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Estrogen protects the blood–brain barrier from inflammation-induced disruption and increased lymphocyte trafficking

E. Maggioli [a,1], S. McArthur [a,b,1], C. Mauro [a], J. Kieswich [a], D.H.M. Kusters [c,d], C.P.M. Reutelingsperger [c], M. Yaqoob [a], E. Solito [a,*]

[a] William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK
[b] Department of Biomedical Sciences, Faculty of Science & Technology, University of Westminster, New Cavendish Street, London W1W 6UW, UK
[c] Cardiovascular Research Institute, Department of Biochemistry, Maastricht University, 6200 Maastricht, The Netherlands
[d] Department of Pathology, University of Michigan Health System, 109 Zina Pitcher Place, 4062 BSRB, Ann Arbor, MI 48109-2200, United States

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ABSTRACT

Sex differences have been widely reported in neuroinflammatory disorders, focusing on the contributory role of estrogen. The microvascular endothelium of the brain is a critical component of the blood–brain barrier (BBB) and it is recognized as a major interface for communication between the periphery and the brain. As such, the cerebral capillary endothelium represents an important target for the peripheral estrogen neuroprotective functions, leading us to hypothesize that estrogen can limit BBB breakdown following the onset of peripheral inflammation.

Comparison of male and female murine responses to peripheral LPS challenge revealed a short-term inflammation-induced deficit in BBB integrity in males that was not apparent in young females, but was notable in older, reproductively senescent females. Importantly, ovariectomy and hence estrogen loss recapitulated an aged phenotype in young females, which was reversible upon estradiol replacement. Using a well-established model of human cerebrovascular endothelial cells we investigated the effects of estradiol upon key barrier features, namely paracellular permeability, transendothelial electrical resistance, tight junction integrity and lymphocyte transmigration under basal and inflammatory conditions, modeled by treatment with TNFα and IFNγ. In all cases estradiol prevented inflammation-induced defects in barrier function, action mediated in large part through up-regulation of the central coordinator of tight junction integrity, annexin A1. The key role of this protein was then further confirmed in studies of human or murine annexin A1 genetic ablation models.

Together, our data provide novel mechanisms for the protective effects of estrogen, and enhance our understanding of the beneficial role it plays in neurovascular/neuroimmune disease.

1. Introduction

Several lines of evidence suggest that the sex differences seen in vascular and neural diseases may be at least partly linked to differing sex hormone complements. In particular, a number of beneficial effects have been attributed to the principal female hormone estrogen, including in conditions as diverse as Parkinson's disease, Alzheimer's disease, head injury and multiple sclerosis (Gillies and McArthur, 2010). One important working hypothesis is that the neuroprotective effects of estrogen may be related to its known anti-inflammatory and immunomodulatory actions (Czlonkowska et al., 2005; Nadkarni and McArthur, 2013).

The endothelium of the blood–brain barrier (BBB) is at the forefront of the defensive features of the central nervous system, regulating its interactions with the immune system. In particular, there is accumulating evidence that BBB function is compromised during peripheral inflammation, leading to inappropriate passage of cells and molecules into the brain parenchyma (Carvey et al., 2009). Estrogen has been shown to exert a variety of anti-inflammatory effects, including reducing iNOS activity (Cignarella et al., 2009), directly regulating the cytokine milieu (Gameiro et al., 2010) and altering expression of vascular and leukocyte adhesion molecules (Dietrich, 2004). Whilst initial studies have identified estrogen as being able to modulate BBB tight junction

* Corresponding author at: Centre for Translational Medicine and Therapeutics, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK.
E-mail address: e.solito@qmul.ac.uk (E. Solito).
[1] EM and SMcA contributed equally to this work.

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proteins such as claudin 5 (Burek et al., 2014), full characterization of the effects of estrogen upon the BBB is lacking, and represents an underexplored aspect of hormonal protection.

We have previously studied the importance of the anti-inflammatory protein annexin A1 (ANXA1) in the BBB, where it plays a major role in the regulation of tight junction expression, contributing to limited barrier permeability (Cristante et al., 2013). Although ANXA1 was originally described as a glucocorticoid second messenger (Flower and Blackwell, 1979), we and others have since reported that it can also be modulated by estrogen (Solito et al., 2003; Davies et al., 2007; Nadkarni et al., 2011), leading us to hypothesize that estrogen may exert protective effects upon the BBB in inflammation through the regulation of this protein.

Using a combined *in vivo*/*in vitro* approach, we examined the response of the BBB to peripheral inflammatory challenge and the ability of the principal estrogen: estradiol (E2) to restore homeostasis in this system. We report here the presence of sexual dimorphism in the response of the BBB to peripheral pro-inflammatory challenge *in vivo*, and a dual protective role for estradiol upon the inflamed cerebral endothelium *in vitro*, mediated through the regulation of ANXA1 and ICAM-1 expression. Together these actions control movement of both small molecules and immune cells across the cerebral endothelium.

2. Materials and methods

2.1. Reagents

All reagents are from Sigma–Aldrich (Poole, UK) unless otherwise stated. Cell culture medium and solutions were purchased from Lonza (Basel, Switzerland). The ERα agonist PPT (4,4′,4″-(4-propyl-(5)-pyrazole-1,3,5-triyl)trisphenol), the ERβ agonist DPN (2,3-bis(4-Hydroxyphenyl)-propionitrile), the GPR30 agonist G-1 ((±)-1-[(3aR*,4S*,9bS*)-4-(6-bromo-1,-3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone), the ERβ antagonist PHTPP (4-[2-phenyl-5,7-bis(trifluoromethyl-)pyrazolo[1,5-a]pyrimidin-3-yl]phenol) and the GPR30 antagonist G-15 ((3aS*,4R*,9bR*)-4-(6-bromo-1,3-benz-odioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone) were purchased from Tocris Bioscience (Bristol, UK). TNFα and IFNγ were purchased from R&D Systems (Abingdon, UK).

2.2. Human recombinant annexin A1 production and purification

cDNA of human ANXA1 carrying a cleavable N-terminal poly-His tag was expressed in *Escherichia coli*. Recombinant protein was purified on IMAC (GE Healthcare), and the poly-His tag was subsequently removed. Purity of recombinant ANXA1 was confirmed by SDS–PAGE and the 4800 MALDI-TOF/TOF (Applied Biosystems), revealing a 38.6-kDa protein that was >95% pure.

2.3. Animal experiments

Male and female 2 and 15 month old C57Bl/6 and AnxA1$^{-/-}$ mice were bred at Charles River under our project QMUL.ES Annexin. Female C57Bl/6 mice were sham-operated or ovariectomised under isoflurane anesthesia with buprenorphine analgesia, and micro-osmotic pumps containing either estradiol or β-cyclodextrin vehicle were implanted subcutaneously (Alzet model 2002; Durect Co., Cupertino, CA, USA); similar ovariectomy has been previously shown to significantly reduce circulating estrogen in ovariectomised animals when compared with sham-operated mice (Villar et al., 2011). Mice were sacrificed by exsanguination. All experiments were performed at the same time of day to avoid the confounding effects of circadian rhythm. In some experiments ovariectomised mice were further treated with intravenous (i.v.) human recombinant ANXA1 before further challenge with 3 mg/kg lipopolysaccharide (LPS).

2.4. In vivo assessment of BBB leakage

Mice were injected i.p. with 3 mg/kg LPS in 100 μl saline vehicle and cerebrovascular permeability was assessed at 4 h, 24 h or 7 days post-injection. One hour before assessment animals were injected i.v. with 100 μl of a 2% (w/v) solution of Evans blue dye in 0.9% saline (Sigma–Aldrich Ltd., Poole, UK). Dye was permitted to circulate for 1 h before animals were transcardially perfused with 0.9% saline at 4 °C to remove circulating dye, as described previously (Cristante et al., 2013); brain Evan's blue content was expressed as μg of dye/mg of brain tissue, normalized to circulating plasma concentrations.

2.5. Murine T lymphocyte transmigration on isolated primary microvascular brain endothelial cells

Murine T cells were isolated from lymph nodes of C57BL/6 WT mice and activated for 3 days in 24-well plates coated with anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) antibodies (BioLegend), and containing IL-2 (10 ng/ml; PeproTech) as previously described (Haas et al., 2015). Capillaries were extracted from the cortex of 3-month-old WT mice as previously described (Cristante et al., 2013). Isolated vessels were put in culture until confluence. Primary brain microvascular endothelial cells were then used at passage one in 24-well plate Polycarbonate transwell inserts (surface: 0.33 cm^2, pore size: 5 μm; Sigma–Aldrich, UK) and put in contact with murine T lymphocytes for the lymphocyte transmigration assay (4 h at 37 °C in 5% CO_2) as described in Section 2.8.

2.6. hCMEC/D3 cells

The hCMEC/D3 cell line used for *in vitro* and molecular analysis was maintained and treated as described previously (Weksler et al., 2005; Cristante et al., 2013). For permeability and imaging experiments cells were either polarized through growth on 12-well plate Polyethylene Terephthalate (PET) transwell inserts (surface: 1.12 cm^2, pore size: 0.4 μm; Sigma–Aldrich, UK) coated with calf-skin collagen and fibronectin (Sigma–Aldrich, UK), or plated on Nunc™ Lab-tek Chamber slide system Permanox Plastic 25 × 75 mm (ThermoScientific, UK).

2.7. In vitro barrier function assessments

Permeability of hCMEC/D3 cell monolayers to 70 kDa FITC-dextran (3 mg/ml) was measured as described previously (Abbott et al., 1992; Coisne et al., 2005; Cristante et al., 2013). Transendothelial electrical resistance (TEER) measurements were performed on 100% confluent cultures using a Millicell-ERS apparatus (Millipore, Watford, UK) and expressed as Ω cm^2. Values obtained from cell-free inserts were subtracted from the total values.

2.8. Lymphocyte transmigration assay

hCMEC/D3 brain endothelial cells were cultured in monolayers on 24-well plate Polycarbonate transwell inserts (surface: 0.33 cm^2, pore size: 5 μm; Sigma–Aldrich, UK) for 48 h prior to assay. After 24 h, TNFα and IFNγ (10 ng/ml) were added to the upper chambers; after 48 h, and just before the assay started, 600 μl of complete EGM-2 MV medium was added to the lower compartment. Peripheral blood mononuclear cells (PBMCs),