression. Findings of the effects of $A\beta_{(1-42)}$ on ABCG2 expression reported in the literature are conflicting. Our findings contrast those of Kania et al. which demonstrated no clear change in ABCG2 expression in hCMEC/D3 cells after exposure to 25 $\mu\text{g/ml}$ $A\beta_{(1-42)}$ for 48 h [16], and those of Carrano et al. which report no alterations in ABCG2 mRNA levels in hCMEC/D3 cells after 24 h exposure to $A\beta_{(1-42)}$ oligomers and fibrils, however the effect of monomeric $A\beta_{(1-42)}$ was not investigated [53]. The difference in findings may be due to the fact that the current studies employed primary porcine brain endothelial cells whilst the above studies used the immortalised human hCMEC/D3 endothelial cell line in which CAR and PXR are barely detectable [54]. Furthermore, whilst in vivo studies report levels of *abcg2* RNA were not affected in the brains of mice following subcutaneous administration of $A\beta_{(1-42)}$ [48], up-regulation of ABCG2 expression has been reported in brains from individuals with Alzheimer's disease [17].

In this study we report $A\beta_{(1-42)}$, at 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$, significantly decreases ABCC5 activity in PBECs. This is the first study to demonstrate $A\beta_{(1-42)}$ -mediated inhibition of ABCC5, and this inhibition can be explained by the fact that, like ABCB1 and ABCG2, ABCC5 demonstrates broad substrate specificity and that the $A\beta_{(1-42)}$ substrate inhibits GS-MF transport, possibly by competitive inhibition. This finding is supported by the fact that $A\beta_{(1-42)}$ is a substrate for another member of the ABC subclass of transporters, namely *abcc1* since, in a mouse model of Alzheimer's disease with a deficiency in *abcc1*, increased cerebral $A\beta_{(1-42)}$ levels were observed [55].

In the current study treatment of PBECs with $A\beta_{(1-42)}$ at 25 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$ for 24 h and 48 h significantly reduced ABCC5 expression levels. Whilst this is the first study to demonstrate an $A\beta_{(1-42)}$ -mediated reduction in ABCC5 expression, it is consistent with findings that $A\beta_{(1-42)}$ reduces expression of the ABCB1 efflux transporter [16,49,50].

The findings that $A\beta_{(1-42)}$ significantly reduces ABCB1, ABCG2 and ABCC5 expression suggest coordination of $A\beta_{(1-42)}$ -mediated regulation of ABC efflux transporters in primary porcine blood-brain barrier endothelial cells. The pregnane X receptor and constitutive androstane receptor have been reported to regulate ABCB1 and ABCG2 expression at the BBB by binding to response elements in the promoter regions of the *ABCB1* and *ABCG2* genes in rat [20,22], and in PBECs [56,57]. Therefore, the ability of $A\beta_{(1-42)}$ to regulate

expression of PXR and CAR, which could subsequently impact ABCB1 and ABCG2 expression, was investigated.

Initial control studies were performed to establish the effect of the PXR ligand rifampicin on ABCB1 and ABCG2 expression and activity in PBECs. Rifampicin significantly increased both ABCB1 and ABCG2 expression and function, (data not shown), indicating expression of functional PXR in the PBECs employed in this study. This finding is similar to that of Ott et al. which reported rifampicin-induced ABCB1 expression and activity in PBECs [57], and to that of Abuznait et al. which demonstrated upregulation of ABCB1 expression in LS-180 cells [58].

This is the first study to provide direct evidence that $A\beta_{(1-42)}$ significantly reduces expression of both PXR and CAR. However, this finding is supported by studies carried out using the Tg2576 transgenic mouse model of Alzheimer's disease in which human amyloid precursor protein (hAPP) is overexpressed. The latter study reports expression of pxr protein in brain capillaries of Tg2576 transgenic mice, was lower than in control mice [59], which can be explained by the fact that high levels of hAPP are processed to $A\beta_{(1-42)}$, which subsequently reduces pxr expression.

The findings of the current study suggest $A\beta_{(1-42)}$ significantly inhibits activities of BBB ABCB1, ABCC5 and ABCG2, and modulates expression of components of their regulatory pathway. $A\beta_{(1-42)}$ therefore has the potential to impact on penetration of ABC transporter substrates, including many therapeutic drugs, across the BBB. Moreover, given that $A\beta_{(1-42)}$ is an ABCB1 substrate and that ABCB1-mediated amyloid- β efflux represents a clearance pathway for removal of amyloid- β from the brain, increased $A\beta_{(1-42)}$ production during Alzheimer's disease may interfere with this clearance mechanism.

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