TNF-α evokes blood-brain barrier dysfunction through activation of Rho-kinase and neurokinin 1 receptor

Xin Gao[1], Ulvi Bayraktutan[1*]

[1]Academic Unit of Mental Health and Clinical Neurosciences, School of Medicine, University of Nottingham, Nottingham, UK

***Corresponding author**

Associate Professor Ulvi Bayraktutan

Academic Unit of Mental Health and Clinical Neurosciences

Queens Medical Centre

School of Medicine

University of Nottingham

Derby Road

Nottingham

NG7 2UH

E-mail: ulvi.bayraktutan@nottingham.ac.uk

Tel: +44 115 8231764

Fax: +44 115 8231767

Abstract

Ischaemic stroke, accompanied by neuroinflammation, impairs blood–brain barrier (BBB) integrity through a complex mechanism involving activation of both RhoA/Rho kinase/myosin light chain-2 and neurokinin 1 receptor (NK1R). Using an in vitro model of human BBB composed of brain microvascular endothelial cells (BMEC), astrocytes and pericytes, this study examined the potential contributions of these elements to BBB damage induced by elevated availability of pro-inflammatory cytokine, TNF-α. Treatment of human BMECs with TNF-α significantly enhanced RhoA activity and the protein expressions of Rho kinase and phosphorylated Ser19MLC-2 while decreasing that of NK1R. Pharmacological inhibition of Rho kinase by Y-27632 and NK1R by CP96345 neutralised the disruptive effects of TNF-α on BBB integrity and function as ascertained by reversal of decreases in transendothelial electrical resistance and increases in paracellular flux of low molecular weight permeability marker, sodium fluorescein, respectively. Suppression of RhoA activation, mitigation of actin stress fibre formation and restoration of plasma membrane localisation of tight junction protein zonula occludens-1 appeared to contribute to the barrier-protective effects of both Y-27632 and CP96345. Attenuation of TNF-α-mediated increases in NK1R protein expression in BMEC by Y-27632 suggests that RhoA/Rho kinase pathway acts upstream to NK1R. In conclusion, specific inhibition of Rho kinase in cerebrovascular conditions, accompanied by excessive release of pro-inflammatory cytokine TNF-α, helps preserve endothelial cell morphology and inter-endothelial cell barrier formation and may serve as an important therapeutic target.

Keywords: TNF-α, brain microvascular endothelial cells, neurokinin 1 receptor, RhoA, Rho kinase, blood-brain barrier, cytoskeleton

Introduction

Neuroinflammation, associated with exaggerated release of pro-inflammatory cytokines, is considered as a key pathology in breakdown of the blood-brain barrier (BBB) in various neurological conditions, including multiple sclerosis and ischaemic stroke. The BBB regulates the selective passage of molecules between the brain and systemic circulation and is formed by a monolayer of brain microvascular endothelial cells (BMECs) surrounded by a continuous astrocyte end-feet, basement membrane and pericytes (Daneman et al., 2015). Inter-endothelial cell tight junctions (TJs) contribute to the tightness of BBB and are established by intimate relationships between actin cytoskeleton and the key transmembrane proteins, zonula occludens-1, claudins and occludin (Dejana, 2004). Hence, any physical, chemical or humoral stimuli capable of altering the availability and localisation of these proteins may also alter the integrity and function of the BBB. TNF-α, a prominent pro-inflammatory cytokine, produced by infiltrated leukocytes and resident microglia during acute neuroinflammation represents one such stimuli (Movat et al., 1987, Beutler et al., 1986, Abdullah et al., 2014). Recent evidence shows that induction of oxidative stress, matrix metalloproteinase-2/9 activity, apoptosis and endothelial cell and astrocyte cytoskeletal reorganisation are involved in TNF-α-mediated BBB disruption (Abdullah et al., 2016, Abdullah et al., 2015).

Dysregulation of small GTP-binding protein RhoA also plays a critical role in neurodegenerative diseases associated with inflammation and BBB disruption (Kimura et al., 2021, Arrazola Sastre et al., 2020). RhoA and its downstream effector Rho-kinase regulate a range of diverse cellular processes, including actin cytoskeleton, cell adhesion and migration. The binding of activated RhoA to Rho-kinase destabilises tight junctions through phosphorylation of myosin light chain (MLC) and decreases the bioavailability of nitric oxide (NO), the most potent endogenous antioxidant and anti-inflammatory agent. Because increased expression and activity of endothelial Rho-kinase have been shown to account for BBB

hyperpermeability in experimental settings of ischaemic injury or hyperglycaemia, it is possible that inhibition of Rho/Rho-kinase pathway may also attenuate or negate the BBB-disruptive effects of TNF-α (Srivastava et al., 2013). Previous reports demonstrating a strong correlation between RhoA or Rho-kinase activation and EC barrier dysfunction support the validity of this hypothesis (Peng et al., 2011, Clark et al., 2015).

Most cytokines, including TNF-α induce the expression of substance P (SP) (Annunziata et al., 2002), a neurotransmitter and a neuromodulator, and its receptor neurokinin 1 (NK1R) (Akasaka et al., 2005). SP and NK1R, in turn, stimulate the expression and activity of almost all cytokines (Ansel et al., 1993, Sipka et al., 2010) and are intimately involved in cell proliferation (Kim et al., 2015), angiogenesis (Um et al., 2016) and inflammation (Eapen et al., 2019, Garcia-Recio et al., 2015). Investigation of the correlations between TNF-α and SP or NK1R at cellular level has revealed that treatment with an SP antagonist markedly attenuates TNF-α-stimulated rat brain endothelium activation and mitigates permeability through in part regulation of endothelial nitric oxide synthase expression (Annunziata et al., 2002). In addition to their well-documented antiemetic, antidepressant and anxiolytic effects, NK1R antagonists have also been shown to suppress TNF-α-mediated tissue damage in various animal models (Bang et al., 2004, Liu et al., 2019a, Un et al., 2020). Taken together, these findings suggest that inhibition of NK1R may be somewhat protective against TNF-α-mediated BBB damage. The putative pathways involved in TNF-α-triggered BBB breakdown is summarised in Figure 1.

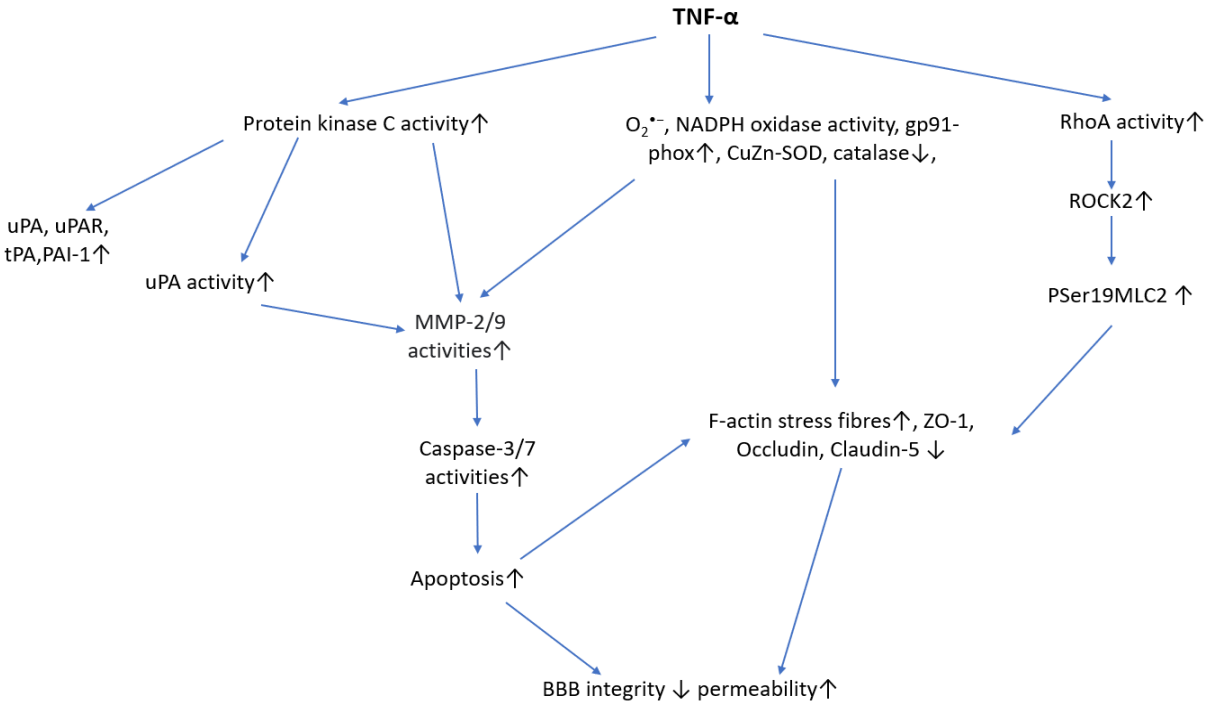


Fig. 1. Potential mechanisms involved in the deleterious effect of TNF-α on BBB integrity and permeability. uPA: urokinase plasminogen activator, uPAR: urokinase plasminogen activator receptor, tPA: tissue-type plasminogen activator, PAI-1: plasminogen activator inhibitor-1, MMP: matrix metalloproteinase, $O_2^{\bullet-}$: superoxide anion, NAPDH: nicotinamide adenine dinucleotide phosphate, CuZn-SOD: Copper Zinc Superoxide Dismutase, gp91-phox: an active subunit of NADPH oxidase, ZO-1: Zonula occludens-1.

Materials and Methods

Cell culture

Primary human brain microvascular endothelial cells (HBMEC), human astrocytes (HA) and human pericytes (HP) were purchased from Neuromics, USA and cultured in respective specific media supplemented with the necessary growth factors and 1% penicillin/streptomycin solution (Sciencell, USA) at 37ºC in 5% CO_2/95% air and constant humidity.

An *in vitro* model of human BBB consisting of HBMEC, HA and HP were established as before (Kadir et al., 2022, Srivastava et al., 2013). Briefly, HA was seeded on the basolateral side of

12-well Transwell inserts (12mm diameter, 0.4 µm pore size, Corning) for 4 h to allow them to attach the membrane. Once HA was ~80% confluent, the HBMEC were seeded on apical side of the inserts and both layers were cultured to full confluence. The inserts were then transferred to fresh 12-well plates containing confluent pericytes to form a triple-cell culture model of human BBB.

In current study, cells were exposed to TNF-α (10 ng/ml, R&D Systems, USA) for 6 h in the absence or presence of inhibitors for NK1R (CP96345, 10 µM, R&D Systems, USA) and Rho-kinase (Y-27632, 2.5 µM, Abcam, UK).

Assessment of BBB integrity and function

The integrity and function of the BBB were studied by measurements of Transendothelial electrical resistance (TEER) and flux of a low molecular weight permeability marker i.e. sodium fluorescein (NaF), respectively. To detect differences in TEER, an EVOM resistance meter (World Precision Instruments, Hertfordshire, UK) was employed as before (Kadir et al., 2022). To measure paracellular flux, NaF was added to apical chamber and incubated at 37^{o}C for 1 h prior to collection of samples from both upper and lower chambers and fluorometric assessment (excitation/emission: 440/525 nm). NaF flux was indicated by following formula, NaF cleared volume (µL) = abluminal reading x 500/luminal reading.

Western blotting

Cells subjected to experimental conditions were trypsinized and washed with phosphate-buffered saline (PBS) before lysis in radioimmunoprecipitation assay buffer containing phenylmethylsulfonyl fluoride (Cell Signalling Technology, USA), PhosSTOP, complete EDTA-free protease inhibitor (Roche Diagnostics GmbH, Germany). The quantity of proteins in samples was determined by bicinchoninic acid assay (Thermo Scientific, USA) using a Microplate reader 680 (Bio-Rad, USA).

Approximately 80 µg of total protein samples were run on 6-12% sodium dodecyl sulfate-polyacrylamide gel. Following transfer of proteins, the membranes were blocked and incubated overnight at 4°C with primary antibodies targeting GAPDH (Sigma Aldrich, UK, HPA040067 and G8795), RhoA (Santa Cruz, USA, sc-418), Rho-kinase 2 (ROCK2, Santa Cruz, USA, sc-398519), MLC2 (Merck Millipore, USA, MABT180), phosphorylated threonine18/serine19 MLC2 (p-Thr18Ser19MLC2, Cell Signalling Technology, USA, #3674), phosphorylated serine19 MLC2 (p-Ser19MLC2, Cell Signalling Technology, USA, #3671), ZO-1 (Invitrogen, USA, #33-9100), occludin (Invitrogen, USA, #71-1500), and claudin-5 (Invitrogen, USA, #35-2500), NK1R (Abcam, UK, ab183713). The membranes were then incubated with secondary antibodies (anti-mouse IgG, 926-68072 and anti-rabbit IgG, 926-32213, Licor Biosciences, UK) for 1 h at room temperature and scanned using Li-Cor Odyssey infrared imaging system (Li-Cor Biosciences, UK). Densitometric analysis of protein bands was performed using the Image Studio Lite software (Li-Cor Biosciences, UK).

Immunofluorescence

HBMECs, grown on coverslips and subjected to experimental conditions, were fixed and permeabilised in 4% paraformaldehyde in PBS (Thermo Fisher, USA) and 0.1% Triton X-100, respectively. To detect actin microfilaments, the cells were incubated with phalloidin-iFluor 647 Reagent (Abcam, UK, ab176759). To visualise tight junction proteins, ZO-1 (Invitrogen, USA, #33-9100), occludin (Invitrogen, USA, #71-1500) and claudin-5 (Invitrogen, USA, #35-2500), cells were stained with relevant primary and secondary antibodies (anti-mouse IgG, ab150113 and anti-rabbit IgG, ab150077, Abcam, UK). Coverslips were then counterstained by Vectashield anti-fade mounting media containing 4,6-diamidino-2-phenylindole for nuclei stain (DAPI, Vector Laboratories, USA). The intensities of protein-specific staining of F-actin in cytoplasm and tight junction proteins in whole cells were quantified using ImageJ software.

Rho activity assay

Rho activity was monitored using a commercial Rho-pull down kit (Merck Millipore, USA, Catalog # 17-294). Briefly, cell lysates (~150 μg) were incubated with Rhotekin Rho-binding peptide immobilised on agarose beads. Activated GTP-RhoA bound to Rhotekin was then detected by Western blotting using an anti-Rho (-A, -B, -C) antibody included in the kit (Srivastava et al., 2013). Total RhoA was detected by RhoA antibody (Proteintech, Cat no: 10749-1-AP).

Statistical analysis

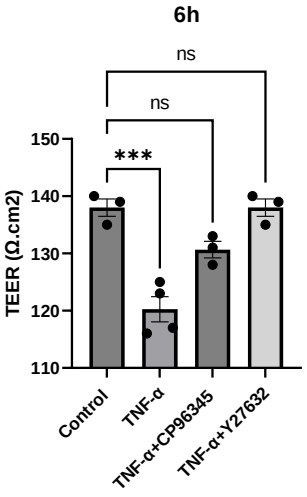
Statistical analyses were conducted using GraphPad Prism 9.4.1 software package. Comparison of the mean values was performed by one-way analysis of variance, followed by Dunnett's *post hoc* testing. $P<0.05$ was considered significant. Data were presented as mean±s.e.m from at least three independent experiments.

Results

Effects of TNF-α on BBB integrity and function

Exposure of BBB to TNF-α for 6 h significantly compromised the integrity and function of BBB as evidenced by decreases in TEER and concurrent increases in NaF flux which were negated by inhibition of Rho-kinase and NK1R with Y-27632 and CP96345, respectively (Fig. 2A-B). The magnitude of improvements in BBB characteristics appeared to be greater with Y-27632 than CP96345.

A.



B.

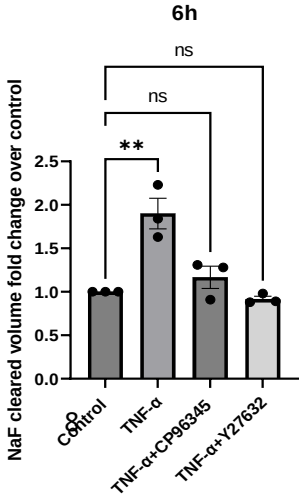
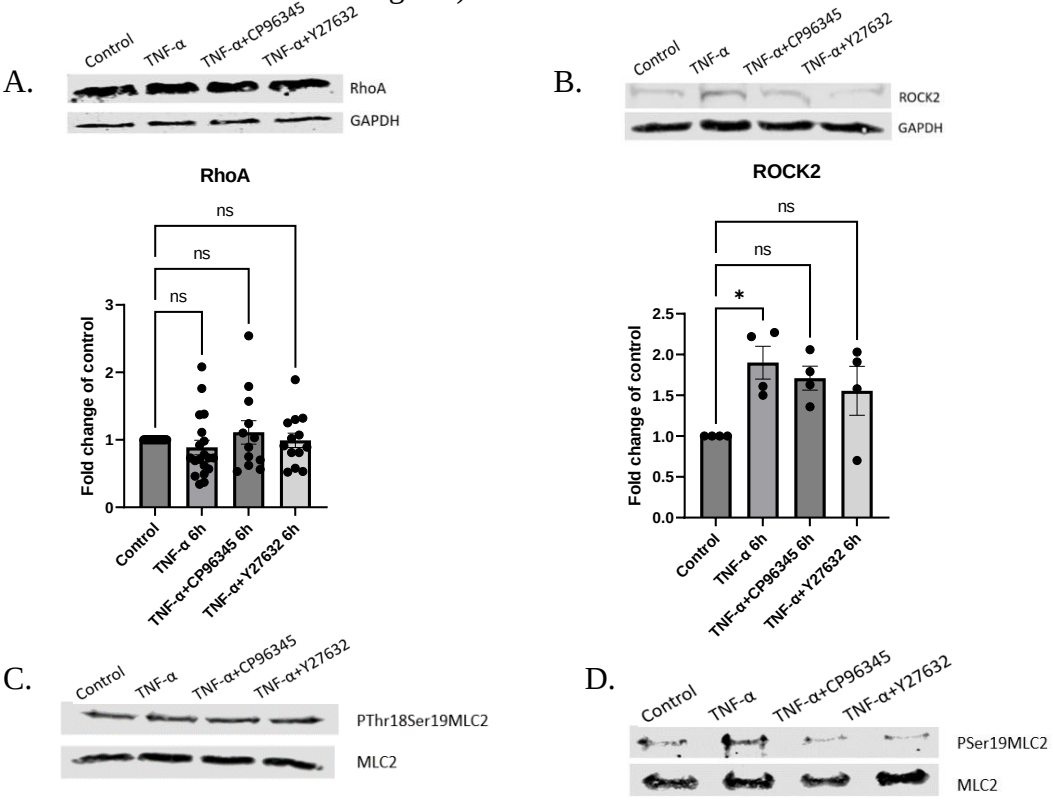


Figure 2. TNF-α impaired the characteristics of blood-brain barrier (BBB) *in vitro*. Treatments with TNF-α for 6 h significantly decreased TEER (A) and increased paracellular flux of NaF (B), hallmarks of integrity and function of BBB. Inhibition of Rho-kinase by Y-27632 and NK1R by CP96345 attenuated the impact of TNF-α on BBB characteristics. Data are expressed as mean±s.e.m. from at least three independent experiments. **p<0.01, ***p<0.001.

Effects of TNF-α on protein expression of RhoA/ROCK/MLC2 pathway components and NK1R

Treatment of HBMECs with TNF-α for 6 h led to significant increases in ROCK2 and p-Ser19MLC2 but not RhoA and p-Thr18Ser19MLC2 protein expressions. Co-treatment of cells with Y-27632 and CP96345 markedly attenuated these increases (Fig. 3A-D). TNF-α, on the other hand, decreased the expression of NK1R protein which was normalised by co-treatments with CP96345 or Y-27632 (Fig. 3E).



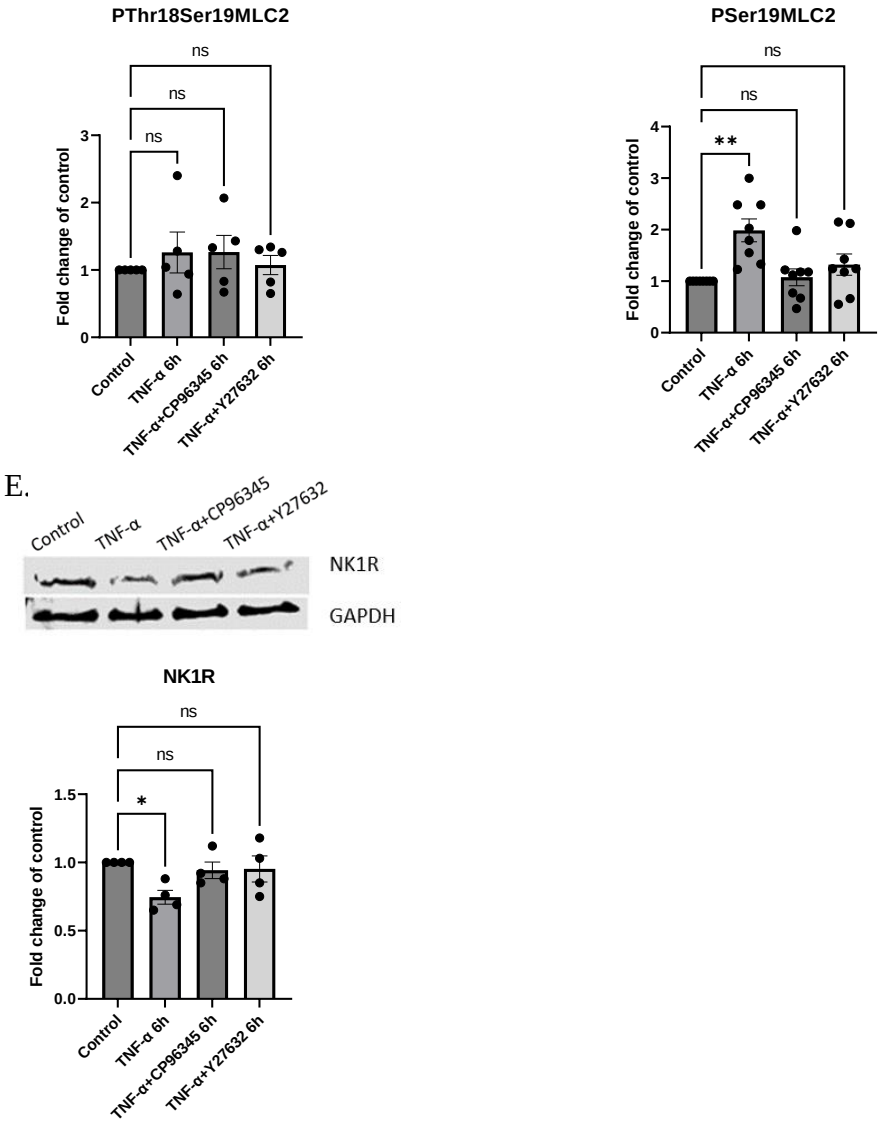
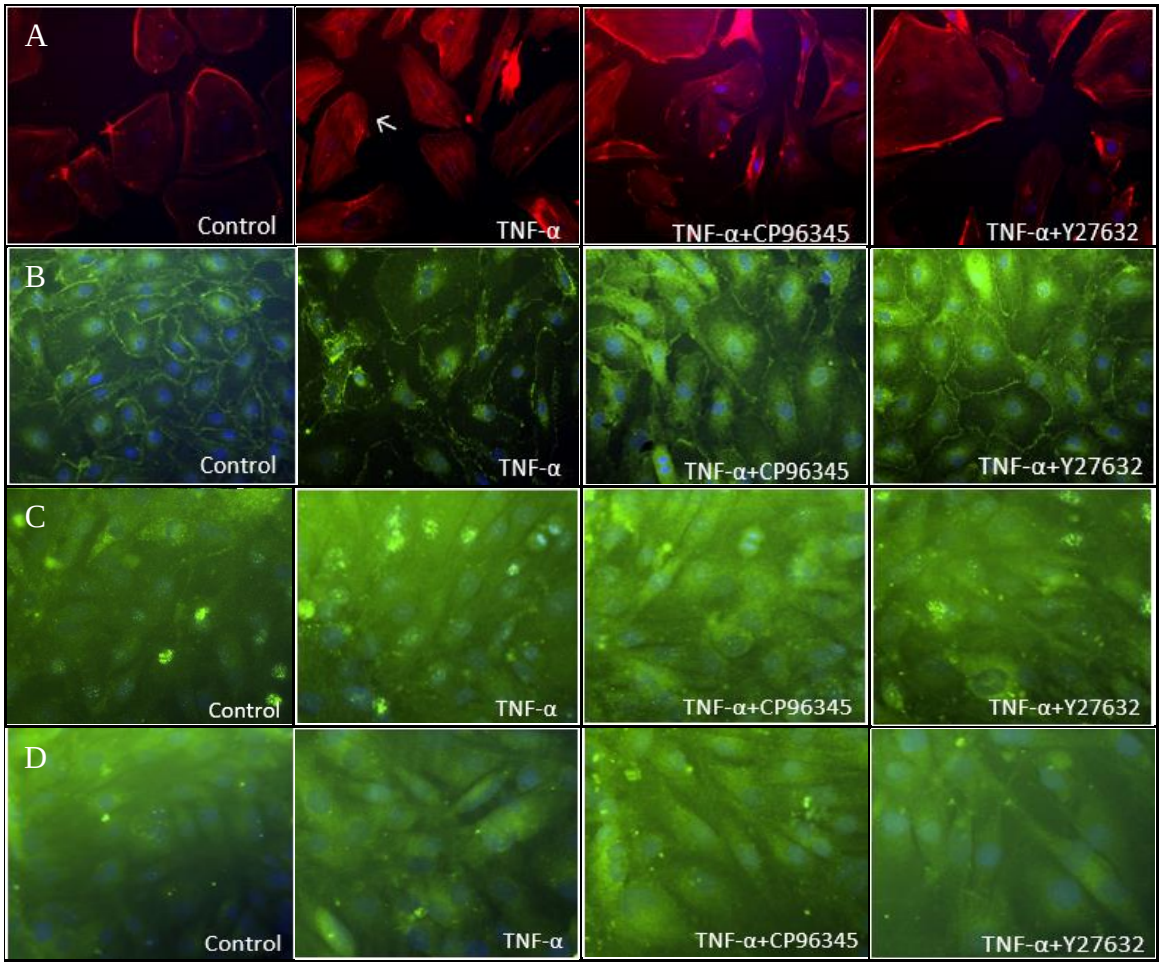


Figure 3. TNF-α distinctly regulated the protein expression of RhoA/ROCK2/MLC2 pathway components and NK1R in HBMEC. Treatment of human brain microvascular endothelial cells with TNF-α for 6 h did not affect the total protein expression of RhoA (A) while increasing those of ROCK2 (B) and pSer19MLC2 (C). TNF-α also failed to influence the protein expression of pThr18Ser19MLC2 (D) while selectively reduced that of NK1R (E). Co-treatment of cells with Rho-kinase inhibitor (Y-27632) and NK1R antagonist (CP96345) negated the effects of TNF-α on both ROCK2, p-Ser19MLC2 and NK1R protein expressions without affecting those of the others. Data are expressed as mean±s.e.m. from at least three different experiments. *$p<0.05$, **$p<0.01$

Effects of TNF-α on endothelial cell cytoskeleton and tight junction protein localisation

Incubation of HBMEC with TNF-α for 6 h substantially enhanced stress fibre formation and led to the translocation of normally plasma membrane-bound actin filaments to cytoplasm

which were prevented by the co-treatment of cells with CP96345 or Y-27632 (Fig. 4A and E). Amongst the three tight junction proteins targeted in this study, only ZO-1 appeared to localise to the plasma membrane in control HBMECs. While treatments with TNF-α disrupted the continuous staining of ZO-1 on HBMEC plasma membrane, co-treatments with CP96345 or Y-27632 effectively attenuated the impact of TNF-α on ZO-1 localisation (Fig.4B). Unlike ZO-1, both occludin and claudin-5 appeared to localise to the cytoplasm in control HBMECs and those treated with TNF-α in the absence or presence of CP96345 or Y-27632 (Fig. 4C-D). No significant differences were observed in the overall staining of any of the aforementioned tight junction proteins in HBMECs subjected to TNF-α alone or with CP96345 or Y-27632 compared to the control cells (Fig.4E).



E

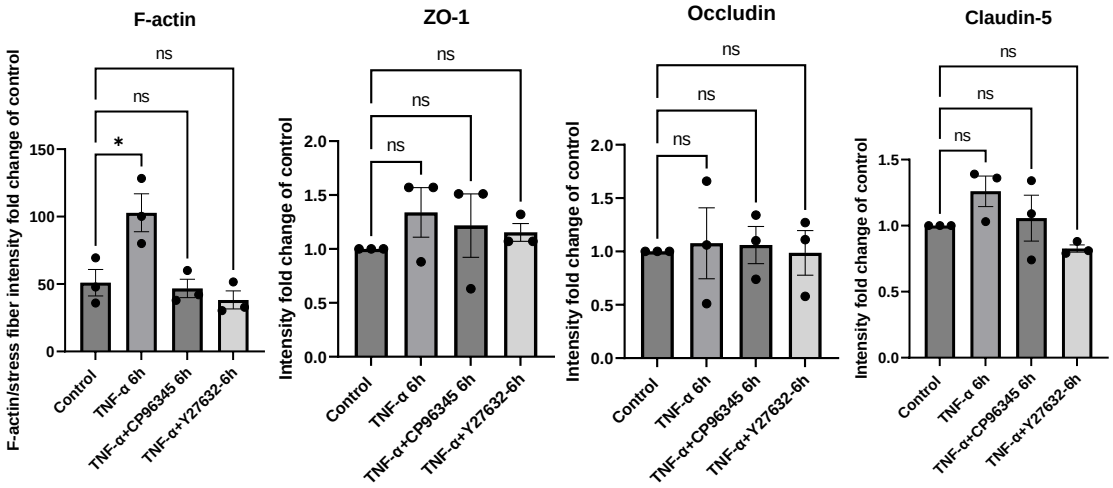
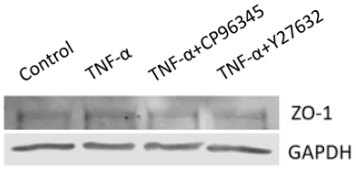


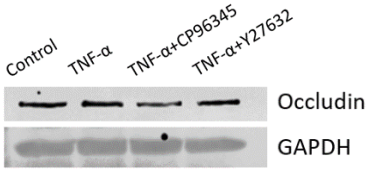
Figure 4. TNF-α influenced the subcellular localisation of actin microfilaments and tight junction proteins in human brain microvascular endothelial cells (HBMECs). In physiological settings, HBMECs manifested a prominent cortical actin staining. In contrast, HBMECs treated with TNF-α displayed significant stress fibre formation. Co-treatment of these cells with an inhibitor of Rho-kinase, Y-27632 or an antagonist of NK1R, CP96345 effectively prevented stress fibre formation (A). TNF-α compromised the plasma membrane localisation of zonula occludens-1 (ZO-1) which was prevented by co-treatments with Y-27632 and CP96345 (B). Cytosolic localisation of occludin and claudin-5 were unaffected by treatments with TNF-α alone or together with Y-27632 or CP96345 (C, D). While suppressing the intensity of stress fibre formation in HBMECs, treatments with Y-27632 or CP96345 had no impact on cellular localisation or abundance of tight junction proteins (E). Data are expressed as mean±s.e.m. from three different experiments. *$p<0.05$.

Furthermore, exposure of HBMECs to TNF-α had no effect on the level of ZO-1, occludin or claudin-5 total protein expressions in the absence or presence of Y-27632 and CP96345 (Fig. 5A-C).

A.



B.



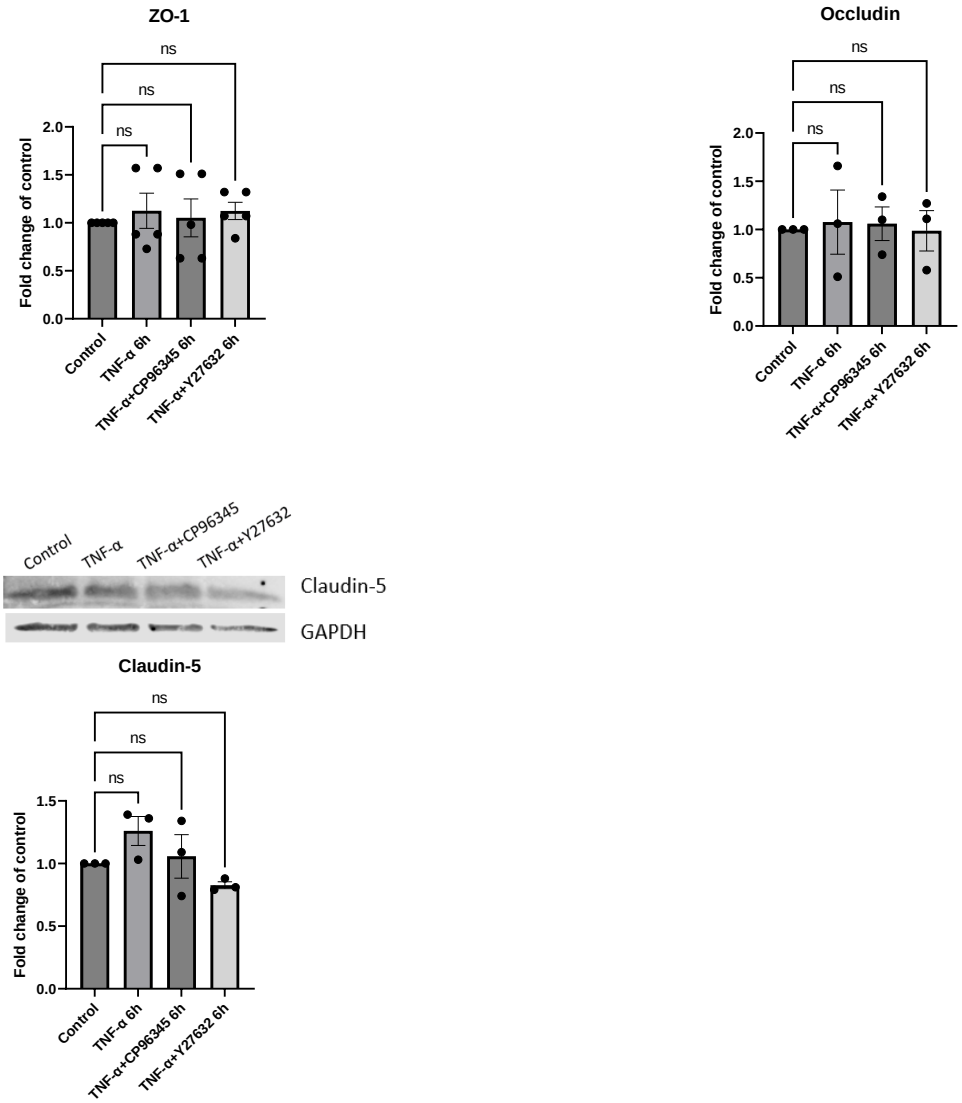
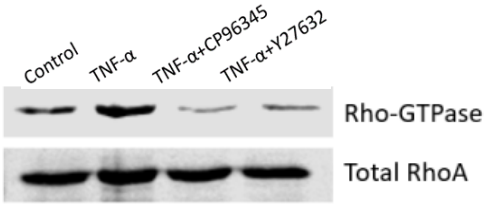


Figure 5. Western blots and corresponding graphs show similar level of tight junction protein zonula occludens-1 (A), occludin (B) and claudin-5 (C) expression in human brain microvascular endothelial cells (HBMECs) subjected to TNF-α alone or with a Rho-kinase (Y-27632) or neurokinin 1 receptor (CP96345) inhibitor for 6 h. Data are expressed as mean±s.e.m. from at least three different experiments.

Impact of TNF-α on RhoA activation in HBMEC

Treatments with TNF-α significantly increased RhoA activity in HBMECs, as evidenced by marked increases in Rho-GTPase protein levels. Suppression of NK1R and Rho-kinase via CP96345 and Y-27632, respectively neutralised the effect of TNF-α on RhoA.activity (Fig. 6).



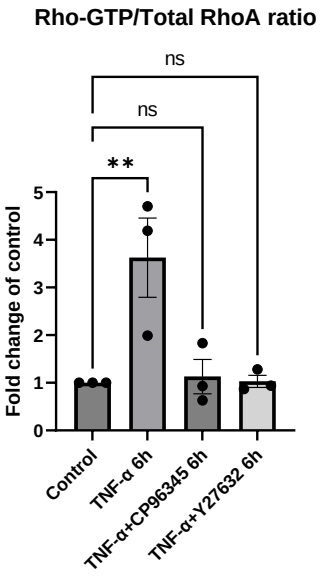


Figure 6. Effect of TNF-α on RhoA activity in human brain microvascular endothelial cells (HBMECs). TNF-α provoked an increase in Rho-GTPase levels in HBMECs which were negated by inhibition of NK1R and Rho-kinase by CP96345 and Y-27632, respectively. Data are expressed as mean±s.e.m. from three different experiments. **p<0.01.

Discussion

Inflammatory reactions characterised by exaggerated release of pro-inflammatory cytokines, notably TNF-α may perturb BBB integrity and thus trigger the formation of brain oedema. Protection of the integrity of a co-culture model of human BBB in experimental settings of ischaemia-reperfusion injury by a specific antibody targeting TNF-α corroborates this notion (Abdullah et al., 2015). Exposure to higher levels of TNF-α affects the functionality of all the major cell lines that contribute to the formation of BBB, in particular BMECs and astrocytes (Abdullah et al., 2015). Although TNF-α causes significant increases in MMP-2 activity, actin stress fibre formation, caspase activity, NADPH oxidase activity and $O_2^{\cdot-}$ formation in both HBMEC and HA, the magnitude of these changes appears to be markedly greater in ECs. Similarly, despite attenuating these effects in both cell lines, the impact of quenching TNF-α activity is considerably greater in HBMECs (Abdullah et al., 2015), adding weight to the notion that endothelial cells constitute the most responsive cells in vasculature (Ulker et al., 2003,

Bayraktutan, 2002). By using a triple culture model of human BBB, the current study has confirmed that TNF-α substantially compromises the integrity and function of cerebral barrier, evidenced by decreases in TEER and concomitant increases in NaF flux, respectively. The model established by concurrent culture of HBMEC, astrocytes and pericytes represents an anatomically and physiologically more relevant *in vitro* model of human BBB and has been proven to generate consistently higher levels of electrical resistance compared to HBMEC monolayers and co-culture models (Allen et al., 2009, Shao et al., 2013). Taken together, these findings imply that factors released by astrocytes and/or pericytes play a crucial role in strengthening the unity and tightness of the triple culture models (DeStefano et al., 2018, Banks et al., 2018) . Significant tightening of endothelial cell monolayer barrier integrity through incorporation of pericytes and astrocytes to the system somewhat validate this hypothesis (Stebbins et al., 2019).

In addition to the mechanisms mentioned above, the current study has also shown that RhoA/Rho-kinase/MLC2 pathway and NK1R may also play pivotal roles in TNF-α-mediated BBB breakdown.

Considering the involvement of these pathways in immune responses and major cellular mechanisms e.g. migration, proliferation and survival and the causative role of inflammatory cytokines in various neurological conditions, elucidation of the nature of correlations between TNF-α and these pathways may have greater implications than presently anticipated (Jagannathan et al., 2009, Pan et al., 2006, Zaremba et al., 2001).

Despite increasing RhoA activity/Rho-GTPase, exposure to TNF-α did not lead to an increase in total RhoA protein expression in HBMEC. Rather, it specifically increased ROCK2 protein expression and Ser19MLC2 phosphorylation. Suppression of RhoA activity, but not total RhoA levels, by the Rho-kinase inhibitor, Y-27632 implies the existence of a feedback

mechanism between RhoA activity and Rho-kinase bioavailability in inflammatory settings. Previous studies documenting a similar relationship between RhoA activity, its protein expression and TNF-α corroborate these observations (McKenzie et al., 2007, Srivastava et al., 2013, Allen et al., 2010). Previous studies also indicate that Rho-kinase may be activated through RhoA-independent mechanisms and Rho-kinase plays an instrumental role in the regulation of various TNF-α-mediated biological effects other than those observed on BBB integrity and function in the current study (McKenzie et al., 2007, Nwariaku et al., 2003). However, the absence of a similar feedback mechanism between RhoA and Rho kinase activity in hyperglycaemic settings signifies the importance of predominant pathological stimulus in dictating the type and extent of correlations amongst RhoA/Rho-kinase/MLC2 pathway components (Srivastava et al., 2013).

In light of the previous evidence, it is reasonable to suggest that TNF-α-evoked selective phosphorylation of Ser19MLC2 may be sufficient to increase BBB permeability (Kazakova et al., 2020, Hirano et al., 2016). The phosphorylation of MLC2 is realised by either primarily calcium-dependent activation of myosin light chain kinase (MLCK) or by Rho-kinase-mediated inhibition of myosin light chain phosphatase (MLCP) which normally triggers cytoskeletal relaxation through dephosphorylation of MLC (Shen et al., 2010, Fukata et al., 2001, Rigor et al., 2013). Similar to MLCK, Rho-kinase can also directly phosphorylate MLC2, primarily at Ser19 which precedes phosphorylation of Thr18 (Amano et al., 1996, Ikebe et al., 1985, Ikebe et al., 1986). As Thr18 phosphorylation requires considerably higher concentrations of MLCK and Rho-kinase plays an essential role in MLCK-induced diphosphorylation of MLC, it is reasonable to suggest that treatment with TNF-α does not elevate the activity of MLCK and/or Rho-kinase in HBMECs to the levels required for Thr18Ser19MLC2 diphosphorylation (Kazakova et al., 2020). Taken together, these findings also suggest that changes observed in actin cytoskeleton, characterised by formation of stress

fibres, are largely dictated by p-Ser19MLC2 in pathological settings associated with exaggerated bioavailability of TNF-α and possibly other pro-inflammatory cytokines. Intriguingly, the molecular weight of the phosphorylated proteins in the present study appeared to be higher than expected which might in part derive from the posttranslational modification of these proteins (Larsen et al., 2006). It is of note that similar size proteins with the same antibodies had also been reported in a previous study (Hirano et al., 2016).

This and the concomitant disappearance of ZO-1 on HBMEC plasma membrane may account for the TNF-α-induced inter-endothelial cell openings and ensuing BBB failure which appeared to be fully mitigated by Y-27632 and CP96345. Similar cytosolic staining patterns observed for occludin and claudin-5 in HBMECs treated with TNF-α (Chen et al., 2020) in the absence or presence of these inhibitors substantiate the crucial involvement of stress fibres and partial loss of ZO-1 in BBB openings. Contrary to our previous studies, no differences in the expression of tight junction proteins were detected in the present study (Abdullah et al., 2014, Abdullah et al., 2016). Although employment of a different batch of HBMECs may somewhat explain this dichotomy, differences in TNF-α concentration and incubation time may also contribute (Rochfort et al., 2016, Chen et al., 2020). However, given the prominent disruption of BBB with TNF-α in the current study, it is likely that the concentration and incubation time employed in the study were sufficient to evoke dramatic increases in oxidative stress, apoptosis, matrix metalloproteinase-2/9, caspase and NADPH oxidase activities as previously documented (Abdullah et al., 2016, Abdullah et al., 2015). Similar to immunoblotting results, comparative analyses of ZO-1 immunostainings in control versus all TNF-α-treated experimental groups also yielded no significant difference in its cellular intensity. Despite reducing TEER and elevating the focal cell junctional disruptions, exposure to TNF-α also failed to alter the overall ZO-1 protein expression and submembrane staining in glomerular endothelial cells (Xu et al., 2015).

Here, prevention of BBB damage with Y-27632 and CP96345 propose Rho-kinase and NK1R as potentially efficacious novel therapeutic targets for mitigation of BBB damage in inflammatory settings (Petrache et al., 2003, Srivastava et al., 2013, Gibson et al., 2014). Indeed, Y-27632 has previously been shown to attenuate TNF-α-mediated hyperpermeability of human lung microvascular endothelial cells (Nwariaku et al., 2003, Shi et al., 2007). It is noteworthy here that inter-endothelial cell openings accompanied by changes in ZO-1, but not occludin or claudin-5, distribution has also been reported in immortalized human brain endothelial cell line hCMEC/D3, rat BMECs and mouse BMECs, namely b.End3 cells exposed to various pro-inflammatory cytokines, notably TNF-α, interferon-γ or interleukin-1β (Lopez-Ramirez et al., 2012, Alluri et al., 2016, Yamamoto et al., 2008).

As alluded above, inhibition of NK1R via CP96345 also prevented the deleterious effects of TNF-α on BBB integrity and function, confirming the critical role of SP in cytokine-stimulated endothelial barrier damage (Annunziata et al., 2002). Since TNF-α failed to augment SP secretion, and *vice versa* in HBMECs (data not shown), the barrier-protective effects of CP96345 were unlikely to stem from the cytokine-mediated putative changes in SP levels. Considering the extremely short half-life of SP in laboratory BBB settings (Freed et al., 2001), it is probable that much of the SP released in response to TNF-α may actually degrade well before the use of a commercial kit to detect it. Hence, investigation of the effect of TNF-α on SP mRNA in this study would have been useful. Previous studies focusing on this issue reveals that TNF-α modulates SP mRNA level. For instance, treatments with TNF-α (50 U/ml) have been shown to increase SP mRNA expression in rat superior cervical ganglia without affecting its protein expression in pure rat superior cervical ganglia culture (Ding et al., 1995, Freidin et al., 1991). TNF-α has also been shown to elevate SP mRNA levels in a promonocytic cell line and a T-cell line compared to their respective untreated controls (Ho et al., 2002). Similarly,

TNF-α-evoked increases in rat brain endothelial cells SP immunoreactivity were accompanied by increases in its mRNA expression compared to untreated cells (Cioni et al., 1998).

The observation of a decreased NK1R protein expression in HBMECs exposed to TNF-α may in part derive from ubiquitination and consequent degradation of NK1R protein by this particular cytokine (Cottrell et al., 2006). Recent evidence reveals that TNF-α may bind to NK1R (Johnson et al., 1991) which suggests that NK1R can actually act as a defence mechanism against the deleterious effects of TNF-α. By preventing the binding of TNF-α to NK1R, receptor antagonists may play a barrier-protective role. Aside from its beneficial effects on BBB, NK1R antagonists also suppress inflammation arising from rheumatoid arthritis (Liu et al., 2019b), lipopolysaccharide (Fulenwider et al., 2018) and prurigo nodularis (Agelopoulos et al., 2019).

Conclusion

In conclusion, this study shows that TNF-α affects the integrity and function of an *in vitro* model of human BBB through activation of RhoA/Rho-kinase/MLC2 and SP/NK1R pathways. The inhibition of Rho-kinase and NK1R attenuates the impact of TNF-α on BBB and may therefore serve as potential therapeutic targets in conditions associated with excessive release of inflammatory cytokines, notably TNF-α.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of interest

Declarations of interest: none

References

ABDULLAH, Z. & BAYRAKTUTAN, U. 2014. NADPH oxidase mediates TNF-alpha-evoked in vitro brain barrier dysfunction: roles of apoptosis and time. *Mol Cell Neurosci,* 61**,** 72-84.

ABDULLAH, Z. & BAYRAKTUTAN, U. 2016. Suppression of PKC-alpha attenuates TNF-alpha-evoked cerebral barrier breakdown via regulations of MMP-2 and plasminogen-plasmin system. *Biochim Biophys Acta,* 1862**,** 1354-66.

ABDULLAH, Z., RAKKAR, K., BATH, P. M. & BAYRAKTUTAN, U. 2015. Inhibition of TNF-alpha protects in vitro brain barrier from ischaemic damage. *Mol Cell Neurosci,* 69**,** 65-79.

AGELOPOULOS, K., RÜLANDER, F., DANGELMAIER, J., LOTTS, T., OSADA, N., METZE, D., LUGER, T. A., LOSER, K. & STÄNDER, S. 2019. Neurokinin 1 receptor antagonists exhibit peripheral effects in prurigo nodularis including reduced ERK1/2 activation. *Journal of the European Academy of Dermatology and Venereology,* 33**,** 2371-2379.

AKASAKA, Y., ABE, K., SATO, T. & INOUE, H. 2005. Regulation of neurokinin-1 receptor messenger RNA expression in synovial fibroblasts of patients with rheumatoid arthritis. *Neuropeptides,* 39**,** 467-74.

ALLEN, C., SRIVASTAVA, K. & BAYRAKTUTAN, U. 2010. Small GTPase RhoA and Its Effector Rho Kinase Mediate Oxygen Glucose Deprivation- Evoked In Vitro Cerebral Barrier Dysfunction. *Stroke,* 41**,** 2056-2063.

ALLEN, C. L. & BAYRAKTUTAN, U. 2009. Antioxidants attenuate hyperglycaemia-mediated brain endothelial cell dysfunction and blood–brain barrier hyperpermeability. *Diabetes, Obesity and Metabolism,* 11**,** 480-490.

ALLURI, H., WILSON, R. L., ANASOOYA SHAJI, C., WIGGINS-DOHLVIK, K., PATEL, S., LIU, Y., PENG, X., BEERAM, M. R., DAVIS, M. L., HUANG, J. H. & THARAKAN, B. 2016. Melatonin Preserves Blood-Brain Barrier Integrity and Permeability via Matrix Metalloproteinase-9 Inhibition. *PLoS One,* 11**,** e0154427.

AMANO, M., ITO, M., KIMURA, K., FUKATA, Y., CHIHARA, K., NAKANO, T., MATSUURA, Y. & KAIBUCHI, K. 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem,* 271**,** 20246-9.

ANNUNZIATA, P., CIONI, C., SANTONINI, R. & PACCAGNINI, E. 2002. Substance P antagonist blocks leakage and reduces activation of cytokine-stimulated rat brain endothelium. *J Neuroimmunol,* 131**,** 41-9.

ANSEL, J. C., BROWN, J. R., PAYAN, D. G. & BROWN, M. A. 1993. Substance P selectively activates TNF-alpha gene expression in murine mast cells. *J Immunol,* 150**,** 4478-85.

ARRAZOLA SASTRE, A., LUQUE MONTORO, M., GALVEZ-MARTIN, P., LACERDA, H. M., LUCIA, A. M., LLAVERO, F. & ZUGAZA, J. L. 2020. Small GTPases of the Ras and Rho Families Switch on/off Signaling Pathways in Neurodegenerative Diseases. *Int J Mol Sci,* 21.

BANG, R., BIBURGER, M., NEUHUBER, W. L. & TIEGS, G. 2004. Neurokinin-1 receptor antagonists protect mice from CD95- and tumor necrosis factor-alpha-mediated apoptotic liver damage. *J Pharmacol Exp Ther,* 308**,** 1174-80.

BANKS, W. A., KOVAC, A. & MOROFUJI, Y. 2018. Neurovascular unit crosstalk: Pericytes and astrocytes modify cytokine secretion patterns of brain endothelial cells. *J Cereb Blood Flow Metab,* 38**,** 1104-1118.

BAYRAKTUTAN, U. 2002. Free radicals, diabetes and endothelial dysfunction. *Diabetes Obesity & Metabolism,* 4**,** 224-238.

BEUTLER, B. & CERAMI, A. 1986. Cachectin/tumor necrosis factor: an endogenous mediator of shock and inflammation. *Immunol Res,* 5**,** 281-93.

CHEN, J., WANG, X., HU, J., DU, J., DORDOE, C., ZHOU, Q., HUANG, W., GUO, R., HAN, F., GUO, K., YE, S., LIN, L. & LI, X. 2020. FGF20 Protected Against BBB Disruption After Traumatic Brain Injury by Upregulating Junction Protein Expression and Inhibiting the Inflammatory Response. *Front Pharmacol,* 11**,** 590669.

CIONI, C., RENZI, D., CALABRO, A. & ANNUNZIATA, P. 1998. Enhanced secretion of substance P by cytokine-stimulated rat brain endothelium cultures. *J Neuroimmunol,* 84**,** 76-85.

CLARK, P. R., KIM, R. K., POBER, J. S. & KLUGER, M. S. 2015. Tumor necrosis factor disrupts claudin-5 endothelial tight junction barriers in two distinct NF-kappaB-dependent phases. *PLoS One,* 10**,** e0120075.

COTTRELL, G. S., PADILLA, B., PIKIOS, S., ROOSTERMAN, D., STEINHOFF, M., GEHRINGER, D., GRADY, E. F. & BUNNETT, N. W. 2006. Ubiquitin-dependent Down-regulation of the Neurokinin-1 Receptor*. *Journal of Biological Chemistry,* 281**,** 27773-27783.

DANEMAN, R. & PRAT, A. 2015. The blood-brain barrier. *Cold Spring Harb Perspect Biol,* 7**,** a020412.

DEJANA, E. 2004. Endothelial cell-cell junctions: happy together. *Nat Rev Mol Cell Biol,* 5**,** 261-70.

DESTEFANO, J., JAMIESON, J., LINVILLE, R. & SEARSON, P. 2018. Benchmarking in vitro tissue-engineered blood–brain barrier models. *Fluids and Barriers of the CNS,* 15.

DING, M., HART, R. P. & MILLER JONAKAIT, G. 1995. Tumor necrosis factor-α induces substance P in sympathetic ganglia through sequential induction of interleukin-1 and leukemia inhibitory factor. *Journal of Neurobiology,* 28**,** 445-454.

EAPEN, P. M., RAO, C. M. & NAMPOOTHIRI, M. 2019. Crosstalk between neurokinin receptor signaling and neuroinflammation in neurological disorders. *Rev Neurosci,* 30**,** 233-243.

FREED, A. L., AUDUS, K. L. & LUNTE, S. M. 2001. Investigation of the metabolism of substance P at the blood-brain barrier using capillary electrophoresis with laser-induced fluorescence detection. *Electrophoresis,* 22**,** 3778-84.

FREIDIN, M. & KESSLER, J. A. 1991. Cytokine regulation of substance P expression in sympathetic neurons. *Proceedings of the National Academy of Sciences,* 88**,** 3200-3203.

FUKATA, Y., AMANO, M. & KAIBUCHI, K. 2001. Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. *Trends in Pharmacological Sciences,* 22**,** 32-39.

FULENWIDER, H. D., SMITH, B. M., NICHENKO, A. S., CARPENTER, J. M., NENNIG, S. E., CHENG, K., RICE, K. C. & SCHANK, J. R. 2018. Cellular and behavioral effects of lipopolysaccharide treatment are dependent upon neurokinin-1 receptor activation. *Journal of Neuroinflammation,* 15**,** 60.

GARCIA-RECIO, S. & GASCON, P. 2015. Biological and Pharmacological Aspects of the NK1-Receptor. *Biomed Res Int,* 2015**,** 495704.

GIBSON, C. L., SRIVASTAVA, K., SPRIGG, N., BATH, P. M. & BAYRAKTUTAN, U. 2014. Inhibition of Rho-kinase protects cerebral barrier from ischaemia-evoked injury through modulations of endothelial cell oxidative stress and tight junctions. *J Neurochem,* 129**,** 816-26.

HIRANO, M. & HIRANO, K. 2016. Myosin di-phosphorylation and peripheral actin bundle formation as initial events during endothelial barrier disruption. *Scientific Reports,* 6.

HO, W. Z., LAI, J. P., LI, Y. & DOUGLAS, S. D. 2002. HIV enhances substance P expression in human immune cells. *Faseb Journal,* 16**,** 616-+.

IKEBE, M. & HARTSHORNE, D. J. 1985. Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. *J Biol Chem,* 260**,** 10027-31.

IKEBE, M., HARTSHORNE, D. J. & ELZINGA, M. 1986. Identification, phosphorylation, and dephosphorylation of a second site for myosin light chain kinase on the 20,000-dalton light chain of smooth muscle myosin. *J Biol Chem,* 261**,** 36-9.

JAGANNATHAN, M., HASTURK, H., LIANG, Y., SHIN, H., HETZEL, J. T., KANTARCI, A., RUBIN, D., MCDONNELL, M. E., VAN DYKE, T. E., GANLEY-LEAL, L. M. & NIKOLAJCZYK, B. S. 2009. TLR cross-talk specifically regulates cytokine production by B cells from chronic inflammatory disease patients. *J Immunol,* 183**,** 7461-70.

JOHNSON, C. L. & JOHNSON, C. G. 1991. Tumor necrosis factor and interleukin-1 down-regulate receptors for substance P in human astrocytoma cells. *Brain Research,* 564**,** 79-85.

KADIR, R. R. A., ALWJWAJ, M. & BAYRAKTUTAN, U. 2022. Establishment of an In Vitro Model of Human Blood–Brain Barrier to Study the Impact of Ischemic Injury. *In:* STONE, N. (ed.) *The Blood-Brain Barrier: Methods and Protocols.* New York, NY: Springer US.

KAZAKOVA, O. A., KHAPCHAEV, A. Y. & SHIRINSKY, V. P. 2020. MLCK and ROCK mutualism in endothelial barrier dysfunction. *Biochimie,* 168**,** 83-91.

KIM, K. T., KIM, H. J., CHO, D. C., BAE, J. S. & PARK, S. W. 2015. Substance P stimulates proliferation of spinal neural stem cells in spinal cord injury via the mitogen-activated protein kinase signaling pathway. *Spine J,* 15**,** 2055-65.

KIMURA, T., HORIKOSHI, Y., KURIYAGAWA, C. & NIIYAMA, Y. 2021. Rho/ROCK Pathway and Noncoding RNAs: Implications in Ischemic Stroke and Spinal Cord Injury. *International Journal of Molecular Sciences* [Online], 22.

LARSEN, M. R., TRELLE, M. B., THINGHOLM, T. E. & JENSEN, O. N. 2006. Analysis of posttranslational modifications of proteins by tandem mass spectrometry. *Biotechniques,* 40**,** 790-8.

LIU, B. K., JIN, X. W., LU, H. Z., ZHANG, X., ZHAO, Z. H. & SHAO, Y. 2019a. The Effects of Neurokinin-1 Receptor Antagonist in an Experimental Autoimmune Cystitis Model Resembling Bladder Pain Syndrome/Interstitial Cystitis. *Inflammation,* 42**,** 246-254.

LIU, X., ZHU, Y., ZHENG, W., QIAN, T., WANG, H. & HOU, X. 2019b. Antagonism of NK-1R using aprepitant suppresses inflammatory response in rheumatoid arthritis fibroblast-like synoviocytes. *Artificial Cells, Nanomedicine, and Biotechnology,* 47**,** 1628-1634.

LOPEZ-RAMIREZ, M. A., FISCHER, R., TORRES-BADILLO, C. C., DAVIES, H. A., LOGAN, K., PFIZENMAIER, K., MALE, D. K., SHARRACK, B. & ROMERO, I. A. 2012. Role of caspases in cytokine-induced barrier breakdown in human brain endothelial cells. *J Immunol,* 189**,** 3130-9.

MCKENZIE, J. A. & RIDLEY, A. J. 2007. Roles of Rho/ROCK and MLCK in TNF-alpha-induced changes in endothelial morphology and permeability. *J Cell Physiol,* 213**,** 221-8.

MOVAT, H. Z. & CYBULSKY, M. I. 1987. Neutrophil emigration and microvascular injury. Role of chemotaxins, endotoxin, interleukin-1 and tumor necrosis factor alpha. *Pathol Immunopathol Res,* 6**,** 153-76.

NWARIAKU, F. E., ROTHENBACH, P., LIU, Z., ZHU, X., TURNAGE, R. H. & TERADA, L. S. 2003. Rho inhibition decreases TNF-induced endothelial MAPK activation and monolayer permeability. *J Appl Physiol (1985),* 95**,** 1889-95.

PAN, X., EATHIRAJ, S., MUNSON, M. & LAMBRIGHT, D. G. 2006. TBC-domain GAPs for Rab GTPases accelerate GTP hydrolysis by a dual-finger mechanism. *Nature,* 442**,** 303-6.

PENG, J., HE, F., ZHANG, C., DENG, X. & YIN, F. 2011. Protein kinase C-α signals P115RhoGEF phosphorylation and RhoA activation in TNF-α-induced mouse brain microvascular endothelial cell barrier dysfunction. *J Neuroinflammation,* 8**,** 28.

PETRACHE, I., CROW, M. T., NEUSS, M. & GARCIA, J. G. 2003. Central involvement of Rho family GTPases in TNF-alpha-mediated bovine pulmonary endothelial cell apoptosis. *Biochem Biophys Res Commun,* 306**,** 244-9.

RIGOR, R. R., SHEN, Q., PIVETTI, C. D., WU, M. H. & YUAN, S. Y. 2013. Myosin light chain kinase signaling in endothelial barrier dysfunction. *Med Res Rev,* 33**,** 911-33.

ROCHFORT, K. D., COLLINS, L. E., MCLOUGHLIN, A. & CUMMINS, P. M. 2016. Tumour necrosis factor-α-mediated disruption of cerebrovascular endothelial barrier integrity in vitro involves the production of proinflammatory interleukin-6. *J Neurochem,* 136**,** 564-72.

SHAO, B. & BAYRAKTUTAN, U. 2013. Hyperglycaemia promotes cerebral barrier dysfunction through activation of protein kinase C-beta. *Diabetes Obesity & Metabolism,* 15**,** 993-999.

SHEN, Q., RIGOR, R. R., PIVETTI, C. D., WU, M. H. & YUAN, S. Y. 2010. Myosin light chain kinase in microvascular endothelial barrier function. *Cardiovasc Res,* 87**,** 272-80.

SHI, J. & WEI, L. 2007. Rho kinase in the regulation of cell death and survival. *Arch Immunol Ther Exp (Warsz),* 55**,** 61-75.

SIPKA, A., LANGNER, K., SEYFERT, H. M. & SCHUBERTH, H. J. 2010. Substance P alters the in vitro LPS responsiveness of bovine monocytes and blood-derived macrophages. *Vet Immunol Immunopathol,* 136**,** 219-26.

SRIVASTAVA, K., SHAO, B. & BAYRAKTUTAN, U. 2013. PKC-beta exacerbates in vitro brain barrier damage in hyperglycemic settings via regulation of RhoA/Rho-kinase/MLC2 pathway. *J Cereb Blood Flow Metab,* 33**,** 1928-36.

STEBBINS, M. J., GASTFRIEND, B. D., CANFIELD, S. G., LEE, M.-S., RICHARDS, D., FAUBION, M. G., LI, W.-J., DANEMAN, R., PALECEK, S. P. & SHUSTA, E. V. 2019. Human pluripotent stem cell–derived brain pericyte–like cells induce blood-brain barrier properties. *Science Advances,* 5**,** eaau7375.

ULKER, S., MCKEOWN, P. P. & BAYRAKTUTAN, U. 2003. Vitamins reverse endothelial dysfunction through regulation of eNOS and NAD(P)H oxidase activities. *Hypertension,* 41**,** 534-539.

UM, J., YU, J., DUBON, M. J. & PARK, K. S. 2016. Substance P and thiorphan synergically enhance angiogenesis in wound healing. *Tissue Eng Regen Med,* 13**,** 149-154.

UN, H., UGAN, R. A., KOSE, D., BAYIR, Y., CADIRCI, E., SELLI, J. & HALICI, Z. 2020. A novel effect of Aprepitant: Protection for cisplatin-induced nephrotoxicity and hepatotoxicity. *Eur J Pharmacol,* 880**,** 173168.

XU, C., WU, X., HACK, B., BAO, L. & CUNNINGHAM, P. 2015. TNF causes changes in glomerular endothelial permeability and morphology through a Rho and myosin light chain kinase-dependent mechanism. *Physiological Reports,* 3**,** e12636.

YAMAMOTO, M., RAMIREZ, S. H., SATO, S., KIYOTA, T., CERNY, R. L., KAIBUCHI, K., PERSIDSKY, Y. & IKEZU, T. 2008. Phosphorylation of claudin-5 and occludin by rho kinase in brain endothelial cells. *Am J Pathol,* 172**,** 521-33.

ZAREMBA, J. & LOSY, J. 2001. Early TNF-alpha levels correlate with ischaemic stroke severity. *Acta Neurol Scand,* 104**,** 288-95.