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Vascular changes in the developing rat retina in response to hypoxia

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ABSTRACT

This study was carried out to investigate the roles of tight junction (TJ) proteins and other factors in the increased permeability of the blood retinal barrier (BRB) affecting the immature neonatal retina following a hypoxic insult. The expression of endothelial TJ proteins such as claudin-5, occludin and zonula occludens-1 (ZO-1) and endothelial cell specific molecule-1 (ESM-1), and associated structural changes in the blood vessels were analyzed in the retinas of 1-day-old Wistar rats subjected to hypoxia for 2 h and subsequently sacrificed at different time points ranging from 3 h to 14 d. The mRNA and protein expression of claudin-5, occludin & ZO-1 was found to be reduced in the hypoxic retina, although, at the ultrastructural level, the TJ between the endothelial cells and retinal pigment epithelial cells appeared to be intact. Following the hypoxic insult vascular endothelial cells frequently showed presence of cytoplasmic vacuoles, vacuolated mitochondria and multivesicular aggregations projecting into the lumen of the capillaries. The expression of ESM-1 in the immature retinas was found to be increased following hypoxic exposure. The structural and molecular changes in the hypoxic neonatal retinas were consistent with a hypoxia induced impairment of the BRB. Hypoxia reduced the expression of TJ proteins in the neonatal retina, but the role of increased ESM-1 expression in this process warrants further investigation.

1. Introduction

Hypoxia is known to cause many changes in the developing neural tissues including the retina. It increases vascular permeability in neural tissues such as the brain and retina. An increased production of factors such as vascular endothelial growth factor (VEGF), nitric oxide (NO), inflammatory cytokines and free radicals has been implicated in this process (Kaur et al., 2008a). The structural and functional integrity of the retina depends on intact inner and outer blood retinal barriers (BRBs). The inner BRB (iBRB) is formed by TJ between the vascular endothelial cells of capillaries in the inner retina whereas TJ between retinal pigment epithelial cells form the outer BRB (oBRB).

We have previously reported an abnormal leakage of intraperitonealy or intravenously administered tracers [horseradish peroxidase (HRP) and rhodamine isothiocyanate (RhIC)] in the retinas of adult and neonatal rats following a hypoxic insult (Kaur et al., 2009, 2007). The leakage of these tracers occurred through the blood vessels forming the iBRB whereas the oBRB remained intact with no evidence of leakage of HRP or RhIC through this route.

The immature retina is extremely vulnerable to hypoxic-ischemic conditions (Di Fiore et al., 2010; Distefano et al., 1998). Premature birth, associated with incomplete vascularization of the retina may result in the development of sight-threatening retinopathy of prematurity (ROP). Fetal hypoxia during gestation can develop from many maternal causes such as diabetes, asthma, multiple pregnancy, anemia, smoking and use of alcohol or drugs. Defective pulmonary function (infantile respiratory distress syndrome), anemia and episodes of bradycardia, all of which induce hypoxia, are risk factors for ROP (Ng et al., 1988). Cyanotic heart disease is also an important, but less common aetiological factor that may result in hypoxia in neonates and subsequently in the development of retinopathy (Johns et al., 1991). Hypoxia may induce damage to or loss of retinal ganglion cells (RGCs) in the

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developing retina with resulting visual impairment. We have shown that RGCs and neurons in the inner nuclear layer (INL) die by apoptosis or necrosis in new born rats subjected to hypoxia (Kaur et al., 2009). Several factors induced by hypoxia such as inflammation, excitotoxicity and oxidative stress can contribute to cell death in the developing retina (Kaur et al., 2012, 2013).

Retinal vascularization is reported to begin in the inner retina at the optic nerve head at around birth (P0) in rodents (Gariano and Gardner, 2005) and reach the retinal periphery around 6–8 days of age (Fruttiger, 2002, 2007). A recent study has reported that the iBRB is formed as early as 3 days of age in mice (Yao et al., 2014). The capillaries forming the iBRB are characterized by lack of fenestrations in their endothelial cells and presence of TJs between them. The endothelial cells rest on a basal lamina which is surrounded by astrocyte processes. Pericytes are also closely associated with the endothelial cells. Transmembrane proteins such as occludin, claudin-5 and cytoplasmic proteins such as zonula occludens-1 (ZO-1) that are constituents of the TJ are structurally and functionally important for maintenance of iBRB integrity. In the adult retina, hypoxia-ischemia has been reported to affect the integrity of the TJ through a reduced expression of TJ-associated proteins ZO-1 and occludin, resulting in increased BRB permeability leading to vasogenic edema (Kaur et al., 2008a; Tong et al., 2013).

In addition to loss of RGCs and damage to the neurons in the INL in the hypoxic developing retina, our recent studies have shown increased production of VEGF, NO and inflammatory cytokines along with increased permeability of blood vessels and presence of edema in the hypoxic neonatal retina (Kaur et al., 2009; Sivakumar et al., 2011). An increased expression of VEGF in astrocytes associated with retinal blood vessels is thought to affect the permeability of blood vessels by binding to and activating two tyrosine kinase receptors, VEGFR1 and VEGFR2 expressed on the endothelial cells, which contribute to disruption of TJ by decreasing occludin and ZO-1 expression (Fischer et al., 2002; Spoerri et al., 2006). Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) have also been documented to play a role in the development of vasogenic edema by inducing TJ disruption (Lv et al., 2010).

It is not known however if the increased vascular permeability in hypoxic conditions is due to damage to the vascular endothelial cells or alterations in the TJ proteins. In view of this, we evaluated the structure of endothelial cells and determined the expression of TJ-associated proteins such as occludin, claudin-5 and ZO-1 in the retinas of neonatal rats subjected to hypoxia. We also determined the expression of endothelial cell-specific molecule-1 (ESM-1), also called endocan, in the retinal vasculature. ESM-1/Endocan has been used to assess the inflammatory events and vascular endothelial dysfunction in patients suffering from gliomas and it has been reported that its expression is up-regulated in response to hypoxia (Maurage et al., 2009).

2. Materials and methods

2.1. Animals

1-day old Wistar rats were used in the present study. Seventy-seven rats were exposed to hypoxia by placing them in a chamber (Model: MCO 18M; Sanyo Biomedical Electrical Co, Ltd, Tokyo, Japan.) filled with a gas mixture of 5% oxygen and 95% nitrogen for two hours. The rats were then allowed to recover under normoxic conditions for 3, 24 h, 3, 7 and 14 d before sacrifice. Another group of 77 rats kept outside the chamber was used as age matched controls. This study was approved by the Institutional Animal Care and Use Committee of National University of Singapore.

2.2. Real time RT-PCR

Retinas were removed from the hypoxic rats ($n = 5$ at each time point) and their corresponding controls ($n = 5$ at each time point). Total RNA was extracted from the retinas using RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol.

Quantitative RT-PCR was carried out on a Light Cycler 2 instrument using a FastStart DNA Master plus SYBR Green I kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Expression of target genes was measured in triplicate and was normalized to β-actin, as an internal control. Forward and reverse primer sequences for each gene and their corresponding amplicon size are provided in Table 1. Gene expression was quantified using a modification of the $2^{-\Delta\Delta ct}$ method as previously described (Livak and Schmittgen, 2001).

2.3. Western blotting

Retinas were removed from the hypoxic rats ($n = 5$ at each time point) and their corresponding controls ($n = 5$ at each time point). The protein was extracted from the retina with protein extraction reagent (Pierce Biotechnology, IL, USA) containing protease inhibitors. Protein concentrations were determined by the Bradford's (1976) method using bovine serum albumin (Sigma–Aldrich) as a standard. Samples of supernatant containing 20 μg protein were heated to 95 °C for 5 min and were separated by sodium dodecyl sulphate-poly-acrylamide gel electrophoresis in 10% gels, in a Mini-Proteon 3 apparatus (Bio-Rad, CA, USA). Protein bands were electroblotted onto 0.45 μm polyvinylindene difluoride membrane and then incubated with polyclonal antibodies directed against rabbit-anti claudin-5 (Santa Cruz Biotechnology, Santa Cruz, USA; 1:500), rabbit-anti occludin (Santa Cruz Biotechnology; 1:500), rabbit-anti ZO-1 (Santa Cruz Biotechnology; 1:1000) and goat-anti ESM-1 (Santa Cruz Biotechnology; 1:3000) in blocking solution overnight at 4 °C. After washing three times, the membranes were then incubated with the respective secondary antibody; HRP conjugated anti-rabbit or goat IgG (GE Healthcare UK Limited, Bucks, UK). Specific binding was revealed by an enhanced-chemiluminescence kit (GE healthcare, UK) following the manufacturer's instructions. For load control, after intensive washing, the membranes were incubated with monoclonal mouse anti-β-actin (1:5000) (Sigma–Aldrich). X-ray films (GE healthcare, UK) were scanned with a computer-assisted G-710 densitometer (Bio-Rad) to quantify band optical density using Quantity One software (Bio-Rad).

2.4. Double immunofluorescence

Rats at 3 d after the hypoxic exposure and their corresponding controls ($n = 6$ in each group) were used for double immunofluorescence. Following deep anesthesia with 6% pentobarbital, the rats

Table 1
Sequence of primers used for estimating mRNA expression of TJ molecules and ESM-1.

Target	Forward primer	Reverse primer	Amplicon size
Claudin-5	5′-ggc-ttg-tgg-cac-tct-ttg-t-3′	5′-act-ccc-gga-cta-gga-tgt-tg-3′	148
Occludin	5′-cag-gat-tgc-gct-gac-tat-ga-3′	5′-gct-agg-gtt-acg-gct-atg-ga-3′	160
ZO-1	5′-agt-tct-gcc-ctc-agc-tac-ca-3′	5′-cag-gtt-tag-ggc-caa-caa-gc-3′	209
ESM-1	5′-cgt-cca-tgc-ctg-aga-ctg-ta-3′	5′-act-gac-cac-gct-cct-gat-tc-3′	184