Microvascular Research 100 (2015) 48–53

Contents lists available at ScienceDirect

Microvascular Research

journal homepage: www.elsevier.com/locate/ymvre

Short Communication

Cytokine-mediated dysregulation of zonula occludens-1 properties in human brain microvascular endothelium

Keith D. Rochfort, Philip M. Cummins *

School of Biotechnology, Centre for Preventive Medicine, Dublin City University, Glasnevin, Dublin 9, Ireland

ARTICLE INFO

Article history:
Accepted 29 April 2015
Available online 4 May 2015

Keywords:
Blood–brain barrier
Cytokine
Endothelial
Zonula occludens-1
NADPH oxidase

ABSTRACT

Zonula occludens-1 (ZO-1) is essential to the proper assembly of interendothelial junction complexes that control blood–brain barrier (BBB) integrity. The goal of the current paper was to improve our understanding of how proinflammatory cytokines modulate ZO-1 properties within the human BBB microvascular endothelium. In this respect, we investigated the effects of TNF-α and IL-6 on ZO-1 using human brain microvascular endothelial cells (HBMvECs). Following treatment of HBMvECs with either cytokine (0–100 ng/ml, 18 h), we observed significantly decreased ZO-1 expression and ZO-1:occludin co-association, in parallel with increased ZO-1 phosphorylation (pTyr and pThr). All effects were dose-dependent. Either cytokine also caused extensive cell-cell border delocalization of ZO-1 in parallel with elevated HBMvEC permeability. Furthermore, pre-treatment of HBMvECs with antioxidants (superoxide dismutase, catalase, apocynin, *N*-acetylcysteine), or employing targeted inhibition of NADPH oxidase activation (NSC23766, gp91/p47 siRNA), were all found to comparably attenuate the cytokine-dependent decrease in ZO-1 protein expression. In summary, we present an *in vitro* model of how different pro-inflammatory cytokines can dysregulate ZO-1 properties in HBMvECs. A causal role for NADPH oxidase activation and oxidant signalling is also confirmed. Our findings add mechanistic depth to current *in vivo* models of BBB injury manifesting ZO-1 dysregulation.

Introduction

Much evidence points to a role for proinflammatory cytokines in the BBB dysregulation associated with neurological disorders (Tuttolomondo et al., 2008). These cytokines originate from cells within the neurovascular unit (*e.g.* endothelial cells, pericytes, astrocytes, neurons) and peripheral sources, whilst numerous studies have attempted to shed light on how cytokines specifically interact with the BBB endothelium. In this respect, most attention has focused on tumour necrosis factor-α (TNF-α), an acute-phase cytokine associated with systemic inflammation, with researchers confirming the ability of TNF-α to increase brain microvascular endothelial cell permeability *in vitro* (Lopez-Ramirez et al., 2012). Interestingly, other proinflammatory cytokines relevant to BBB dysregulation such as interleukin-6 (IL-6) for example, have received considerably less attention within the literature on this topic, an issue that we address in this short paper.

A critical determinant of interendothelial junction integrity within the BBB is ZO-1. This cytosolic scaffolding protein is associated with both adherens and tight junction complexes, where it serves to intracellularly tether transmembrane junction proteins to the actin cytoskeleton (Fanning and Anderson, 2009). Using the transient middle cerebral artery occlusion (tMCAO) model, researchers have recently demonstrated *in vivo* how ischemic stroke conditions disrupt ZO-1 expression and localization at the cell-cell border in cerebral microvessels, correlating with barrier failure and extravasation (Huang et al., 2013; Zehendner et al., 2013), an inflammatory process in which multiple cytokines are implicated. Consistent with this notion, the ability of TNF-α to disrupt ZO-1 localization to the cell-cell border in microvascular endothelial cells *in vitro* for example, has previously been reported (Lutgendorf et al., 2014), whilst other recent microvascular endothelial studies have demonstrated how ZO-1 dysregulation may accompany injury-specific induction of IL-6 (Chaudhuri et al., 2008; Labus et al., 2014). Notwithstanding these observations however, a fuller understanding of the relationship between proinflammatory cytokines and ZO-1 within the BBB microvascular endothelium would complement the existing knowledge base.

To address this, the present short paper employs HBMvECs to investigate the effects of both TNF-α and IL-6 on ZO-1 gene expression, tight junction localization, and phosphorylation state, in parallel with effects on HBMvEC permeability. Moreover, as cytokine-driven neuroinflammatory disorders frequently manifest elevated levels of reactive oxygen species (ROS) (Frey et al., 2009), the relative contribution of NADPH oxidase to cytokine-mediated effects on ZO-1 is also examined.

* Corresponding author. Fax: +353 11 3531 7005412.
E-mail address: phil.cummins@dcu.ie (P.M. Cummins).

http://dx.doi.org/10.1016/j.mvr.2015.04.010
0026-2862/

Materials and methods

Materials

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Dublin, IRL). Apocynin, NSC23766, TNF-α and IL-6 were from Millipore (Cork, IRL). Primary antisera were from: Anti-ZO-1 IgG, anti-occludin IgG (Bio-Sciences, Dublin, IRL); Anti-GAPDH IgG (Santa Cruz Biotechnology, CA, USA); Anti-pTyr IgG, anti-pThr IgG (Life Technologies, UK). HRP-conjugated secondary antisera were from both Cell Signaling Technologies Inc. (MA, USA) and Sigma-Aldrich. siRNA constructs for gp91 (SC35503, RefSeq NM_000397.3) and p47 (SC29422, RefSeq NM_000265.5) were from Santa Cruz Biotechnology.

Cell culture

Primary-derived human brain microvascular endothelial cells (HBMvECs) were obtained from Cell Systems Corporation (WA, USA – Certified ACBRI 376) and routinely grown in EndoGRO MV Basal Medium (Millipore) as previously described (Rochfort et al., 2014).

For experimental purposes, confluent cells were treated with either TNF-α or IL-6 at a concentration of 0–100 ng/ml (18 h), a concentration range routinely employed in other studies. Treatment of HBMvECs with up to 100 ng/ml of either cytokine for 18 h had no injurious effects on cell viability or apoptosis. Post-treatment, cells were harvested for analysis. Cell lysate preparation, protein assay, and lysate storage have been described previously (Rochfort et al., 2014).

Pharmacological agents were employed to dissect the role of NADPH oxidase and ROS in mediating cytokine-dependent effects on ZO-1. These included: 200 units/ml superoxide dismutase (SOD); 200 units/ml catalase (CAT); 1 mM *N*-acetylcysteine (NAC); 10 μM apocynin (APO); and 50 μM NSC23766. Cells were typically pretreated with these enzymes and compounds for 1 h in advance of cytokine treatment (to facilitate uptake – *e.g.* Waelti and Barton, 2006), and subsequently for the duration of cytokine treatment. These compounds have been widely used for probing oxidant signalling pathways in endothelial cells and were pre-screened in our laboratory at the aforementioned concentrations to rule out any negative effects on HBMvEC viability.

Western immunoblotting

Cell lysates were resolved by 10% SDS-PAGE under reducing conditions, and electroblotted as previously described (Rochfort et al., 2014; Guinan et al., 2013). Primary antisera: 0.5 μg/ml anti-ZO-1 mouse monoclonal IgG, 0.5 μg/ml anti-occludin mouse monoclonal IgG, and 0.2 μg/ml anti-GAPDH rabbit monoclonal IgG. Secondary antisera: 1:2000 HRP-conjugated goat anti-mouse IgG (ZO-1, occludin) and 1:3000 HRP-conjugated goat anti-rabbit IgG (GAPDH). Membranes were developed using a Luminata Western HRP kit (Millipore). Scanning densitometry of blots was performed using NIH ImageJ software, with glyceraldehyde phosphate dehydrogenase (GAPDH) routinely employed as a loading control.

Immunocytochemistry

HBMvECs were seeded at a density of 1×10^6 cells/ml onto attachment factor-coated glass coverslips and grown to confluency. Post-cytokine treatment, coverslips were slide-mounted and cells washed with pre-warmed PBS before 10 min fixation *in situ* with ice-cold 3.7% paraformaldehyde. The cells were then washed twice with PBS before adding 50 mM ammonium chloride for 10 min. Following two more PBS washes, the fixed cells were treated with a permeabilizing/blocking solution (0.1% saponin, 0.25% fish gelatine, and 0.02% sodium azide in PBS) for 30 min prior to overnight incubation with primary antisera (diluted 1:50 in permablock solution) at 4 °C. The following day, cells were washed twice with permablock solution prior to incubation with secondary antisera (diluted 1:500 in permablock) for 1 h in the dark. Cells were also routinely counterstained for actin and nuclei using 1:50 Alexa Fluor 488/546 Phalloidin (Life Technologies) and 1:2000 DAPI, respectively, prior to a final wash with permablock and addition of DAKO fluorescent mounting media (DAKO, CA, USA). Slides were stored at 4 °C until visualized by confocal microscopy.

Immunoprecipitation (IP)

Column IP was employed in conjunction with Western immunoblotting to monitor changes in ZO-1 phosphorylation and ZO-1:occludin co-association. All IPs were performed using a Co-IP Kit (Pierce, Cheshire, UK) and all relevant beaded agarose columns were prepared in accordance with manufacturer instructions. Briefly, post-treatment HBMvECs were harvested, lysed, and quantified for protein. All sample lysates were routinely volume-adjusted to give a total protein concentration of 200 μg/300 μl using IP Lysis/Wash Buffer. Lysates were then transferred to individual pre-equilibrated IP columns (containing either ZO-1 or occludin antisera linked to agarose beads), which were subsequently sealed and rotated for 4 h at 4 °C. Following incubation, columns were placed in fresh collection tubes and centrifuged at 1000 ×*g* for 1 min. Columns were then washed thrice with 200 μl of IP Lysis/Wash Buffer with each wash collected by centrifugation (1000 ×*g* for 1 min). Columns were then transferred to fresh collection tubes and 60 μl of Elution Buffer was added for 5 min and centrifuged accordingly. Collected IP eluents were then stored at −80 °C for subsequent Western analysis.

For monitoring pTyr/Thr-ZO-1 levels, ZO-1 IP samples were immunoblotted (equal protein loading) and probed with 1 μg/ml of mouse anti-pTyr or anti-pThr monoclonal IgG, respectively. Membranes were then stripped using Restore Western Blot Stripping Buffer (Thermo Scientific, UK) and re-probed with anti-ZO-1 mouse monoclonal IgG as described above. For monitoring ZO-1:occludin co-association, occludin IP samples were immunoblotted and probed with anti-ZO-1 mouse monoclonal IgG. Membranes were then stripped using Restore Western Blot Stripping Buffer (Thermo Scientific, UK) and re-probed with anti-occludin mouse monoclonal IgG.

Quantitative RealTime PCR

Post-treatment, HBMvECs were harvested for total RNA extraction and analysis of ZO-1 mRNA expression as previously described (Guinan et al., 2013) with minor modifications. PCR reaction conditions were: denaturation at 95 °C for 10 min followed by 40 cycles of (i) denaturation at 95 °C for 15 s, (ii) annealing at 59 °C for 60 s, and (iii) elongation at 72 °C for 15 s. Each cDNA sample was assayed ×3 and results analysed by the comparative C_T method. GADPH was used for normalization. ZO-1 (218 bp): Forward 5′-gaacgaggcatcatccctaa-3′; Reverse 5′-ccagcttctcgaagaaccac-3′; GAPDH (238 bp): Forward 5′-gagtcaacggatttggtcgt-3′; Reverse 5′-gagtcaacggatttggtcgt-3′.

Transfection

Transfection of siRNA, as well as validation of siRNA efficacy towards the intended targets, has been described previously by our group (Rochfort et al., 2014). For siRNA transfections (gp91, p47, and scrambled siRNA constructs at a final concentration of 50 nM), the Microporator Mini MP-100 system (Life Technologies, Paisley, UK) was used.

Transendothelial permeability assay

Analysis of HBMvEC permeability following cytokine treatments employed transwell diffusion of fluorescein isothiocyanate (FITC)-labelled 40 kDa dextran, and has been previously described (Rochfort et al., 2014).