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RESEARCH PAPER

Transport of interleukin-1 across cerebromicrovascular endothelial cells

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Background and purpose: The inflammatory cytokine interleukin-1 (IL-1) has profound actions in the brain, causing neuronal cell death and exacerbating brain damage. While circulating levels are normally low, IL-1 can be produced on the vascular side of the brain endothelium, and within the brain. The naturally occurring IL-1 receptor antagonist has been administered peripherally in a Phase II trial in acute stroke patients; understanding how IL-1 and IL-1 receptor antagonist penetrate the brain is, therefore, of considerable importance.
Experimental approach: An *in vitro* blood–brain barrier model was generated by co-culture of porcine brain microvascular endothelial cells with astrocytes. The mechanisms of transcellular transport of IL-1β and IL-1 receptor antagonist were characterized in this model, using endocytosis inhibitors and IL-1 receptor-blocking antibodies.
Key results: Transcellular IL-1β and IL-1 receptor antagonist transport was temperature-dependent and IL-1β was transported with higher affinity than IL-1 receptor antagonist. IL-1β inhibited IL-1 receptor antagonist transport more potently than IL-1 receptor antagonist inhibited IL-1β transport. Transport of IL-1β and IL-1 receptor antagonist was not via adsorptive-mediated endocytosis, although inhibition of microtubule assembly significantly attenuated transport of both cytokines. An antibody directed to the type II IL-1 receptor significantly reduced IL-1β transport.
Conclusions and implications: These results are consistent with IL-1 and IL-1 receptor antagonist being transported across cultured cerebromicrovascular endothelial cells and suggest that IL-1β transport may occur via a type II IL-1 receptor-dependent mechanism. Understanding IL-1 transport into the brain may have benefits, particularly in enhancing penetration of IL-1 receptor antagonist into the brain.
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Keywords: interleukin-1; *in vitro* blood–brain barrier model; transcytosis; microtubule

Abbreviations: AME, absorptive-mediated endocytosis; BBB, blood–brain barrier; IL-1, interleukin-1; LDL, low-density lipoprotein; PBEC, porcine brain endothelial cells

Introduction

Interleukin-1 (IL-1) is a pro-inflammatory cytokine that exerts numerous actions on the brain, including mediation of key host defence responses, and is associated with acute and chronic central nervous system (CNS) disorders (Rothwell and Luheshi, 2000). The IL-1 family comprises three members: the agonists IL-1α and IL-1β, that act by binding to a transmembrane receptor known as the type I IL-1 receptor (Sims *et al.*, 1988), and the naturally occurring IL-1 receptor antagonist (Dinarello, 1996) that blocks all known actions of IL-1α and IL-1β. IL-1 receptor antagonist has been used therapeutically in rheumatoid arthritis and tested in an early clinical trial in acute stroke (Emsley *et al.*, 2005; Clark *et al.*, 2007). A second receptor, type II IL-1 receptor that binds IL-1 (and shows higher affinity for IL-1β), is referred to as a 'decoy receptor' because it has a short intracellular domain that does not initiate signal transduction and its biological function is largely unknown.

IL-1 receptor antagonist shares 26% sequence identity with IL-1β (Eisenberg *et al.*, 1990) and both proteins are reported to possess a β-trefoil topology consisting of a β-barrel, composed of six β-strands, closed at one end by a further six β-strands (Vigers *et al.*, 1994). Structural analyses indicate that IL-1 receptor antagonist demonstrates the same overall fold pattern as IL-1β. However, significant sequence and structural differences between IL-1 receptor antagonist and IL-1β are observed in the region containing the N-linked glycosylation

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site (residues 83–95 of IL-1 receptor antagonist). Additionally, a second divergent region is located at the top of the β-barrel, an area implicated in the interaction of IL-1β with the type I IL-1 receptor (Vigers *et al.*, 1994).

The mechanisms through which systemic infection or inflammation influence CNS function are unknown. Circulating levels of IL-1 are normally very low, although the cytokine can be produced on the vascular side of the cerebral endothelium [e.g. by atherosclerotic plaques (Moyer *et al.*, 1991)], and thus can potentially enter the brain. IL-1 is also expressed by brain cells (Blasi *et al.*, 1999; Pearson *et al.*, 1999). While penetration of peripheral IL-1 into the brain may have detrimental effects, there is considerable interest in facilitating entry of IL-1 receptor antagonist, such that systemic administration of the antagonist may have therapeutic benefits (Allan *et al.*, 2005; Clark *et al.*, 2007; Gueorguieva *et al.* 2007).

Previous *in vivo* studies in mice indicated that IL-1 and IL-1 receptor antagonist are transported into the brain either via multiple carriers with overlapping affinities or by a single carrier capable of transporting both IL-1β and IL-1 receptor antagonist as well as IL-1α (Banks *et al.*, 1991). Uptake of other large molecules such as transferrin (Roberts *et al.*, 1993), insulin (Miller *et al.*, 1994) and low-density lipoprotein (LDL) (Dehouck Fenart Dehouck *et al.*, 1997) at the blood–brain barrier (BBB) is reported to involve receptors. As yet, the precise mechanism of IL-1 and IL-1 receptor antagonist entry into the brain is unknown, but the involvement of type I or type II IL-1 receptors is a possibility which we tested here.

The objective of this study was to test the hypothesis that IL-1 and IL-1 receptor antagonist are transported across an *in vitro* model of the BBB, and to investigate the mechanisms of this transport.

Methods

Cerebromicrovascular endothelial cell isolation

Cerebromicrovascular endothelial cells were isolated based on the method of Rubin *et al.* (1991) with modifications. Porcine brains were transported from the abattoir in L-15 medium containing 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin. Brain hemispheres (10–12) were then washed in phosphate-buffered saline (PBS), cleared of meninges and placed in ice-cold PBS. The white matter was removed and the remaining brain tissue chopped into smaller pieces and passed through a 50 mL syringe into MEM/HEPES containing 10% (v/v) foetal calf serum (FCS) (10 mL brain tissue into 35 mL medium). Cortical grey matter was gently homogenized with two pestles (89–127 μm clearance, 15 strokes and 25–76 μm clearance, 15 strokes) and sequentially filtered first through a 150 μm nylon mesh and then through a 60 μm nylon mesh. The material on the 60 μm mesh was digested in 80 mL M119 medium containing 10% (v/v) FCS, 100 U·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin, 210 U·mL^{-1} collagenase, 114 U·mL^{-1} DNase I and 91 U·mL^{-1} trypsin for 1 h at 37°C. Material was washed off the mesh using MEM/HEPES, the digest mix centrifuged for 10 min at 1000× *g* and the pellet containing cerebromicrovessels resuspended in 10 mL growth medium [Dulbecco's modified Eagle's medium (DMEM) plus 10% (v/v) plasma derived serum, 100 U·mL^{-1} penicillin, 100 μg·mL^{-1} streptomycin, 2 mmol·L^{-1} glutamine and 125 μmol·L^{-1} heparin]. One mL aliquots were added to individual wells of 6-well plates pre-coated with rat tail collagen (100 μg·mL^{-1}) and with human fibronectin (50 μg·mL^{-1}) and maintained in growth medium at 37°C in a humidified atmosphere of 5% CO_2 in air.

Astrocyte isolation

Mixed glial cell cultures were prepared from the brains of 0- to 2-day-old rat pups as described previously (McCarthy and de Vellis, 1980). The two cortices were removed and rolled on a piece of sterile filter paper to remove the meninges. Cortices were dissociated through an 80 μm nylon mesh, the filtrate collected and centrifuged for 10 min at 200× *g*. Supernatant was removed and the pellet resuspended in 10 mL DMEM supplemented with 10% (v/v) FCS, 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin. Cells were seeded at 5×10^5 cells·mL^{-1} in 75 cm^2 tissue culture flasks coated with poly-D-lysine, and grown in a 5% CO_2 humidified atmosphere. The culture medium was changed after 5 days and then every 3 days until the cells reached confluency (12–13 days). Mixed glial cell cultures in 75 cm^2 tissue culture flasks were shaken at 37°C overnight at 245 rpm and the culture medium containing microglia discarded. Astrocytes were then dissociated using trypsin and collected by centrifugation (200× *g*, 5 min). The cells were re-suspended in DMEM with 10% (v/v) FCS, 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin, seeded at 1×10^5 cells·mL^{-1} onto 12-well plates and cultured for 10 days.

In vitro BBB model

The *in vitro* BBB model was prepared on rat-tail collagen type I and fibronectin coated Transwell® polycarbonate inserts (surface area 1 cm^2; pore size 0.4 μm). Porcine brain endothelial cells (PBEC), grown on 6-well plates to 70% confluency, were washed twice in PBS, then once in PBS containing 0.2 mg·mL^{-1} EDTA and harvested by incubating with trypsin at 37°C. PBEC were seeded at a density of 8×10^4 cells per insert, and the inserts placed in 12-well plates containing confluent rat astrocytes for 3 days. 24 h prior to use, the medium in the apical and basal compartments was replaced with serum-free medium supplemented with 312.5 μmol·L^{-1} 8-4-chlorophenylthio-cAMP, 17.5 μmol·L^{-1} RO-20-1724 and 55 nmol·L^{-1} hydrocortisone, which are reported to increase transcellular electrical resistance (Rubin *et al.*, 1991). Transendothelial electrical resistance (TEER) was monitored daily using EVOM chopstick electrodes (World Precision Instruments, Sarasota, FL, USA). Monolayer TEER was calculated by subtracting the value of a blank Transwell® insert from total resistance obtained for the endothelial monolayer and insert. The average net TEER of cell monolayers was 394 ± 81 Ω·cm^2 and monolayers with a resistance of less than 250 Ω·cm^2 were discarded.

IL-1 transport studies

In order to measure IL-1β and IL-1 receptor antagonist transport across the *in vitro* BBB model, cells were incubated at

37°C with IL-1β (100 ng·mL^{-1}) and IL-1 receptor antagonist (1 μg·mL^{-1}) in the apical compartment and a 100 μL sample taken from the basal compartment after 1 h. TEER was measured immediately prior to each sampling. Samples were stored at –80°C and IL-1β and IL-1 receptor antagonist concentrations subsequently quantified by ELISA. In order to assess if transport of IL-1β and IL-1 receptor antagonist was temperature-dependent, transport was measured at 4°C and 37°C. To investigate the mechanism of cytokine transcytosis, the following reagents were added to the apical compartment (at the final concentrations indicated) 30 min prior to transport studies: sodium azide (1 mmol·L^{-1}), 2-deoxyglucose (1 mmol·L^{-1}), protamine (10 μg·mL^{-1}), sheep anti-rat type I IL-1 receptor (5 μg·mL^{-1}), sheep anti-rat type II IL-1 receptor (5 μg·mL^{-1}), sheep IgG (5 μg·mL^{-1}) colchicine (10 μmol·L^{-1}), cytochalsin D (0.5 μmol·L^{-1}) and IL-1β and IL-1 receptor antagonist (at concentrations specified in figure legends). Transport via the paracellular pathway was assessed by addition of 2.5 μmol·L^{-1} fluorescein isothiocyanate (FITC)-labelled dextran (20 kDa) to the apical compartment. Samples were taken from the basal compartment after 1 h and fluorescence measured (excitation wavelength 475 nm; emission wavelength 538 nm) using a 96-well spectrofluorometer.

ELISA

Interleukin-1β and IL-1 receptor antagonist were quantified by ELISA. Immunosorbent 96-well flat-bottomed plates were coated overnight at 4°C with mouse monoclonal anti-human IL-1β (4 μg·mL^{-1}) or IL-1 receptor antagonist (1.25 μg·mL^{-1}) in PBS, pH 7.4, washed in wash buffer [0.5 mol·L^{-1} NaCl, 2.5 mmol·L^{-1} NaH_2PO_4, 7.5 mmol·L^{-1} Na_2HPO_4, 0.1% (v/v) Tween-20], and blocked overnight at 4°C with PBS containing 0.5% (w/v) BSA and 200 mg·mL^{-1} sodium azide. Medium samples (100 μL) from transport studies were added to pre-coated, blocked plates and plates incubated overnight at 4°C. Standard curves for IL-1β and IL-1 receptor antagonist were obtained by serial dilution of recombinant protein. For IL-1β ELISA, following three washes with wash buffer, plates were incubated with polyclonal goat anti-human IL-1β serum (1 μg·mL^{-1}) in assay buffer [150 mmol·L^{-1} NaCl, 20 mmol·L^{-1} Tris pH 7.4, 0.1% (w/v) bovine serum albumin and 0.05% (v/v) Tween-20] supplemented with 1% (v/v) mouse serum, for 2 h at room temperature. Plates were then washed and incubated with horseradish peroxidase-labelled donkey anti-goat IgG (0.2 μg·mL^{-1}) for 2 h at room temperature. For IL-1 receptor antagonist ELISA, following three washes with wash buffer, plates were incubated with biotinylated mouse monoclonal anti-human IL-1 receptor antagonist (0.2 μg·mL^{-1}) in assay buffer supplemented with 1% (v/v) mouse serum for 2 h at room temperature. Following washing, samples were incubated with horseradish peroxidase-labelled streptavidin (312.5 μg·mL^{-1}) for 30 min at room temperature. For both IL-1β and IL-1 receptor antagonist ELISAs, following further washing, plates were incubated with 2.5 mg·mL^{-1} *ortho*-phenylenediamine dihydrochloride and 30% (v/v) hydrogen peroxide. Colour formation was quenched by addition of 2 mol·L^{-1} H_2SO_4 (25 μL) and optical density measured at 490 nm using a 96-well spectrometer.

Western blot analysis

Porcine brain endothelial cells were harvested into cell lysis buffer [50 mmol·L^{-1} Tris-HCl pH 7.5, 50 mmol·L^{-1} NaF, 1 mmol·L^{-1} phenylmethylsulphonyl fluoride, 50 mmol·L^{-1} β-glycerophosphate, 5 mmol·L^{-1} Na_3VO_4, 1% (v/v) Triton X-100] and protein content determined using the BCA protein assay reagent. Cellular proteins (10 μg per lane), were resolved by electrophoresis on a 10% (w/v) SDS-polyacrylamide gel and proteins transferred onto nitrocellulose membranes. Membranes were incubated for 1 h in PBS containing 2.5% (w/v) BSA and 0.1% Tween-20 prior to overnight incubation at 4°C with either mouse anti-human type I IL-1 receptor (R&D Systems, 1:100), mouse anti-human type II IL-1 receptor (R&D Systems, 1:100) or sheep anti-rat type II IL-1 receptor [National Institute for Biological Standards and Control (NIBSC), 1:1000] antibodies in PBS containing 1 % (w/v) skimmed milk powder and 0.1 % Tween-20. Following washing, membranes were incubated with horse-radish peroxidase-conjugated anti-mouse (1:2000) or anti-sheep IgG antibodies (1:2000) (Dako) and bands were detected by enhanced chemiluminescence using Hyperfilm (GE Healthcare).

Statistical analysis

Data were expressed as mean (± standard error), and analysed and presented using GraphPad Prism (version 4.0). Groups of two were analysed using Student's *t*-test; groups of three or more were analysed using either one-way analysis of variance (ANOVA) with a Dunnett's *post hoc* test or, if multiple variables were involved, two-way ANOVA with Bonferroni *post hoc* test. Values were considered to be significantly different when the probability that differences were not due to chance alone was less than 5% ($P < 0.05$).

Nomenclature

Receptor nomenclature is as defined by Alexander *et al.* (2008)

Materials

Cell culture plastic ware, rat tail collagen type I and human fibronectin were obtained from Becton Dickinson (Oxford, UK). DMEM, penicillin/streptomycin and L-glutamine were purchased from Invitrogen (Paisley, UK), FCS from PAA (Yeovil, UK) and plasma-derived serum from First Link (Birmingham, UK). NIBSC (Potters Bar, UK) supplied recombinant human IL-1β, IL-1 receptor antagonist, sheep anti-rat type I IL-1 receptor antibody and sheep anti-rat type II IL-1 receptor antibody. Mouse anti-human type I IL-1 receptor and mouse anti-human type II IL-1 receptor antibodies used in Western blot analysis as well as ELISA reagents were obtained from R&D Systems (Oxford, UK). The phosphodiesterase inhibitor RO20-1724 was obtained from Merck (Nottingham, UK). Trypsin, collagenase I and DNase I were purchased from Worthington Lorne Laboratories (Twyford, UK). Enhanced chemiluminescence reagents and nitrocellulose were from GE Healthcare (Little Chalfont, UK). The BCA protein assay reagent was obtained from Pierce Biotechnology (Cramling-

ton, UK). All other materials were purchased from Sigma-Aldrich (Poole, UK), unless stated otherwise.

Results

Characterization of IL-1β and IL-1 receptor antagonist transport across cultured cerebromicrovascular endothelial cell monolayers

In order to establish the concentration of IL-1β to be used experimentally, initial studies investigated the effect of IL-1β on endothelial monolayer integrity by measuring TEER. Over the course of 1 h, exposure of cells to 100 ng·mL^{-1} IL-1β in the apical compartment did not significantly affect TEER; however, following incubation for 2 h, there was a significant decrease ($P < 0.05$) in resistance (Figure 1A). Treatment with IL-1β (1000 ng·mL^{-1}) significantly reduced TEER, even after 1 h exposure (Figure 1A). In subsequent transport studies, cells were therefore routinely incubated with 100 ng·mL^{-1} IL-1β for 1 h. Transport of IL-1 receptor antagonist could not be detected at 100 ng·mL^{-1}. TEER was not significantly affected by either 100 ng·mL^{-1} IL-1 or 1000 ng·mL^{-1} IL-1 receptor antagonist (Figure 1B); hence, all IL-1 receptor antagonist studies were performed by incubating cells with 1000 ng·mL^{-1} IL-1 receptor antagonist for 1 h.

Both IL-1β and IL-1 receptor antagonist were transported across the *in vitro* BBB model in a temperature-dependent manner. Transport of IL-1β across the cell monolayer at 37°C was significantly higher ($P < 0.01$) than at 4°C, indicating IL-1β transport to be temperature sensitive (Figure 2A). Similarly, transport of IL-1 receptor antagonist across the *in vitro* BBB model at 37°C was significantly higher ($P < 0.01$) than at 4°C (transport not detectable) (Figure 2B). Importantly, there was no significant difference observed in the transport of the paracellular marker FITC-dextran (20 kDa) at 37°C and 4°C (data not shown). These findings suggest that a temperature-sensitive mechanism(s) is involved in transporting IL-1β and IL-1 receptor antagonist across cultured cerebromicrovascular endothelial cells.

In an attempt to assess if IL-1β and IL-1 receptor antagonist transport were active processes, endothelial cells were pre-treated with the respiratory inhibitors sodium azide (1 mmol·L^{-1}) and 2-deoxyglucose (1 mmol·L^{-1}) prior to trans-

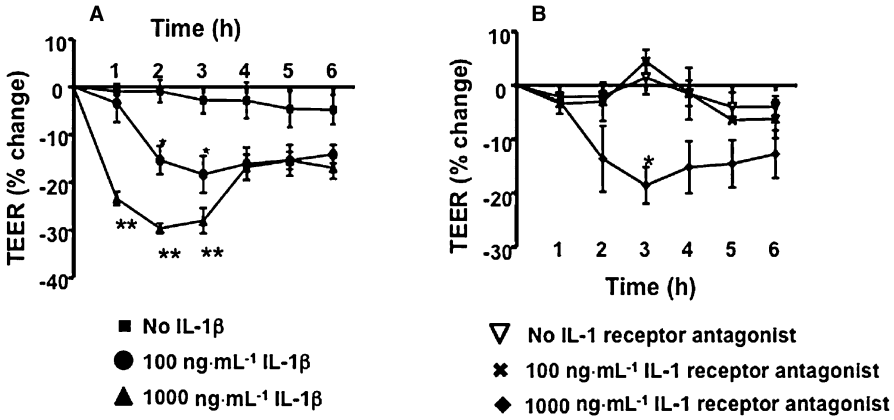

Figure 1 Effect of IL-1β and IL-1 receptor antagonist on transendothelial electrical resistance. Porcine brain endothelial cells on Transwell® inserts were incubated with either IL-1β (A) or IL-1 receptor antagonist (B) in the apical compartment at 37°C. Data are presented as mean ± s.e. of 5 Transwell® inserts performed across two independent experiments. Statistical significance was determined using two-way ANOVA followed by Bonferroni *post hoc* test (*$P < 0.05$, **$P < 0.01$). IL-1, interleukin-1; TEER, transendothelial electrical resistance.

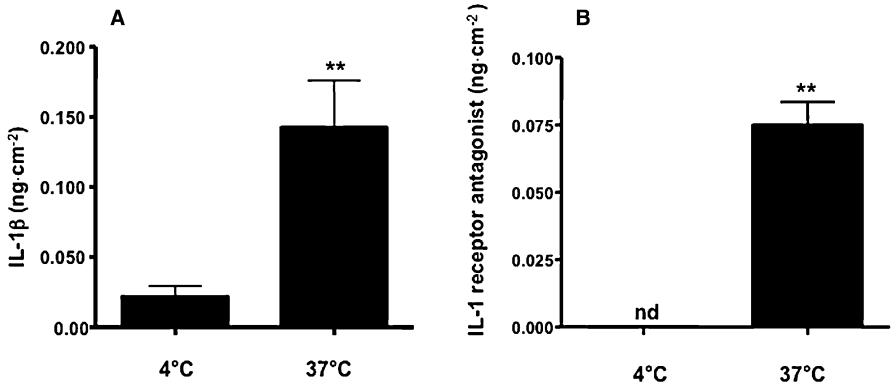

Figure 2 Effect of temperature on IL-1β and IL-1 receptor antagonist transport. Porcine brain endothelial cells on Transwell® inserts were incubated with either 100 ng·mL^{-1} IL-1β, (A) or 1 μg·mL^{-1} IL-1 receptor antagonist, (B) in the apical compartment for 1 h at 4°C and 37°C. A 100 μL sample was removed from the basal compartment following incubation, and IL-1β and IL-1 receptor antagonist quantified by ELISA. Data are presented as mean ± s.e. of 10 Transwell® inserts performed across two independent experiments and were analysed by Student's *t*-test, **$P < 0.01$. IL-1, interleukin-1.

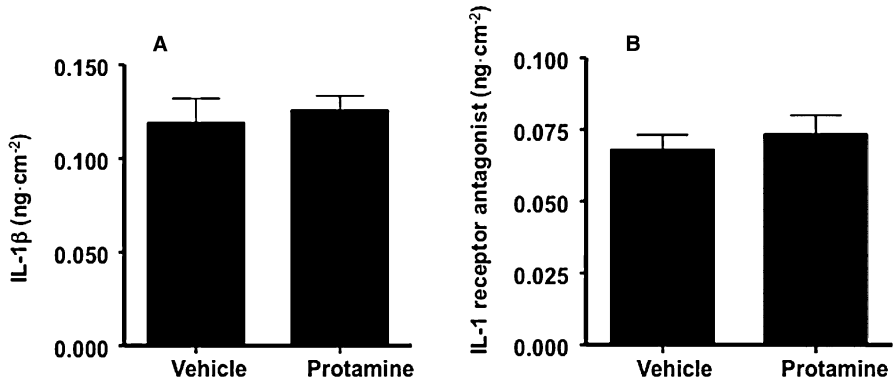

Figure 3 Lack of effect of inhibiting adsorptive-mediated endocytosis on IL-1β and IL-1 receptor antagonist transport. Porcine brain endothelial cells on Transwell® inserts were pre-incubated at 37°C with either vehicle or 10 $\mu g \cdot mL^{-1}$ protamine, in the apical compartment prior to transport analysis. Cells were incubated with either 100 $ng \cdot mL^{-1}$ IL-1β, (A) or 1 $\mu g \cdot mL^{-1}$ IL-1 receptor antagonist, (B) in the apical compartment for 1 h at 37°C. A 100 μL sample was removed from the basal compartment following incubation and IL-1β and IL-1 receptor antagonist quantified by ELISA. Data are presented as mean ± s.e. of 7 Transwell® inserts performed across two independent experiments and were analysed by Student's *t*-test. IL-1, interleukin-1.

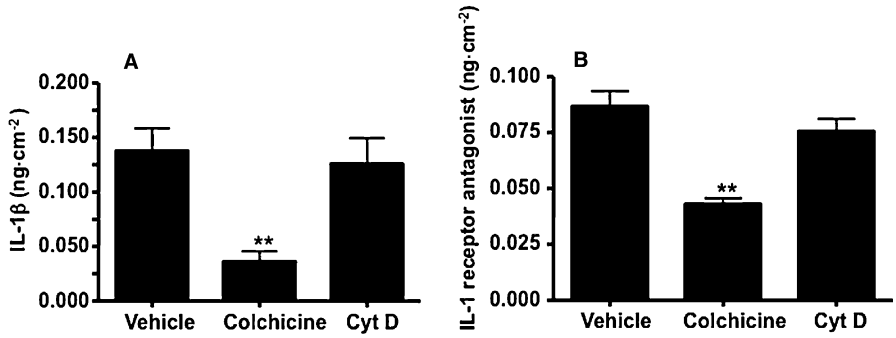

Figure 4 Role of microtubules in IL-1β and IL-1 receptor antagonist transport. Porcine brain endothelial cells on Transwell® inserts were pre-incubated at 37°C with either vehicle, colchicine (10 $\mu mol \cdot L^{-1}$) or cytochalasin D (0.5 $\mu mol \cdot L^{-1}$) for 30 min. Cells were incubated with either IL-1β (100 $ng \cdot mL^{-1}$) (A) or IL-1 receptor antagonist (1 $\mu g \cdot mL^{-1}$) (B) in the apical compartment for 1 h. A 100 μL sample was removed from the basal compartment following incubation and IL-1β and IL-1 receptor antagonist quantified by ELISA. Data are presented as mean ± s.e. of 10 Transwell® inserts performed across two independent experiments and were analysed by one-way ANOVA followed by Dunnett's multiple comparison of means, $^{**}P < 0.01$. IL-1, interleukin-1.

port. However, treatment resulted in loss of monolayer integrity as reflected by significant decreases in TEER. Consequently, transport studies using cells treated with these inhibitors could not be performed.

In order to establish if IL-1β and IL-1 receptor antagonist were transported across cerebromicrovascular endothelial cells by adsorptive-mediated endocytosis (AME), cells were pretreated with the AME inhibitor protamine. Neither IL-1β (Figure 3A) nor IL-1 receptor antagonist (Figure 3B) transport was significantly attenuated by pretreatment with protamine. No significant change in TEER was observed with protamine treatment (pretreatment TEER, 485 ± 68 $\Omega \cdot cm^2$; post-treatment TEER, 500 ± 58 $\Omega \cdot cm^2$).

In order to investigate the role of microtubules and actin in IL-1 transport, PBEC were pretreated for 30 min with either vehicle (0.1% (w/v) BSA in PBS), the microtubule assembly inhibitor colchicine or the actin depolymerizing agent cytochalasin D. Transport of both IL-1β (Figure 4A) and IL-1 receptor antagonist (Figure 4B) was significantly reduced ($P < 0.01$) by the microtubule assembly inhibitor colchicine but not by cytochalasin D.

Further characterization of IL-1β and IL-1 receptor antagonist transport by inhibitory studies revealed that IL-1β (100 $ng \cdot mL^{-1}$) transport across the PBEC monolayer was significantly inhibited only by exposure to a thousand-fold excess of IL-1 receptor antagonist (100 $\mu g \cdot mL^{-1}$) (Figure 5A). However, transport of IL-1 receptor antagonist (1 $\mu g \cdot mL^{-1}$) was significantly inhibited by IL-1β at a concentration of 1 $\mu g \cdot mL^{-1}$ (Figure 5B).

Role of IL-1 receptors

Initially, to assess the potential involvement of the type I and II IL-1 receptors in receptor-mediated transport of IL-1β and IL-1 receptor antagonist across the *in vitro* BBB, receptor expression in PBEC was investigated by Western blot analysis. Studies revealed both type I and type II IL-1 receptors were expressed in brain endothelial cells. The mouse anti-human type I IL-1 receptor antibody (R&D Systems) detected expression of this receptor with an apparent molecular weight of 68 kDa (Figure 6A). This antibody also potently inhibited IL-1β-induced IL-2 release from LBRM-33 Tg6 cells (data not

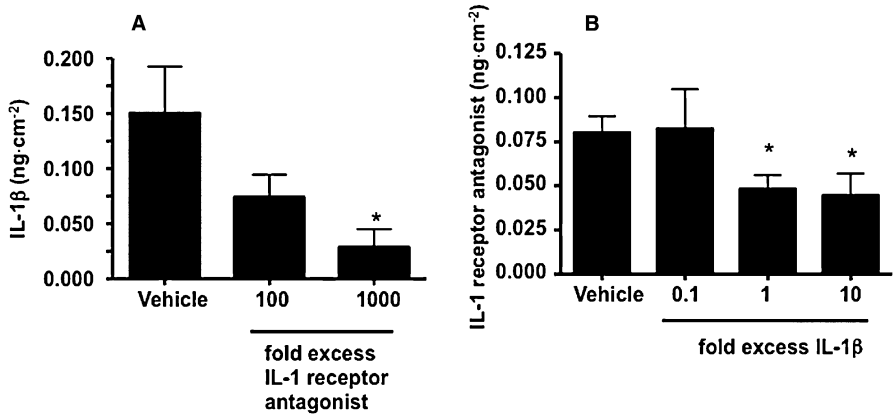

Figure 5 Relative inhibitory potencies of IL-1β and IL-1 receptor antagonist on each other's transport. Porcine brain endothelial cells were grown on Transwell® inserts. Cells were pretreated for 30 min with 100 (10 μg·mL^{-1}) or 1000 (100 μg·mL^{-1})-fold excess of IL-1 receptor antagonist, and incubated with IL-1β (100 ng·mL^{-1}) at 37°C. After 1 h, a 100 μL sample was removed from the basal compartment and IL-1β quantified by ELISA (A). Cells were pretreated for 30 min with 0.1 (0.1 μg·mL^{-1}), 1 (1 μg·mL^{-1}) and 10 (10 μg·mL^{-1})-fold excess of IL-1β, and incubated with IL-1 receptor antagonist (1 μg·mL^{-1}) at 37°C. After 1 h, a 100 μL sample was removed from the basal compartment and IL-1 receptor antagonist quantified by ELISA (B). Data are presented as mean ± s.e. of 10 Transwell® inserts performed across three independent experiments and were analysed by one-way ANOVA followed by Dunnett's multiple comparison of means, *$P < 0.05$. IL-1, interleukin-1.

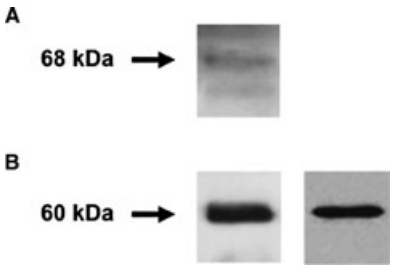

Figure 6 Interleukin-1 (IL-1) receptor expression in porcine brain endothelial cells. Cellular proteins (10 μg per lane) were separated by SDS-polyacrylamide gel electrophoresis, electrotransferred to nitrocellulose membrane and incubated with (A) mouse anti-human type I IL-1 receptor, (B) mouse anti-human type II IL-1 receptor (left panel) or sheep anti-rat type II IL-1 receptor (right panel) antibodies. Following incubation with either horse-radish peroxidase-conjugated anti-mouse IgG or anti-sheep IgG, IL-1 type I and type II receptors were detected by enhanced chemiluminescence.

shown) confirming its specificity. Both mouse anti-human type II IL-1 receptor (R&D Systems) and sheep anti-rat type II IL-1 receptor (NIBSC) antibodies revealed expression of the type II IL-1 receptor with an apparent molecular weight of 60 kDa (Figure 6B). The type II IL-1 receptor appeared to be expressed at much higher level than the type I receptor in these cerebromicrovascular endothelial cells.

Treatment of endothelial cell monolayers with sheep anti-rat type II IL-1 receptor antibody had no effect on the TEER (pretreatment TEER, 516 ± 25 Ω·cm^2; post-treatment TEER, 562 ± 90 Ω·cm^2), but significantly decreased IL-1β transport ($P < 0.01$) in the apical to basal direction (Figure 7A). No significant reduction of IL-1β transport was observed in response to treatment with sheep anti-rat type I IL-1 receptor antibody (Figure 7A). IL-1 receptor antagonist transport was not significantly affected by either antibody (Figure 7B). Sheep IgG antibody was included as a control and did not significantly reduce transport of either IL-1β (Figure 7A) or IL-1 receptor antagonist (Figure 7B) and had no significant effect on TEER.

Discussion and conclusions

A low permeability *in vitro* model consisting of porcine brain microvascular endothelial cells, co-cultured with rat astrocytes, has been used to investigate the mechanism of IL-1β and IL-1 receptor antagonist transport across endothelial cells. Both IL-1β and IL-1 receptor antagonist were transported across this *in vitro* BBB model. Whereas IL-1β transport was observed using an initial apical IL-1β concentration of 100 ng·mL^{-1}, transport of IL-1 receptor antagonist was observed only when using an initial IL-1 receptor antagonist concentration of 1 μg·mL^{-1}, with transport of IL-1 receptor antagonist being undetectable at 100 ng·mL^{-1}. This suggests that IL-1β is transported across the cell barrier with higher affinity than IL-1 receptor antagonist. Our findings revealed that while transport of IL-1 receptor antagonist (1 μg·mL^{-1}) across the cell monolayer was significantly inhibited by IL-1β at a concentration of 1 μg·mL^{-1}, transport of IL-1β (100 ng·mL^{-1}) was significantly inhibited only by a thousand-fold excess of IL-1 receptor antagonist (100 μg·mL^{-1}). These results further suggest that IL-1β is transported across the cell monolayer with a higher affinity than IL-1 receptor antagonist; such differences in ligand selectivity are characteristic of saturable transport systems. These observations that transport of IL-1β is inhibited by IL-1 receptor antagonist and IL-1 receptor antagonist transport inhibited by IL-1β are consistent with the findings of Gutierrez *et al*. (1994), who showed that in mice, although IL-1 receptor antagonist, given peripherally, inhibited IL-1β entry into the brain, IL-1β inhibited entry of IL-1 receptor antagonist more efficiently than either IL-1α or IL-1 receptor antagonist. Sequence and structural differences between IL-1β and IL-1 receptor antagonist in domains of the proteins implicated in interaction with the receptor (Vigers

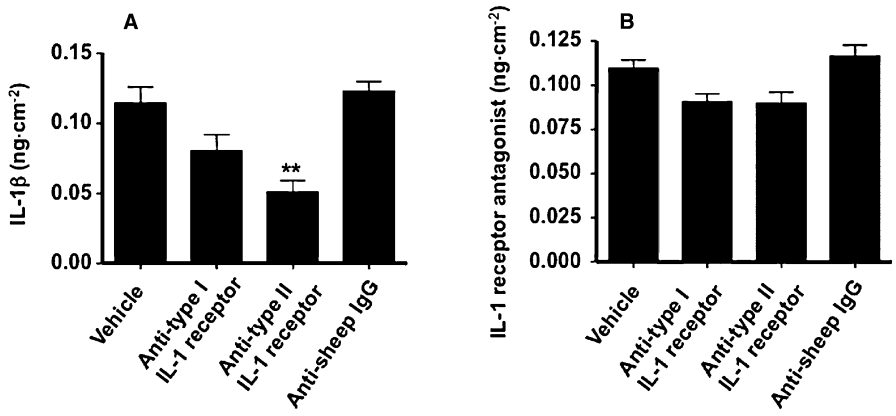

Figure 7 Porcine brain endothelial cells on Transwell® inserts were pre-incubated for 30 min at 37°C with either vehicle, sheep anti-rat type I IL-1 receptor antibodies (5 μg·mL^{-1}), sheep anti-rat type II IL-1 receptor antibodies (5 μg·mL^{-1}) or sheep IgG (5 μg·mL^{-1}). Cells were incubated with either IL-1β (100 ng·mLl^{-1}) (A) or IL-1 receptor antagonist (1 μg·mL^{-1}) (B) in the apical compartment for 1 h. A 100 μL sample was removed from the basal compartment following incubation and IL-1β and IL-1 receptor antagonist quantified by ELISA. Data are presented as mean ± s.e. of 10 Transwell® inserts performed across two independent experiments and were analysed by one-way ANOVA followed by Dunnett's multiple comparison of means, $^{**}P < 0.01$. IL-1, interleukin-1.

et al., 1994) may potentially contribute to differential interactions between IL-1 ligands and receptor.

Transport of molecules via transcellular pathways (e.g. receptor-mediated endocytosis and transcytosis) is temperature-dependent (Demeule *et al.*, 2002; Miller *et al.*, 1994). Our findings indicate transport of both IL-1β and IL-1 receptor antagonist across the *in vitro* BBB model is highly temperature sensitive, with significantly less cytokine being transported at 4°C compared with 37°C. The endothelial cell monolayers used in our studies were intact, highly restrictive and demonstrated significant TEER. Transport of relatively large molecules such as IL-1β (17 kDa) and IL-1 receptor antagonist (18 kDa) via the paracellular pathway is therefore very unlikely. To assess the effect of temperature on diffusion via the paracellular pathway, transport of the known paracellular marker FITC-dextran (20 kDa) was studied at 4°C and 37°C. Unlike the transport of both IL-1β and IL-1 receptor antagonist, transport of FITC-dextran was not temperature-dependent, suggesting IL-1β and IL-1 receptor antagonist were not transported via the paracellular pathway. These findings are consistent with reports that transport of proteins, for example, transferrin (Raub and Newton, 1991), LDL (Dehouck Fenart Dehouck *et al.*, 1997) and lactoferrin (Fillebeen *et al.*, 1999), is significantly reduced at 4°C in *in vitro* models of the BBB. In addition, our findings are supported by the *in vivo* studies of Hashimoto *et al.* (1991) which report internalization of IL-1 into intracellular vesicles by cerebromicrovascular endothelial cells.

A potential mode of initial cellular entry is absorptive-mediated endocytosis (AME), a temperature-dependent process (Drin *et al.*, 2003), which has been reported in isolated brain capillaries (Kumagai *et al.*, 1987). Our studies revealed, however, that transport of neither IL-1β nor IL-1 receptor antagonist was significantly attenuated by the AME inhibitor protamine (Sai *et al.*, 1998), suggesting transport of the cytokines was not via this process.

Transport of large molecules, for example, low-density lipoprotein, across the BBB is often via receptor-mediated endocytosis and transcytosis (Dehouck Fenart Dehouck *et al.*, 1997). Transcytosis across endothelial cells has been shown to be microtubule-dependent (Antohe *et al.*, 1993). Our studies revealed that transport of both IL-1β and IL-1 receptor antagonist across cultured cell monolayers was significantly reduced by the microtubule assembly inhibitor colchicine, supporting involvement of microtubules in IL-1β and IL-1 receptor antagonist transcytosis. This finding is consistent with that of Ostlund *et al.* (1979) who demonstrated that colchicine significantly reduced the rate of low-density lipoprotein endocytosis in cultured cells.

Our findings that both IL-1β and IL-1 receptor antagonist are transported in a temperature- and microtubule-dependent manner and that IL-1β and IL-1 receptor antagonist inhibit the other's transport with differing potency suggest the cytokines may be transported via a transport system (potentially receptor-mediated endocytosis) as proposed from *in vivo* studies by Banks *et al.* (1991) and Gutierrez *et al.* (1994).

It is possible that IL-1 receptors are involved in the transport of IL-1 across the *in vitro* model of the BBB. Using Western blot analysis, we first showed that both type I and type II IL-1 receptors were expressed on porcine cerebromicrovascular endothelial cells, which is consistent with the findings of Konsman *et al.* (2004) who reported type I receptor reactive vessels throughout the rat brain, and those of Docagne *et al.* (2005) which showed expression of the type II receptor on brain endothelial cells. The type I IL-1 receptor, which appeared to be expressed at a much lower level than the type II receptor, possessed an apparent molecular weight of 68 kDa. This finding is consistent with the apparent molecular weights of 60–68 kDa reported in previous studies (Matsushima *et al.*, 1986; Horuk *et al.*, 1987; Bomsztyk *et al.*, 1989; Fasano *et al.*, 1991). The apparent molecular weight of the type II IL-1 receptor as determined in our study was 60 kDa as previously published (Docagne *et al.*, 2005). Importantly, we demonstrate that the sheep anti-rat type II IL-1 receptor antibody (specificity demonstrated as stated above) significantly decreased IL-1β transport across the cell monolayer but had no significant effect on IL-1 receptor antagonist

transport. In contrast, the sheep anti-rat antibody to the type I IL-1 receptor [specificity demonstrated previously (Dunn *et al*., 2002)] did not significantly affect transport of either IL-1β or IL-1 receptor antagonist.

These findings suggest the involvement of type II IL-1 receptors in the internalization and/or transport of IL-1β in the *in vitro* BBB model. This is consistent with the finding that IL-1β is internalized by neutrophils via type II IL-1 receptor but not type I IL-1 receptor, which then co-localizes with the early endosome to enable receptor recycling (Bourke *et al*., 2003). In addition, a recent report suggested that the type II IL-1 receptor acts as a chaperone protein for the intracellular actions of IL-1 (Kawaguchi *et al*., 2006).

The type II IL-1 receptor is traditionally known as a decoy receptor that dampens the inflammatory response. If it also functions as a transport mechanism in cerebrovascular endothelial cells, then this could also reduce the inflammatory response. It appears that IL-1 can signal to the brain more efficiently via type I IL-1 receptor binding and the subsequent release of secondary mediators, than by IL-1 transport. Hence, the transport of IL-1 across the BBB via type II IL-1 receptors could serve to reduce the potency of the inflammatory response. An immune response that is not excessive or prolonged is often beneficial. Therefore, type II IL-1 receptor may act to attenuate the IL-1-induced inflammatory response by either binding IL-1, so it cannot trigger type I IL-1 receptor signalling, or transporting it across the BBB. Discovering the distribution of the type I IL-1 receptor and the type II IL-1 receptor in cerebral vasculature would help in disclosing the relative roles of type I IL-1 receptor signalling and type II IL-1 receptor-mediated transport.

These results illustrate that IL-1β and IL-1 receptor antagonist are transported across cultured cerebromicrovascular endothelial cells via a temperature sensitive, microtubule-dependent mechanism. The data also suggest that type II IL-1 receptors could have a new role in mediating IL-1 transport, by internalizing IL-1 or by binding/chaperoning IL-1 once within the cell. Thus, manipulation of type II IL-1 receptor expression or turnover could modify the transport of IL-1 into or out of the brain.

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Conflicts of interest

None.

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