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Assessment of zonula occluden-1 expression by Wnt/β-catenin pathway activators in hCMEC/D3 cells

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Background

The canonical Wnt/β-catenin pathway plays a key role in the induction and regulation of the blood-brain barrier (BBB) during brain development.[1] Neuronal cells and astrocytes produce Wnt ligands (Wnt7a and Wnt7b) which bind to their receptors on endothelial cells. This binding recruits the β-catenin destruction complex to the plasma membrane and inhibits the glycogen synthase kinase 3b (GSK3b - which phosphorylates β-catenin inducing its ubiquitination), hence stabilizing β-catenin. Upon stabilization, the increased level of β-catenin in the cytosol leads to its translocation into the nucleus. Here, it interacts with the T-cell factor/lymphoid enhancing factor (TCF/LEF) inducing the expression of target genes such as claudin-3, GLUT-1 and P-glycoprotein (P-gp), essential for BBB functioning.[1,2] Hence, impairment in the Wnt/β-catenin pathway results in several central nervous system (CNS) disorders including stroke and brain tumours.[2]

Zonula occluden (ZO) proteins (ZO-1,-2 and -3) are part of the membrane-associated guanylate kinase (MAGUK) family and need for tight junctions (TJs) formation in the BBB, playing a key role in anchoring claudin and occluding proteins to the actin cytoskeleton [3,4]. Indeed, ZO proteins contain domain organizations made of conserved protein-protein interactions (PDZ-1, -2 and -3, SH3 and GuK) which allow the simultaneous binding to several adhe-

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sion receptors such as claudins and actin proteins, forming the so-called "junctional plaque".

Investigations on the regulation and function of the BBB for therapeutic purposes rely on the development of a realistic *in vitro* model of the BBB [3] and the immortalized human cerebral microvascular endothelial cell line (hCMEC/D3) has been established for this purpose.[5] The expression of TJs *in vitro* can be achieved by treating endothelial cells with activators of the Wnt/β-catenin pathway, including lithium chloride (LiCl), simvastatin and epidermal growth factor (EGF).[6–8] However, the interaction of β-catenin with the expression of ZO-1 in hCMEC/D3 cells has been poorly explored. Therefore, the aim of this work was to activate the Wnt/β-catenin pathway in the hCMEC/D3 cell line using simvastatin, LiCl and EGF so as to assess a possible implication with the expression of ZO-1, for therapeutic purposes.

Methods

Cell culture

The hCMEC/D3 cell line (purchased by Cedarlane) was received at passage number 26 and used for experiments until passage 35. Cells were grown in Endothelium Basal Medium (EBM) (Merck, UK) supplemented with 2.5% FBS, 10 mM HEPES, 1 ng/mL bFGF and 5 µg/mL ascorbic acid, on rat tail collagen type I (0.1 mg/mL) coated T75 flasks.

Development of an in-house in vitro model of BBB

The in vitro model of the BBB was developed according to Kafa *et al.*[9] Finally, 1 nM simvastatin or 10 mM lithium chloride or 5 ng/mL EGF were added separately or cells were left untreated (control).

Immunocytochemistry

Cells were fixed and permeabilized with ice-cold methanol for 6 min, blocked in 2% bovine serum albumin (BSA) in PBS for 1 h, prior incubation with the ZO-1 primary antibody (ThermoFisher, UK: polyclonal), diluted 1:100 in blocking buffer, for 1 h at RT. Cells were then washed with 2% BSA/PBS, followed by treatment with the secondary antibody (ThermoFisher, UK: 2 µg/mL goat anti-rabbit IgG (H+L) cross-adsorbed secondary, DyLight 550) for 2 h at RT. Nuclei were stained with Hoechst 33342 and inserts were mounted on microscope slides using ProLong™ Gold Antifade Mountant (ThermoFisher). TJs formation was

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assessed by confocal microscopy (Zeiss Axiovert 200M) using a 40× oil immersion objective and fixed laser settings.

Quantification of ZO-1 expression

ImageJ software (NIH, USA) was used to quantify the signal intensity corresponding to ZO-1 as described by Shihan *et al.*[10]

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Data were analysed by one-way ANOVA with post-hoc Tukey's test (Minitab©). A p-value ≤ 0.05 was considered statistically significant.

Results

Expression of ZO-1 increases in presence of Wnt/β-catenin pathway activators

We investigated the expression of ZO-1 in hCMEC/D3 cells with no Wnt/β-catenin activators and found it to be poorly expressed. Indeed, a week signal intensity associated with ZO-1 was detected at the junctional level between hCMEC/D3 cells (Figure 1B). When cells were treated with 1 nM simvastatin, we detected a stronger immunoreactivity of the protein (Figure 1C) which translated in significantly higher signal intensity compared to the control ($*p < 0.05$) (Figure 1A). The signal intensity was localized not only on the junctional level but also intracellularly, as shown by the numerous yellow dots found in the cytosol of cells. Upon treatment of cells with LiCl and EGF, the staining of ZO-1 became even more pronounced at the junctional level (Figure 1D and E, respectively), with a significant increase in the signal intensity compared to simvastatin ($\#p < 0.05$) (Figure 1A). Especially, the signal intensity was detected at a higher level than simvastatin intracellularly, suggesting an increased expression of the ZO-1 in the cytosol of the cells, not yet involved in the formation of TJs.

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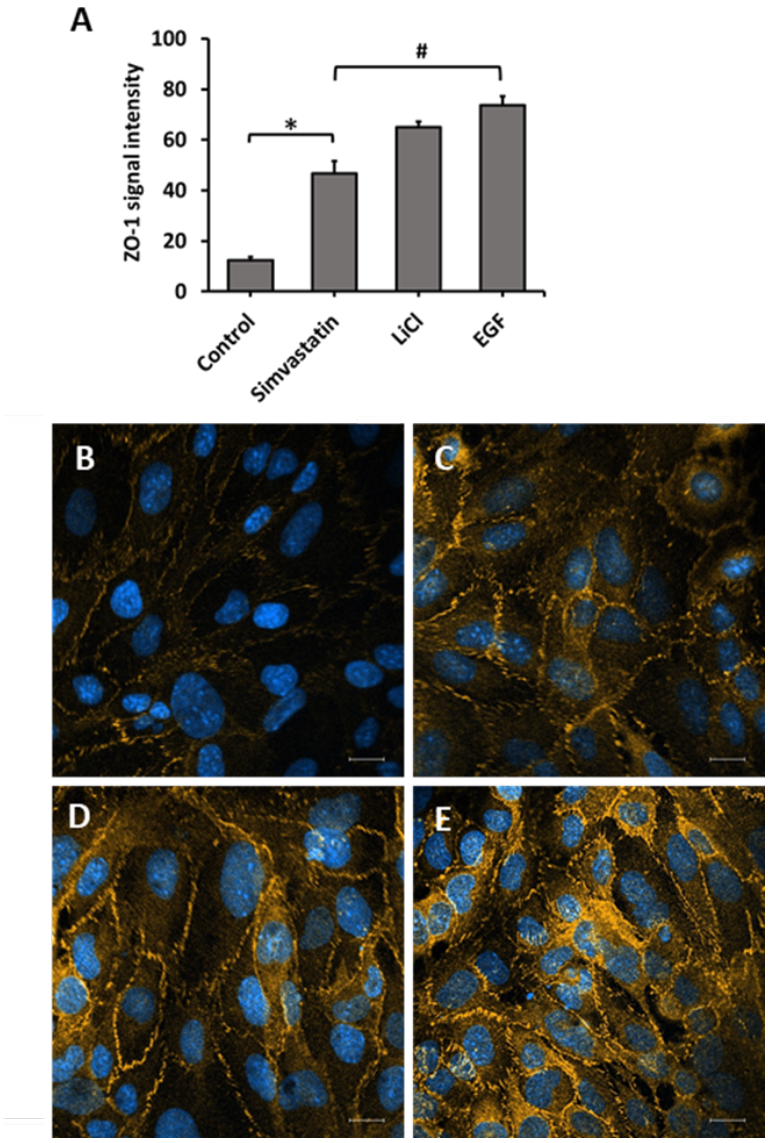

Figure 1 Expression of ZO-1 in hCMEC/D3 cells upon treatment with Wnt/β-catenin pathway activators. A: ZO-1 signal intensity quantification by Image J software. Data represent the mean ± SD (n=6); *p < 0.05 vs control; #p < 0.05 vs simvastatin; B-E: immunolocalization of ZO-1 in untreated cells (B), cells treated with 1 nM simvastatin (C) or 10 mM LiCl (D) or 5 ng/mL EGF (E). Cells were immunostained with an antibody to the ZO-1 protein and visualized with goat anti-rabbit IgG (H+L) conjugated to DyLight 550. Scale bar: 20 μm.

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Discussion

The aim of this study was to characterize ZO-1 in hCMEC/D3 cells following Wnt/β-catenin pathway activation using simvastatin, LiCl and EGF. Our results support the following conclusions:

1. ZO-1 is poorly expressed at the junctional level when no activators were added.
2. Simvastatin increases the expression of ZO-1 at both junctional and intracellular levels.
3. LiCl and EGF induces a higher expression of ZO-1 than simvastatin.

Liebner *et al.*[11] suggested that the therapeutic activation of the Wnt/β-catenin pathway may mitigate the BBB breakdown, hence the development of neurological diseases. However, it was also shown that temozolomide, a commonly used anti-glioma therapeutic agent,[12] acted by lowering the synthesis of Wnt3a ligand by brain cells and β-catenin underwent proteasomal degradation. This reduced the transcription of genes encoding the P-gp so to increase the BBB permeability to drugs that are normally blocked by the efflux P-gp pump.[13]

Several Wnt/β-catenin pathway activators have been used in this study and these may have different mechanism of action. Simvastatin is a hydroxymethylglutaryl coenzyme A reductase inhibitor. It is a lipophilic member of statin family able to cross the BBB [14], hence widely used for the treatment of neurological disorders since modulating the Wnt/β-catenin pathway in neural cells.[15,16] Investigations at the molecular level suggested that simvastatin played a synergistic effect in combination with Wnt3a, hence enhancing Wnt signalling when the pathway was poorly activated.[15] Several reports have shown that simvastatin improves the BBB endothelium features, though not through claudin-3 expression.[8,17] Especially, Ifergan *et al*.[18] showed that no alterations in the expression of ZO-1 in human brain microvascular endothelial cells occurred upon simvastatin treatment, although the permeability properties of the endothelium were improved.

LiCl is an inhibitor of the GSK3b and a widely used psychoactive drug. Its effects on the CNS were associated with the Wnt signalling enhancement.[19] Ramirez *et al*.[20] investigated the effect of several GSK3b inhibitors on the expression of some key TJs proteins in BMVEC cells and a correlation between β-catenin and ZO-1 was found.[20] A similar effect was also shown by Zhang *et al.*[21] on a kidney epithelial cell line (MDCK) where inhibition of the GSK3b induced accumulation of ZO-1 at the junctional level. In a study performed by Paolinelli *et al.*,[22] several GSK3b inhibitors were used on immortalized mouse endothelial cells to assess the BBB properties and an easy-to-handle model of BBB was developed. However, no further investigations were performed on the association of β-catenin with the

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expression of ZO-1. Similarly, Laksitorini *et al*. [23] characterized the hCMEC/D3 cell line properties upon treatment with several GSK3b inhibitors but no further investigations were done regarding the expression of ZO-1.

EGF is a receptor tyrosine kinase (RTK) that was shown to activate β-catenin signalling by the extracellular-signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway.[24] Indeed, it was previously demonstrated that the ERK/MAPK activates the Wnt/β-catenin pathway by phosphorylation of the Wnt co-receptor lipoprotein receptor-related protein 6 (LRP6).[25] Therefore, RTKs activate Wnt/β-catenin signalling by the ERK/LRP6 pathway inducing direct phosphorylation of β-catenin [24]. This mechanism was demonstrated using EGF for the activation of β-catenin in human mesenchymal stem cells.[6] Further, EGF was shown to retain endothelial integrity in Caco-2 cells and prevent TJs disruption, especially occludin and ZO-1, upon oxidative stress.[26] Consistent with our findings, EGF was reported to upregulate the expression of claudin-5 and ZO-1 in bEnd3 cells, an *in vitro* model of BBB.[27]

In conclusion, data in this study suggest that the expression of ZO-1 increases upon treatment with Wnt/β-catenin activators (especially LiCl and EGF) in hCMEC/D3 cells. ZO-1 plays a key role in the formation of TJs since it anchors extracellular TJs to the actin cytoskeleton, hence its expression is of vital importance for ensuring BBB integrity. Future studies aim to apply these findings in the context of BBB disruption for the treatment of brain disorders.

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