Inflammatory activation of the cerebrovascular endothelium in response to oxygen-glucose deprivation

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

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Sophie Francesca Leow-Dyke
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### Abbreviations

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<td>OGD</td>
<td>Oxygen-glucose deprivation</td>
</tr>
<tr>
<td>Papp</td>
<td>Apparent permeability</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Polycarbonate</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDS</td>
<td>Plasma derived serum</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PET</td>
<td>Polyethylene terephthalate</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin-streptomycin</td>
</tr>
<tr>
<td>PSGL</td>
<td>P-selectin glycoprotein ligand</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation, normal T-cell expressed and secreted</td>
</tr>
<tr>
<td>RNI</td>
<td>Reactive nitrogen intermediate</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SGLT</td>
<td>Sodium-glucose transporter</td>
</tr>
<tr>
<td>SS&lt;sub&gt;high/low&lt;/sub&gt;</td>
<td>Side scatter&lt;sub&gt;high/low&lt;/sub&gt;</td>
</tr>
<tr>
<td>TEER</td>
<td>Transendothelial electrical resistance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>TNFR1/2</td>
<td>Tumour necrosis factor receptor 1/2</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR1/2</td>
<td>Vascular endothelial growth factor receptor-1 / 2</td>
</tr>
<tr>
<td>Veh</td>
<td>Vehicle</td>
</tr>
<tr>
<td>VLA-1</td>
<td>Very late antigen-1</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrand factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
<tr>
<td>ZO</td>
<td>Zonula occluden</td>
</tr>
</tbody>
</table>
Abstract
The University of Manchester
Sophie Leow-Dyke
Doctor of Philosophy
2011

Inflammatory activation of the cerebrovascular endothelium in response to oxygen-glucose deprivation

There is increasing evidence that inflammatory processes play a pivotal role in the pathophysiology of ischaemic brain injury. Cerebrovascular endothelial cells that form the blood-brain barrier are critical for maintaining brain homeostasis, however, during cerebral ischaemia they contribute to the post-ischaemic inflammatory responses. It is not yet fully understood how different cerebral cells interact during this inflammatory response. This study aimed to test the hypothesis that oxygen-glucose deprivation (OGD) induces the inflammatory activation of the cerebrovascular endothelium and glial cells in vitro and that intercommunication between these cells regulate their responses to OGD.

Primary murine brain endothelial cells (MBECs) monocultures, murine mixed-glial monocultures and MBEC-glial co-cultures were exposed to OGD for up to 24 hours (h), then reperfused cultures were returned to normoxia for a further 24 hours.

MBECs and glia remained viable over a 24 h OGD exposure and during reperfusion. OGD induced a time-dependent increase in MBEC glucose transporter 1 (GLUT-1) expression but a time-dependent decline in expression and secretion of monocyte chemoattractant protein-1 (MCP-1). A significant increase in keratinocyte-derived chemokine (KC) secretion by MBEC monocultures was observed during reperfusion after prolonged exposure (18-24 h) to OGD whereas, KC secretion by co-cultured MBECs was increased during reperfusion after short exposure (4 h) to OGD. Co-cultured MBECs displayed a significant increase in intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression in response to a short or prolonged exposure to OGD with 24 h of reperfusion. Neither OGD nor reperfusion had any effect on permeability of the MBEC monolayer.

OGD induced a time-dependent increase in nuclear stabilisation of hypoxia inducible factor-1 alpha (HIF-1α) in glial cells which correlated to vascular endothelial growth factor (VEGF) secretion during OGD and subsequent reperfusion. Nuclear stabilisation of the nuclear factor kappa B (NFκB) p65 subunit by glial cells was dependent upon the duration of OGD. Reperfusion induced a significant increase in KC secretion by co-cultured glial cells after short exposure to OGD.

Inflammatory activation of co-cultured MBECs and glia after 4 or 24 h OGD caused a significant increase in neutrophil transendothelial migration which correlated with MBEC expression of ICAM-1 and VCAM-1. A combination of these cell adhesion molecules with neutrophil integrins and soluble glial-derived mediators contributed to neutrophil transendothelial migration.

These studies provide evidence that combined hypoxia and glucose withdrawal induces the activation of MBECs and glial cells in vitro. Cross-talk between these two cell types may further regulate their activation. As a result of this inflammatory activation, soluble MBEC and glial-derived mediators may contribute to neutrophil transendothelial migration through the regulation of MBEC cell adhesion molecule expression.
Lay abstract
The University of Manchester
Sophie Leow-Dyke
Doctor of Philosophy
2011

Understanding the responses of brain cells to stroke

Stroke is one of the leading causes of death and disability worldwide. A stroke occurs when there is a loss in blood flow to the brain that can be caused by a blockage of blood vessels supplying the brain (ischaemic stroke) or when a blood vessel supplying the brain bursts and bleeds (haemorrhagic stroke). The brain needs a constant supply of blood to provide oxygen and nutrients for healthy function. Therefore any interruption in blood flow leads to damage and death of brain cells resulting in substantial injury to the brain with possibly a devastating impact on the patient. There is increasing evidence that inflammation which we normally associate with diseases like arthritis is important in the brain after stroke and causes a lot of the damage. Therefore understanding how inflammation causes damage is very important for the development of new treatments for stroke and related diseases.

The experiments reported in this PhD thesis investigated the responses of the brain endothelial cells which form the inner lining of blood vessels and other supporting cells of the brain when starved of oxygen and glucose in a test tube to mimic the lack of blood flow that occurs during a stroke. It was found that withdrawal of oxygen and glucose resulted in production of molecules that cause inflammation by these cells and this was dependent upon the duration of oxygen and glucose deprivation. Furthermore, communication between these cells played a role in regulating their inflammatory responses.

This study has identified many potential cellular targets for therapeutic intervention, forming the foundation for further research to identify the mechanisms mediating these responses.
I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Sophie Leow-Dyke

Date
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Dedication

To Mum, Dad, Alicia and Natalie
Chapter 1
Introduction
1. Introduction

1.1. The Blood-brain barrier (BBB): Structure and function

The blood-brain barrier (BBB) is a structural and metabolic barrier between the peripheral circulation and the central nervous system (CNS) and is essential for maintenance of brain homeostasis. The BBB (Figure 1.1) is formed by a monolayer of tightly-sealed specialised endothelial cells bound to the underlying basal lamina forming the intraluminal surface of the cerebral vessels.

The BBB is responsible for regulating the uptake of nutrients from the blood as well as efflux of waste products and toxic metabolites from the brain. Under normal physiological conditions the BBB restricts the paracellular and transcellular passage of hydrophilic molecules and ions (Abbott et al., 2006). Tight junction proteins between endothelial cells form a diffusion barrier restricting paracellular permeability of substances while minimal endocytic and pinocytic activity limits transcellular transport across the BBB (Sandoval and Witt, 2008; Engelhart and Coisne, 2011). The transmembrane tight junction proteins occludin and claudin and the junctional adhesion molecules (JAMs) belonging to the immunoglobulin superfamily of cell adhesion molecules have been identified to be the major components of the tight junctions of the BBB (Figure 1.1). Oligomers of occludin have been identified to have a role in regulation of tight junction complexes. Changes in phosphorylation state and disruption to the covalent bonds of the occludin oligomers can affect the ability of tight junction complexes to limit paracellular diffusion (Hawkins and Davis, 2005; Lochhead et al., 2010). Claudins belong to a multigene family containing over 20 proteins. Of these, claudin-5 and -12 have been identified in brain capillary endothelial cells of rodents (Morita et al., 1999; Ohtsuki, S. et al., 2007). These claudins primarily limit the movement of ions across the endothelial monolayer resulting in the high transendothelial electrical resistance (TEER) of the BBB. TEER of the brain endothelium of rodents may exceed 800 ohm.cm$^2$ compared to 50 ohm.cm$^2$ of peripheral capillaries (Butt et al., 1990; Rubin et al., 1991). The JAMs JAM-A, JAM-B and JAM-C are involved in the assembly and maintenance of tight junction proteins (Abbott et al., 2006). The tight junction accessory proteins; the zonula occludens (ZO) stabilise tight junction
proteins by anchoring them to the actin cytoskeleton. ZO-1 and ZO-2 have been identified to connect occludin to actin (Mark and Davis, 2001).

**Figure 1.1. The blood-brain barrier.** A single monolayer of endothelial cells surrounds the capillary lumen. Pericytes within the basal lamina cover a proportion of the abluminal endothelium. Astrocytes end-feet surround the abluminal surface of the endothelium. Endothelial cells are connected by tight junction proteins forming a selective diffusion barrier. The transmembrane tight junction proteins claudin, occludin and JAMs connect adjacent endothelial cells forming a selective diffusion barrier. The cytoplasmic regions of these tight junction proteins are attached to the actin cytoskeleton via the tight junction accessory proteins ZO-1 and ZO-2. Adherens junctions below the tight junction complexes near the abluminal surface of the endothelial cells have a role in localisation and stabilisation of the tight junctions. Adherens junctions are linked to the actin cytoskeleton via α, β and γ-catenins.
In addition to limiting paracellular permeability, endothelial tight junction complexes segregate apical and basal membranes creating polarisation of the endothelial cells. BBB polarity is characterised by the expression of specific transporters and receptors on the apical and basal membranes that also contribute to the selective permeability of the BBB by facilitating the uptake of specific nutrients or efflux of potential harmful compounds (Abbott et al., 2006). Multidrug resistance transporters and drug efflux pumps such as P-glycoprotein (P-gp) are enriched at the luminal membrane (Schinkel, 1999) whereas the excitatory amino acid transporters 1-3 (EAAT-1-3) are concentrated on the abluminal membrane (O’Kane et al., 1999). Some transporters including the glucose transporter 1 (GLUT-1) and L-system amino acid transporter-1 (LAT1) are present on both the luminal and abluminal membranes for the bidirectional transport of substances (Abbott et al., 2006; Nakagawa et al., 2009). Additionally, the uptake of certain substances such as insulin, low density lipoprotein and iron-transferrin complexes are regulated by receptor-mediated transcytosis (Tuma and Hubbard, 2003). Thus, endothelial polarity of receptors, channels and transporters are critical for establishing a selective transport barrier essential for maintaining brain homeostasis.

The notion that the brain is an “immune privileged” organ, unable to elicit an immune response or contribute to inflammatory responses was coined in the late 19th Century by Paul Ehrlich. Injection of water soluble dyes into the peripheral circulation stained all organs in the body except the brain, indicating the brain is an anatomically distinct compartment. However, over the past two decades the concept of the immune privileged brain has been challenged. Animal models of multiple sclerosis; experimental autoimmune encephalomyelitis, have shown that T lymphocytes injected into the peripheral circulation of rats are able to cross the BBB (Ben-Nun et al., 1981). These early animal studies led to the identification that the healthy CNS is continuously screened by circulating immune cells that includes polymorphonuclear (PMN) cells, T-lymphocytes and monocytes performing routine immunosurveillance of the CNS for recognition of tissue damage or infection (Engelhart and Coisne, 2011). The role of circulating immune cells in immune surveillance has led to incorporation of these cells into the neurovascular unit (NVU) (Neuwelt et al., 2011).
1.2. The Neurovascular unit (NVU)

The BBB is an integral part of the conceptual NVU that also comprises of the basal lamina, pericytes, glial cells and neurones. In recent years the concept of the NVU has emerged to also include circulating immune cells, in particular polymorphonuclear (PMN) cells, T-lymphocytes and monocytes that function for homeostatic immune surveillance (Neuwelt et al., 2011) (Figure 1.2). The basal lamina consists of collagen type IV, laminins, fibronectin, and heparan sulphate proteoglycans (del Zoppo et al., 2009). The basal lamina provides a substratum for cell attachment, is a physical barrier to the passage of solutes and is involved in the regulation of cell signalling (Tilling et al., 2002). Pericytes located within the basal lamina and perivascular glial cells including perivascular microglia are closely juxtaexposed with the abluminal surface of the endothelium (Hirschi and D’Amore, 1996). Astrocytes extend long processes with terminal end-feet that surround a significant proportion of the abluminal surface of the endothelium and contact neurones, providing a link between neuronal activity and cerebral blood supply. Cell-cell signalling between the different cellular components of the NVU underlies the health and function of the CNS. Permutations to this homeostatic cellular coupling that consequently alter NVU function contribute to the development and progression of many CNS diseases. In particular, inflammatory responses of the cellular components of the NVU initiated by cerebral ischaemia reflect dysfunctional signalling within the NVU that exacerbates injury.
Chapter 1

Introduction

1.3. Inflammation in cerebral ischaemia

Cerebral ischaemia occurs when there is a cessation or reduction in blood supply to part or parts of the brain. Clinically defined as stroke, cerebral ischaemia can be classified as focal cerebral ischaemia, when blood flow to a specific region of the brain is affected or global cerebral ischaemia when blood flow to the whole brain is lost. Strokes are classified as either ischaemic or haemorrhagic. Ischaemic stroke, accounting for approximately 80% of strokes can be caused by a blockage in one of the blood vessels supplying the brain as a result of either a thrombus or embolism (Van Elzen et al., 2008). Haemorrhagic stroke occurs when a blood vessel supplying the brain ruptures. Aneurysms are a common cause of haemorrhagic stroke as well as vascular structural abnormalities such as arteriovenous malformation. Other causes of cerebral ischaemia include compression of blood vessels, cerebral vasospasm, cardiac arrest or head injury (Van Elzen et al., 2008).
Ischaemia-reperfusion can result in a biphasic injury profile as well as mediating tissue repair and recovery. Early cerebral damage is initiated by the immediate energy depletion caused by the loss in cerebral blood flow that subsequently causes dysfunctional cellular biochemical reactions. Alterations in cellular gene expression lead to inflammatory reactions within the brain that contribute to long term responses that can exacerbate initial injury and may mediate tissue repair and recovery.

### 1.3.1. Early cellular responses to cerebral ischaemia

Normal cerebral blood flow of humans is 60 - 100 mL/100 g/min. A reduction in cerebral blood supply to less than 16 mL/100 g/min results in irreversible neuronal damage (Heiss, 2000). Within minutes of cessation or reduction in cerebral blood flow, the fall in energy leads to perturbation of ATP-dependent ionic pumps that results in depolarisation of neuronal and cerebral cell membranes causing an increase in intracellular calcium (Ca\textsuperscript{2+}), accumulation of intracellular sodium (Na\textsuperscript{+}) and excess potassium (K\textsuperscript{+}) efflux. Elevated intracellular Ca\textsuperscript{2+} results in the release of excitotoxic amino acid neurotransmitters such as glutamate, activation of calcium dependent enzymes such as proteases, endonucleases and cyclooxygenase and free radical generation that lead to neuronal death (Stoll et al., 1998; Pober and Sessa, 2007). The release of excitatory neurotransmitters and cellular biochemical changes cause the rapid induction of early response genes. Increased expression of c-Fos and c-Jun with enhanced activating protein-1 (AP-1) binding activity is detected in the ischaemic hemisphere of rats after experimentally-induced transient cerebral ischaemia (An et al., 1993). Downstream targets of the AP-1 transcription factor include glial fibrillary acidic protein (GFAP), nerve growth factor (NGF) and neutrotropins (Akins et al., 1996) which have been implicated in contributing to further damage and recovery in animal models of cerebral ischaemia. This modulation of early gene expression induces a second wave of responses that include inflammatory activation of resident cerebral cells that further exacerbates injury.

### 1.3.2. Secondary inflammatory responses to cerebral ischaemia

In the majority of focal stroke patients, the small area of brain tissue supplied by the occluded or haemorrhaged vessel is irreversibly damaged. The tissue surrounding this ischaemic core, the penumbra, exhibits functional impairment associated with
the loss of electrical activity and membrane ionic gradients but maintains a critical threshold of cerebral blood flow for conserved oxygen and energy metabolism for morphological preservation of tissue (Astup et al., 1981). Thus the penumbral tissue is potentially salvageable with timely reperfusion or therapeutic intervention. However secondary brain injury can develop within minutes of an ischaemic episode, evolving over hours to days as a result of further excitotoxic cell damage, loss of vascular autoregulation of cerebral blood flow, oedema, cerebral metabolic dysfunction or post-ischaemic inflammation within the penumbral tissue. Consequently, the penumbral tissue may become incorporated into the irreversibly damaged ischaemic core. It has been suggested that targeting this delayed inflammatory response may be a potential avenue for therapeutic intervention.

The modulation in activity of a number of transcription factors contributes to the post-ischaemic inflammatory processes. The redox-sensitive transcription factor nuclear factor kappa B (NFκB) is activated within minutes of reperfusion after temporary cerebral ischaemia in rats and in cultured human cerebral endothelial cells during reperfusion following hypoxia in vitro (Carroll et al., 1998; Howard et al., 1998). NFκB is a dimeric transcription factor consisting of the five proteins p50, RelA/p65, c-Rel, RelB, and p52. NFκB activation initiates transcription of several genes including inducible nitric oxide synthase (iNOS), cyclo-oxygenase enzymes, matrix metalloproteinases (MMPs) and cell adhesion molecules, all of which are implicated in the post-ischaemic inflammatory response.

Induction of hypoxia inducible factor-1 (HIF-1) in cerebral cells in response to hypoxic insults is well characterised (Sharp and Bernaudin, 2004). HIF-1 is a heterodimer composed of a constitutively expressed HIF-1β subunit and the oxygen sensitive HIF-1α subunit (Van Elzen et al., 2008). Under normoxic conditions HIF-1α is hydroxylated by prolyl-4-hydroxylase and is targeted for degradation by the proteasomal system. A decline in cellular oxygen inhibits prolyl-4-hydroxylase and subsequently HIF-1α is stabilised and is translocated to the nucleus to dimerise with HIF-1β to become transcriptionally active (Sharp and Bernaudin, 2004; Van Elzen et al., 2008). HIF-1α regulates the transcription of many genes including erythropoietin, vascular endothelial cell growth factor (VEGF) and endothelial nitric
oxygen synthase (eNOS) (Yang et al., 2007) which will be discussed in later sections.

One of the consequences of this modulation in gene expression is the inflammatory activation of the glial cells and endothelial cells that involves the production of cytokines, chemokines, growth factors and proteolytic enzymes. Cytokines such as interleukin-1\(\beta\) (IL-1\(\beta\)) and tumour necrosis factor-\(\alpha\) (TNF\(\alpha\)) induce upregulation in endothelial expression of the selectins and the immunoglobulin (Ig) family of cell adhesion molecules. An increase in chemokines and endothelial cell adhesion molecules mediates leukocyte infiltration across the BBB into the brain parenchyma. Activated endothelial cells, glia and leukocytes exacerbate injury through further production of pro-inflammatory and cytotoxic mediators, proteolytic enzymes and reactive oxygen species (ROS). As a consequence of post-ischaemic inflammatory responses, structural and functional disruption to the blood-brain barrier (BBB) can lead to cerebral oedema and haemorrhage. Figure 1.3 summarises the sequence of cellular inflammatory responses following cerebral ischaemia. Vascular, glial and neuronal responses to ischaemic injury and recruitment of immune cells are likely to be interconnected, thus the NVU as a whole must be considered concerning the responses to and the impact of ischaemic injury.
Figure 1.3. Inflammatory responses of the neurovascular unit following cerebral ischaemia.
Inflammatory responses of cellular components of the NVU following cerebral ischaemia. The loss in cellular energy within minutes of a reduction in cerebral blood flow leads to increased intracellular Ca\textsuperscript{2+} levels and generation of ROS in ischaemic cells that lead to neuronal injury and/or death. Necrotic cells, ROS and alteration in gene expression lead to the inflammatory activation of glial cells and the endothelium that develops over hours to days. Endothelial activation includes upregulation in expression of cell adhesion molecules that subsequently contributes to increased leukocyte adhesion and infiltration into the brain parenchyma. Activated glia, leukocytes and endothelial cells generate various inflammatory mediators that contribute to the development of post-ischaemic injury. Variability occurs in the time-frame of responses and mediators depending upon the severity and duration of ischaemic injury and the region of brain affected.

1.4. Vascular responses to cerebral ischaemia
Cerebral ischaemia is considered to be a vascular disorder, in which the endothelial cells at the interface between the blood and the brain are the first cells to sense the loss in blood flow. Subsequently, the responses of the endothelium are considered to play a significant role in post-ischaemic inflammation. However, as shown in figure 1.3, cerebral ischaemia initiates a complex inflammatory cascade that involves all the cell types within the brain. Thus, inflammatory responses can be broadly divided into vascular responses by the endothelium and associated pericytes or non-vascular responses such as those by the glial cells and neurones. These will now be discussed in more detail.
1.4.1. Endothelial cells

Under normal physiological conditions the endothelium exhibits anti-thrombotic and anti-inflammatory functions regulating blood flow and permeability of the microvessel (del Zoppo and Mabuchi, 2003; Pober and Sessa, 2007). Endothelial cells of the BBB are the most resistant to cerebral ischaemia, accounting for less than 2% of the cells within the ischaemic core displaying evidence of DNA damage after experimentally-induced ischaemia in both rodents and primates (Tagaya et al., 1997). Nevertheless, endothelial dysfunction after ischaemia plays a major role in the pathophysiology of cerebral ischaemia. Endothelial expression and secretion of a plethora of pro-inflammatory mediators is induced in response to the ischaemic insult and in response to glial and neuronal-derived mediators.

1.4.1.2. Endothelial glucose transporter expression

Under the conditions of hypoxia and/or hypoglycaemia, endothelial cells display changes in the expression of nutrient transporters, in particular, glucose transporters that transport glucose from the blood across the BBB into the brain (Yang et al., 2007). Expression of the glucose transporter, GLUT-1, is both vascular and non-vascular, the latter including astrocytes and neurones. Neurones also express GLUT-3 (Vannucci et al., 1998b). GLUT-1 transcription is under the control of HIF-1. Following hypoxia, there is amplification in GLUT-1 mRNA and subsequent enhancement in expression on the luminal and abluminal side of endothelium and on astrocytes. This upregulation in expression is global, not only confined to the region of the brain supplied by the occluded vessel (Vannucci et al., 1998a). Regulation of GLUT-1 may also be post-translational and increased GLUT-1 mRNA stabilisation has been reported upon glucose deprivation (Vannucci et al., 1998a). GLUT-3 expression is enhanced in the penumbra but reduced in the ischaemic core; this latter observation correlates with neurones undergoing necrosis in this region (Vannucci et al., 1998b). Additionally, the sodium-glucose transporter (SGLT) that is not normally expressed under normal conditions is expressed on the luminal and abluminal side of the endothelium in ischaemia (Vemula et al., 2008). The combined upregulation of these glucose transporters may represent protective responses to increase cerebral glucose to compensate that lost from the reduction in blood flow.
1.4.1.2. Alterations to the integrity of the blood-brain barrier

In animal studies, there is a biphasic disruption to BBB integrity during permanent cerebral ischaemia or during reestablishment of cerebral blood flow after a transient ischaemic episode. Although the exact timings of barrier disruption depend on the animal species and duration of ischaemia, an initial early increase in permeability may occur within five hours of reperfusion, after which there is a restoration in permeability. A secondary phase of disruption from 24 hours may last for days leading to oedema and haemorrhage (Sandoval and Witt, 2008). Measurements of TEER, uptake of radiolabelled or fluorescent tracers or leakage of serum albumin or plasma fibrinogen are used as markers of BBB integrity in animal studies and in confluent endothelial cell monolayers in vitro.

Disruption to the BBB in vivo and in vitro is associated with relocalisation and reduced expression of the tight junction proteins occludin, claudin-5 and the tight junction accessory proteins ZO-1 and ZO-2. These junctional proteins display a more diffuse expression rather than at cell perimeters that correlate to increased phosphorylation state of these proteins (Mark and Davis, 2001; Fischer et al., 2002). Actin expression also increases, forming stress filaments, which, together with the changes in phosphorylation states of the tight junction proteins may contribute to relocalisation of the tight junction complexes (Mark and Davis, 2001; Witt et al., 2003). Several factors may contribute to the disruption to BBB integrity including nitric oxide (NO), MMPs, VEGF and leukocyte-derived products which will be discussed in more detail in subsequent sections. Loss of microvascular integrity is also accompanied by a selective loss of certain matrix binding integrins on the abluminal side of endothelial cells and from the end-feet of astrocytes. These integrins mediate interactions between the endothelial cells and astrocytes to the basal lamina contributing to maintenance of the BBB barrier (Defilippi et al., 1992). Oxygen-glucose deprivation (OGD) reduces $\alpha_1\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrin expression by primary murine cerebral endothelial cells and $\alpha_4$ and $\alpha_6$ integrin subunits from murine astrocyte end-feet, the latter corresponds to loss of $\alpha_6\beta_4$ integrin from astrocytes in nonhuman primate models following middle cerebral artery occlusion (MCAo) as an in vivo model for ischaemic stroke (Milner et al., 2008; Sandoval et al., 2008). Dystroglycan, another family of matrix receptors present on astrocyte
processes are also downregulated in mice under such conditions (del Zoppo, 2009). Cytokines appear to contribute to integrin downregulation. Loss of the $\alpha_6\beta_1$ integrin on endothelial cells has been shown to be mediated by TNF-$\alpha$ and IL-1 (Defilippi et al., 1992). Although integrin loss is observed, only the $\alpha$ subunits of integrins are downregulated while $\beta_1$ subunit expression is maintained. This reduction in the $\alpha$ subunits is proposed to be due either to changes in transcriptional regulation or reduced stability of mRNA (Defilippi et al., 1992).

1.4.1.3. Endothelial cell adhesion molecule expression

Endothelial cell adhesion molecules mediate leukocyte adhesion to the cerebral endothelium and their infiltration into the brain parenchyma during reperfusion. There are three sets of adhesion molecules: selectins, immunoglobulin (Ig) superfamily and integrins. The Ig superfamily expressed by the endothelium include intracellular cell adhesion molecule-1 and 2 (ICAM-1 and ICAM-2), vascular cell adhesion molecule-1 (VCAM-1), platelet cell adhesion molecule-1 (PECAM-1) and mucosal addressin cell adhesion molecule-1 (MadCAM-1). Of these cell adhesion molecules, ICAM-1 and VCAM-1 have been most extensively identified in cerebral ischaemia. Integrins are heterodimers consisting of a common $\beta$ subunit and a variable $\alpha$ subunit. There are three subfamilies of $\beta$ subunit, denoted $\beta_{1-3}$. The $\beta_2$ subunit (CD18) is involved in leukocyte adhesion to the endothelial Ig superfamily of cell adhesion molecules.

Unstimulated human and rodent cerebral cells in culture do not display any surface expression of P or E-selectin. Following MCAo in vivo in rodents or treatment of endothelial cells in culture with inflammatory cytokines such as IL-1$\beta$ induces exocytosis of Wiebel-Palade bodies that rapidly delivers P-selectin to the luminal endothelial surface and de novo transcription of E-selectin is induced (Ley et al., 2007; Pober and Sessa, 2007). Selectins mediate the initial tethering and rolling of leukocytes on the luminal surface of the endothelium. P- and E-selectin bind the sialyl-Lewis X tetrasaccharide on P-selectin glycoprotein ligand (PSGL) expressed by leukocytes. L-selectin is shed from the leukocyte surface membrane following transmigration across the endothelium (Ohmori et al., 2000). Mice overexpressing P-selectin have larger infarct volumes compared to WT mice. P-selectin deficient mice
or administration of antibodies directed against P-selectin results in reduced infarct volume and improved neurological outcome following MCAo (Huang et al., 2006). Microvascular expression of E-selectin is induced as early as two hours after experimentally-induced ischaemia in rats and lasts for up to 70 hours (Zhang et al., 1996). Intravenous infusion of anti-E-selectin antibody to mice, either immediately before and 90 min after MCAo or 3 h after MCAo increased cortical cerebral blood flow, reduced infarct volume and mortality and reduced neutrophil accumulation of the vasculature (Huang et al., 2000).

Elevated plasma levels of soluble ICAM-1 and VCAM-1 have been measured in patients after acute ischaemic stroke, indicative of an inflammatory response after acute stroke (Simundic et al., 2004). Exposure of cerebral endothelial cell cultures to hypoxia/OGD-reperfusion cause upregulation of ICAM-1 from basal, low level expression during the reperfusion period (Clark et al., 1995; Stanimirovic et al., 1997). Increased production of cytokines such as IL-1β, TNF-α and interleukin-6 (IL-6) after cerebral ischaemia induces the upregulation in endothelial expression of ICAM-1 and VCAM-1 (Amantea et al., 2008). In vitro, administration of exogenous IL-1 increases cerebral microvascular endothelial ICAM-1 expression and treatment with interleukin-1 receptor antagonist (IL-Ra) or transcription inhibitors attenuates this (Stanimirovic et al., 1997). Further studies suggest cytokine-mediated upregulation in ICAM-1 is due to enhanced protein transcription (Stanimirovic et al., 1997). ICAM-1 predominantly mediates the binding of neutrophils via the neutrophil integrin lymphocyte function associated antigen-1 (LFA-1; CD11a/CD18). The increase in ICAM-1 expression is concomitant with an increase in neutrophil adhesion to the endothelium which can be blocked by anti-ICAM antibodies or anti-LFA-1 antibodies (Stanimirovic et al., 1997). Although VCAM-1 has high affinity for T lymphocytes and monocytes, the role of VCAM-1 in cerebral ischaemia is not fully understood. Justicia et al. (2006) showed that intravenous injection of anti-VCAM-1 after transient MCAo in rats decreased the number of monocytes and macrophages in the ischaemic hemisphere but did not reduce the amount of lymphocytes. Infarct volumes and brain swelling was also greater in these animals compared to isotype control treated rats suggesting that targeting VCAM-1 does not confer neuroprotection after ischaemic injury.
1.4.1.4. Leukocyte migration across the blood-brain barrier

In the healthy brain, the cerebral endothelium has minimal interaction with circulating leukocytes for regulatory immune surveillance, however, within hours of reperfusion after the onset of ischaemia in rodents and primates, leukocyte accumulation occurs on the endothelium (Vexler et al., 2006). Subsequent leukocyte infiltration into the brain parenchyma is a key feature of the inflammatory response that occurs during ischaemic insults. Neutrophils are the first hemopoietic cells to penetrate the BBB after experimentally-induced cerebral ischaemia in rodents and primates. Monocyte/macrophage and T lymphocyte extravasation occurs later in the post-ischaemic period (Mabuchi et al., 2000; Staničević et al., 1997). Temporal and spatial accumulation of neutrophils in the brain correlates to ischaemic infarct size and neurological damage in rodents (Lipton, 1999). Neutrophils aggravate the pathology of injury by obstructing blood flow, release of vasoconstrictive mediators such as endothelin-1 (ET-1), release of toxic mediators that include ROS, reactive nitrogen intermediates (RNI) and cytotoxic and proteolytic enzymes including MMPs, lysozymes and elastase which lead to disruption to BBB permeability (Yamaski et al., 1997; del Zoppo, 2008). Preventing neutrophil migration in rats reduces the post-ischaemic damage. Intraperitoneal injection of an anti-neutrophil antibody that depletes neutrophils in circulation or an anti-cytokine-induced neutrophil chemoattractant (CINC) antibody immediately after MCAo reduces the size of ischaemic lesions, decreases oedema formation and the extent of neuronal damage in these rats (Yamaski et al., 1997; McColl et al., 2007). Garau, et al. (2006) developed inhibitors of the chemokine receptors CXCR1 and CXCR2 present on neutrophils which reduced infarct volume and improved neurological function following MCAo in rats (Garau et al., 2006).

Figure 1.4 illustrates that leukocyte infiltration across the cerebral endothelium into the brain parenchyma is a regulated sequence of events, involving leukocyte rolling, arrest with strengthening of adhesion, intraluminal crawling and transmigration across the endothelium that may occur by paracellular (between adjacent endothelial cells, at cell-cell junctions) or transcellular routes (through the endothelial cells themselves) (Ley et al., 2007). Initial tethering and rolling of leukocytes on the endothelium are mediated by E- and P-selectin expressed by the endothelium and L-selectin expressed by leukocytes. Rolling of leukocytes on the luminal endothelial
surface is suggested to enable leukocytes to find permissive sites for transendothelial migration. The transition between leukocyte rolling and firm adhesion involves formation of high affinity integrins to bind their corresponding endothelial cell adhesion molecules (Cinamon et al., 2004). Chemokines immobilised on the luminal surface of the endothelium are responsible for mediating firm arrest of leukocytes. Chemokines signal through G-protein coupled receptors (GPCRs) expressed by leukocytes. This results in integrin clustering and induces a fully extended conformation of integrins which have high affinity for their Ig superfamily ligands on the endothelium. (Cinamon et al., 2001; Chesnutt et al., 2006). Chesnutt et al., (2006) showed that the chemokine keratinocyte-derived chemokine (KC) immobilised on the luminal endothelial surface of mice induced firm adhesion of neutrophils which could be correlated to the extended conformation of LFA-1. Additionally, monocyte chemoattractant protein-1 (MCP-1) immunoreactivity on the luminal surface of the endothelium implies MCP-1 produced by the endothelial cells become immobilised on the endothelium for presentation to leukocytes (Pratt et al., 2001).

Although the mechanisms of leukocyte rolling and adhesion in cerebral ischaemia have been well characterised, the mechanisms and route by which leukocytes transverse the cerebral endothelium is still not fully understood. Considering the anatomical structure of the BBB, leukocytes migrating across the BBB must migrate across the endothelial monolayer, penetrate the basal lamina and then migrate between astrocyte endfeet.

The interaction of neutrophil integrins, PSGL-1 and L-selectin with their corresponding endothelial ligands as well as signalling through their GPCRs induces respiratory burst and degranulation of neutrophils. Elastase and cathepsins released from primary granules, collagenase and lysozymes released from secondary granules and MMP-9 from tertiary granules are implicated in mediating neutrophil transendothelial migration (DiStasi and Ley, 2009). Moll et al. (1998) demonstrated that PMN cells adhered to endothelial monolayers in vitro induced degradation of endothelial β-catenins. This may have implications for the junctional integrity of the cadherins. Chimeric knockout (KO) mice lacking MMP-9 in leukocytes exhibit a reduction in leukocyte adherence, capillary plugging and infiltration into the brain
during reperfusion following MCAo (Gidday et al., 2005). By contrast, Allport et al. (2002) showed that MMP-9 or elastase deficient neutrophils were able to roll, adhere and transmigrate across the activated murine endothelium to the same extent as WT neutrophils. Differences between experimental models may account for these discrepancies. The release of glutamate from PMN leukocytes has also been shown to increase BBB permeability in mice after hypoxic exposure (Collard et al., 2002).

There is much evidence that leukocytes can migrate across the endothelium in vivo and in vitro between endothelial cells via tight junction complexes (paracellular pathway) or through the endothelial cells (transcellular pathway). However the route of neutrophil migration across the BBB after cerebral ischaemia has not been investigated extensively. Studies of neutrophil, monocyte and lymphocyte migration across cytokine activated human umbilical vein endothelial cells (HUVECs) is mediated by clustering of ICAM-1 on the endothelial surface that form microvilli-like projections that surround the adhered leukocyte (Carman et al., 2003). Furthermore, lymphocytes have been found to extend actin-rich protrusions that palpate and extend into the endothelial cytoplasm, indicating lymphocyte trafficking involves the formation of transcellular pores (Carman et al., 2007). Additionally, electron microscopy revealed an enrichment of endothelial vesicles close to sites of lymphocyte protrusions, further evidence of a transcellular pathway (Carman et al., 2007). Evidence for leukocytes migrating across the BBB via a paracellular pathway is conflicting. Cowan and Easton (2010) showed that neutrophils restored the increase in permeability of the human endothelial cell line, hCMEC/D3 induced by OGD. Conversely, in vivo studies have also demonstrated that neutrophil recruitment across the cerebral vasculature of rats is associated with loss of occludin and ZO-1 and reorganisation of the adherens junction protein vinculin (Bolton et al., 1998).

The knowledge that leukocyte recruitment into the ischaemic brain exacerbates injury indicates the need for further research into the mechanisms of leukocyte transendothelial migration across the BBB after cerebral ischaemia.
Adapted from Ley et al. (2007)

Figure 1.4. Leukocyte infiltration across the BBB. Major steps in leukocyte infiltration across the endothelium include initial capture or tethering of circulating leukocytes, rolling, arrest followed by strengthening of adhesion, intraluminal crawling and finally transmigration.

Few *in vitro* studies have considered leukocyte transendothelial migration under flow conditions. Shear flow has been implicated to have a role in adhesion of leukocytes. Shear flow induces formation of slip-catch bonds between selectins and their endothelial ligands where the force of flow increases the strength of these bonds (Ley et al., 2007). Using a flow chamber assay, Cinamon et al. (2001) was able to distinguish discrete steps in the interactions between lymphocytes and human endothelial cells including lymphocyte rolling, arrest, spreading and firm adhesion.

1.4.1.5. Endothelial-platelet interactions

Experimentally-induced ischaemia in rodents induces platelet activation that results in platelet aggregation, generation of eicosanoids and the release of growth factors and cytokines from intracellular granules (Turčáni et al., 1988; Lindemann et al., 2001). Platelet activation and adhesion to the endothelium observed during ischaemia-reperfusion contributes to the pathophysiology of ischaemia; affecting vascular permeability and adhesive properties of the endothelium (Turčáni et al., 1988; Gawaz et al., 2000). Patients receiving anti-platelet agents within 24 hours of acute stroke show reduced infarct volumes compared to non-treated patients (Ovbiagele et al., 2008). *In vivo*, gerbils treated with neuraminidase to reduce systemic platelet counts displayed less water accumulation in the cerebral
hemispheres following occlusion of the common carotid artery compared to untreated groups. This is suggestive of the potential involvement of platelets in the development of ischaemic brain oedema (Turčáni et al., 1988). Platelets have a role in leukocyte recruitment to the endothelium. Depletion of circulating platelets in mice via anti-platelet antibodies significantly reduces leukocyte adhesion (Carvalho-Tavares et al., 2000).

The mechanism of platelet adhesion to the endothelium is not fully understood and it may be that several factors are involved. Ishikawa et al. (2004) demonstrated a role of P-selectin in platelet adhesion to the cerebral microvasculature of mice. Platelet adhesion was significantly reduced in P-selectin deficient mice and mice treated with and anti-P-selectin antibody after MCAo. The platelet ligand GPIIb/IIIa also mediates platelet binding by acting as a linking molecule. GPIIb/IIIa binds fibronectin, fibrinogen and von Willebrand Factor (vWF) expressed or secreted by activated platelets which in turn bind to ICAM and/or $\alpha_v\beta_3$ integrin (vitronectin) present on the surface of the endothelium (Bombeli et al., 1998). Neutralising antibodies against GPIIb/IIIa, fibrinogen, fibronectin or vWF all result in a reduction in the number of platelets adhering to endothelial cell monolayers (Bombeli et al., 1998). GPI$\alpha$ expressed by endothelial cells is also suggested to be involved in platelet adhesion (Bombeli et al., 1998). Activated platelets induce NF$\kappa$B activation in endothelial cells speculated to be mediated by IL-1. ICAM-1 expression and MCP-1 secretion from cultured murine endothelial cells increases in the presence of activated and adhered platelets, leading to leukocyte recruitment which are attenuated following addition of IL-1Ra or anti-IL-1 antibody, supporting the involvement of IL-1 in leukocyte migration (Gawaz et al., 2000). Further to this, platelets have been identified to be a source of IL-1$\alpha$ that induces murine endothelial expression of cell adhesion molecules and chemokines (Thornton et al., 2010).

1.4.2. Pericytes
Pericytes present on the abluminal surface of the endothelial cells of the BBB have a range of roles in brain homeostasis. The contractile capacity of pericytes, attributed to their expression of $\alpha$-smooth muscle actin ($\alpha$-SMA) and receptors for vasodilatory and vasoconstrictive substances are involved in contributing to the regulation of
blood flow (Kamouchi et al., 2010). Expression of transforming growth factor-β (TGF-β), VEGF and angiopoietin-1 and 2 by pericytes have been implicated in angiogenesis (Balabanov and Dore-Duffy, 1998; Fisher, 2009). The close contact between pericytes and the abluminal membrane of endothelial cells is crucial for the formation of tight junction proteins by the endothelial cells and paracellular and transcellular permeability of the BBB (Kamouchi et al., 2010). The importance of pericyte regulation of BBB integrity has been identified from studies showing that rat brain endothelial cells co-cultured with rat pericytes exhibit higher transendothelial electrical resistance and lower permeability to fluorescein compared to endothelial monocultures (Nakagawa et al., 2007). The role of pericytes in ischaemic injury has largely been overlooked, partly because of the lack of pericytes-specific markers. Pericytes have been shown to be more resistant than endothelial cells and astrocytes to hypoxia in vitro (Al Ahmad et al., 2009). Furthermore, pericytes promote endothelial survival during hypoxia by inhibition of endothelial caspase-3 (Al Ahmad et al., 2009).

1.5. Non-vascular responses to cerebral ischaemia

1.5.1. Neurones

Neurones are the most sensitive cell type to ischaemic insults. The accumulation of intracellular Ca\(^{2+}\), generation of ROS and cytotoxic enzymes after initial energy decline has a number of deleterious effects on neurones including increased membrane permeability, decreased protein synthesis, damage to the cytoskeleton and mitochondria dysfunction (Lipton, 1999). Such structural and functional changes lead to death of neurones and may have an indirect effect on the cerebral endothelium. Neuronal death may occur by necrotic and apoptotic pathways that may be dependent upon the region of brain affected, severity and/or duration of ischaemia. As part of the early response to ischaemia, neuronal necrosis is induced by excitotoxic damage (Leonardo and Pennypacker, 2009). Apoptotic neurones are observed at the border of the infarct and may remain there for weeks from the onset of ischaemia (Stoll et al., 1998). Mitogen activated protein kinase (MAPK) signalling pathways may participate in regulating neuronal death. Extracellular signal regulated kinase (ERK) and p38 activation in neurones within the penumbra and transient c-Jun N-terminal kinase (JNK) immunoreactivity in neurones of the core infarct with
later expression in neurones of the penumbra has been reported (Ferrer et al., 2003; Repici et al., 2007). JNK activation has been shown to correlate with activation of caspase-3. Administration of a permeable JNK inhibitor prior to MCAo in rats reduces size of ischaemic lesions (Repici et al., 2007).

Leukocyte accumulation and pro-inflammatory cytokines generated from endothelial cells, glia and neurones themselves following cerebral ischaemia can have direct effects on neurones, contributing to neuronal injury (del Zoppo, 2009). IL-1 produced by neighbouring microglial cells increases synaptic inhibition and \( \text{Ca}^{2+} \) entry which contributes to neuronal death (Allan et al., 2005). IL-1 is not neurotoxic to primary cultures of pure rat neurones, inducing production of NGF that supports neurone viability (Strijbos and Rothwell, 1995). However, treatment of mixed glial-neuronal or astrocyte-neuronal co-cultures with IL-1\( \beta \) significantly increases neuronal death compared to neuronal monocultures (Thornton et al., 2006). This indicates IL-1 may mediate its neurotoxic effects through other cells of the neurovascular unit. Mabuchi et al. (2005) reported that neurones located furthest from the occluded vessel after MCAo in primates were the most sensitive to ischaemic injury. This cannot be attributed to invading leukocytes which would affect those closest to the vessel or cytokines which would affect all neurones within the vicinity. This is further suggestive of the involvement of other cerebral cells in mediating neuronal injury. Cerebral endothelial cells may have a role in promoting neuronal survival. Conditioned media from hypoxic-hypoglycemic murine endothelial cells was shown to reduce neuronal death; the endothelial cell-derived neuroprotectant was identified to be BDNF (Guo et al., 2008).

While neuronal death is widely characterised during ischaemic insults, the precise mechanisms and involvement of other cerebral cells in neuronal death is unclear. Similarly, the effects of neuronal responses and death on the endothelium have yet to be extensively examined.

1.5.2. Astrocytes

Astrocytes have a critical role in the maintenance of the BBB. Astrocytes are involved in the maturation of endothelial cells and have a key role in regulating the
tight barrier properties of the BBB (Prat et al., 2001; Sandoval and Witt, 2008). In vitro, direct contact between endothelial cells and astrocyte-derived soluble factors has been shown to maintain high TEER (Hatherell et al., 2011). Furthermore, Tao-Cheng (1987) proposed that the intervening basal lamina between the endothelium and astrocyte endfeet concentrates soluble mediators secreted by glia that regulate BBB permeability. Astrocytes provide functional support for neurones and regulate the extracellular environment through uptake of excess glutamate to prevent excitotoxicity, provide glycogen as an energy reserve and scavenge free radicals (Panickar and Norenberg, 2005). Astrocytes also contribute to neuronal regulation of cerebral blood flow through their engagement between neurones and the microvasculature (del Zoppo, 2009). A recent study showed astrocyte control of vascular tone in rat brain slice cultures exposed to conditions of limited oxygen. In a low oxygen environment an increase in $\text{Ca}^{2+}$ in astrocyte end-feet leads to the generation and release of the vasodilator prostaglandin $\text{E}_2$. Concomitantly, astrocyte glycolysis is triggered, resulting in the generation of lactate. Lactate prevents reuptake of prostaglandin $\text{E}_2$ allowing its accumulation and subsequent maintenance of vasodilation (Gordon et al., 2008).

Experimentally-induced focal and global ischaemia in animals activates astrocytes. Activated astrocytes undergo proliferation (astrogliosis) (Allan et al., 2005). Hypertrophy of astrocytes adjacent to the ischaemic core is documented, with an increase in the number of mitochondria and rough endoplasmic reticulum and enlargement of nuclei and Golgi (Panickar and Norenberg 2005). Additionally, an increase in the intermediate filaments glial fibrillary acidic protein (GFAP) and vimentin, results in thicker and longer cytoplasmic processes that form a “glial scar” at the periphery of the lesion (Panickar and Norenberg, 2005). The significance of the glial scar is not fully understood. Badan et al. (2003) showed that the formation of the glial scar in rats after transient MCAo correlated with poor behavioural recovery. They suggested that reactive astrogliosis may impede functional recovery of neighbouring nervous tissue. Other in vitro studies in rodents and primates have shown that glial scarring may act to isolate the ischaemic tissue from surrounding viable tissue (Leonardo and Pennypacker, 2009). GFAP is postulated to have a bearing on neurone viability. GFAP KO mice are unable to express the glutamate transporters EAAT-1 and EAAT-2 on the surface of astrocytes and subsequently
unable to concentrate excess extracellular glutamate which is likely to contribute to the development excitotoxicity that initially occurs within minutes of ischaemia (Panickar and Norenberg, 2005). Astrocyte swelling occurs early after ischaemia which is attributed partially to an increase in the expression of the water transporting protein aquaporin-4 (AQP4). Elevated AQP4 expression is also thought to facilitate transport of water from blood vessel into the brain parenchyma, contributing to oedema formation (Kaur et al., 2006). OGD induces loss of matrix-binding integrins from astrocyte end-feet which results in separation of astrocytes from the basal lamina, contributing to the breakdown of the BBB (Sandoval and Witt, 2008).

Under hypoxic conditions, astrocytes generate both harmful substances including IL-1, IL-6, TNF-α, MCP-1, MMPs and NOS, and protective factors including nerve growth factor (NGF), brain-derived neutrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) (Allan et al., 2005). Of these protective factors, GDNF acts in an autocrine and a paracrine manner preventing apoptosis in astrocytes. Ischaemia-induced NT-3, a NGF, from rat astrocytes rescues neurones from apoptosis and protects microglia from ischaemia in vitro (Lin et al., 2006). Astrocyte-derived TGF-β1 also has a protective effect on microglia. Concomitantly, expression of GDNF receptors on astrocytes, NT-3 receptors on neurones and NT-3 and TGF-β1 receptors on microglia are upregulated during in vitro simulated ischaemia (Lin et al., 2006). In vivo and in vitro hypoxia increases nuclear stabilisation of HIF-1α by astrocytes with a concomitant increase in VEGF mRNA, protein expression and secretion (Sinor et al., 1998; Kaur et al., 2006; Schmid-Brunclik et al., 2008). Enhanced VEGF expression during ischaemic injury has both detrimental and beneficial effects, primarily through mediating actions on the vasculature. The use of immunofluorescence labelling reveals VEGF immunoreactivity is localised to the terminal end-feet of astrocytic processes after experimentally-induced ischaemia in rats (Kaur et al., 2006), implicating a role of VEGF in directly modulating the BBB. Further to VEGF actions on the vasculature, endogenous VEGF secretion by astrocytes has been identified to promote astrocyte proliferation and survival during hypoxic injury (Schmid-Brunclik et al., 2008).
As early as two hours after MCAo in the rat, astrocytes expressing IL-1β within the penumbral tissue are detected (Amantea et al., 2010). Zhang et al., (2000a) demonstrated that cultured human astrocytes subjected to hypoxia produced IL-1β. Conditioned media from these astrocytes added to endothelial cultures upregulated expression of ICAM-1, MCP-1, interleukin-8 (IL-8) and IL-1β, all of which were abrogated by prior treatment of astrocytes with anti-IL-1β antibody or treatment of endothelial cells with IL-1Ra. This suggests that hypoxic astrocytes produce IL-1β which then evokes activation of the endothelium. Several days after the onset of ischaemic damage, astrocytes producing IL-1β have been observed within and around the lesion, thus they may be involved in development of the lesion and/or recovery processes (Pearson et al., 1999).

Endothelial cells also modulate responses of astrocytes. Endothelial cells stimulate astrocytes to produce laminin-5, aiding repair of the basal lamina (Wagner and Gardner, 2000). Endothelial cells increase glutamine synthase activity of astrocytes to convert excess glutamate to glutamine. ET-1 produced by hypoxic endothelial cells stimulates astrogliosis consistent with the finding that the endothelin receptor, ETB is expressed by glia (Cardell et al., 1994).

1.5.3. Microglia
Microglial cells are resident macrophages of the CNS found within close proximity of neurones. A subset of microglia, the perivascular microglia, are in close association with the abluminal surfaces of blood vessels and are likely to have a role in regulating endothelial responses to ischaemic insults (Stoll et al., 1998). Like astrocytes, microglia can exert both beneficial and deleterious actions in response to ischaemia. Early after onset of ischaemic injury, microglia become activated where they undergo proliferation, chemotaxis and morphological transition from a quiescent ramified state to an ameboid state displaying large rounded cell bodies and short processes (Stoll et al., 1998). Ameboid microglial cells are phagocytic, phagocytosing cellular debris and generate cytotoxic and protective substances (Yenari et al., 2006). Activated microglia release the pro-inflammatory cytokines IL-1β and TNF-α, glutamate, proteolytic and lipolytic enzymes and the angiogenic factor erythropoietin following stabilisation of HIF-1α (Mabuchi et al., 2000; Yenari
et al., 2006; Yang et al., 2007). Erythropoietin can induce synthesis of TGF-β and further production of IL-1. TGF-β, is thought to have anti-inflammatory actions in cerebral ischaemia. Gross et al., (1994) demonstrated that infusion of TGF-β immediately after clot embolisation in rabbits caused a significant reduction in neutrophil accumulation in the brain although this was not associated with any improvement in neurological outcome. Microglia-derived IL-1 and GDNF may act on astrocytes to produce NGF and protect them from ischaemia-induced damage respectively (Panickar and Norenberg, 2005).

The deleterious effects of microglia are attributed to the production of TNF-α, superoxides, IL-1β, MMPs and NO and the release of glutamate which contributes to excitotoxicity (Panickar and Norenberg, 2005). Yenari et al. (2006) demonstrated that microglia increase cell death of endothelial cell-astrocyte co-cultures via the production of TNF-α, superoxides and hydrogen peroxide. Cell death was reduced by addition of minocycline, an inhibitor of microglia activation. This was demonstrated in vivo in which treatment of minocycline after MCAo and reperfusion in mice reduced infarct volumes, haemorrhagic transformation and improved neurological function (Yenari et al., 2006).

Expansion of the ischaemic lesion can occur over several days after the onset of ischaemia. Microglia can contribute to lesion expansion. Proliferating hyperramified and ameboid microglia are observed at the periphery of the lesion that subsequently becomes infarcted (Mabuchi et al., 2000). Production of IL-1β by ameboid microglia is speculated to contribute to enlargement of the lesion (Mabuchi et al., 2000; Pearson et al., 1999). Ameboid microglia also express major histocompatibility complex (MHC) class-I and MHC class-II suggestive of a role in antigen presentation to CD8+ T cells and CD4+ T cells respectively (Stoll et al., 1998). This is in accordance with observations of T cells within the infarct region (Stoll et al., 1998) which may further exacerbate the inflammatory response. Thus, preventing microglial activation after ischaemia may reduce the extent of BBB disruption and subsequent oedema and haemorrhagic transformation.
1.5.4. Oligodendrocytes
Much less is known about responses of oligodendrocytes to ischaemic insults. IL-1 generated from initial ischaemic insult induces differentiation and maturation of oligodendrocyte progenitor cells (Allan et al., 2005). Viable oligodendrocytes are detectable within the infarct up to 24 hours post-cerebral insult. Beyond 24 hours, co-localisation of oligodendrocytes with macrophages and granulocytes within the core of the infarct together with DNA fragmentation of the oligodendrocytes suggests oligodendrocytes are initially resistant to ischaemic–induced damage but subsequently destroyed by infiltrating inflammatory cells (Mabuchi et al., 2000).

1.6. Inflammatory mediators in cerebral ischaemia
Ischaemia-induced activation and injury of both the vascular endothelial cells and brain parenchymal cells results in the production of a myriad pro-inflammatory mediators that exacerbate post-ischaemic injury as well as conferring protection from further damage. As illustrated in Figure 1.3, these mediators include cytokines that have multicellular actions, chemokines that mediate the infiltration of circulating cells, and MMPs and certain growth factors that have vascular-specific actions.

1.6.1. Cytokine production
A number of cytokines are upregulated in the ischaemic brain including IL-1, TNF-α, IL-6, IL-10 and transforming growth factor-beta (TGF-β) (Tarkowski et al., 1997). Of these cytokines, IL-1 and TNF-α have been identified to play a major role in exacerbation of ischaemic injury.

1.6.1.1. Interleukin-1
The pro-inflammatory cytokine IL-1 is a major mediator of the post-ischaemic inflammatory cascade and progression of neuronal death and injury in response to cerebral ischaemia. The IL-1 family consists of two isoforms, IL-1α and IL-1β and the endogenous IL-1 receptor antagonist, IL-1Ra. IL-1α and IL-1β act though the receptors IL-1R1 and IL-1R2 (Allan et al., 2005). IL-1β has been well characterised in ischaemic injury. IL-1β is synthesised as an inactive precursor, pro-IL-1β that is cleaved by caspase-1 to its active form. Within hours of reperfusion after permanent or temporary MCAo in rodents, caspase-1 expression is increased and subsequently
IL-1β mRNA and protein levels are rapidly upregulated by microglia, astrocytes, endothelial cells and neurones (Buttini et al., 1994; Wang et al., 1994). OGD exposure of human cerebral endothelial cells in vitro also induces increased expression of caspase-1 and IL-1β (Zhang et al., 2000a). Amantea et al. (2010) identified the cellular and temporal profile of IL-1β expression in the brains of rats after ischaemia-reperfusion. By two hours reperfusion, IL-1β expression by astrocytes within the penumbral tissue and by activated microglia within the ischaemic core was evident. After 22 hours reperfusion microglia expressing IL-1β were present in the core infarct and more intensely in the penumbral region.

The contribution of IL-1α to ischaemic injury is controversial. IL-1α mRNA is constitutively expressed in WT mice that do not significantly change after MCAo. Furthermore, IL-1α KO mice exhibit the same degree of cerebral infarction as WT mice after MCAo (Boutin et al., 2001). However, an upregulation in murine astrocyte IL-1α mRNA is detected within hours of exposure to hypoxia in vitro (Yu and Lau, 2000). Thus, role of IL-1α in ischaemia injury requires further investigation.

As well as being a source of IL-1, cerebral endothelial cells are also a target for IL-1, indicating autocrine actions of IL-1. IL-1R1 and IL-1R2 have been identified on both rat and human cerebral endothelial cells (Van Dam et al., 1996; Tomita et al., 1998). IL-1β increases endothelial expression of ICAM-1 (Stanimirovic et al., 1997), cyclooxygenase-2, and prostaglandins (Cao et al., 1996) and induces release of IL-8 and monocyte chemoattractant protein-1 (MCP-1) (Zhang et al., 2000a). Thornton et al. (2010) identified that activated mouse platelets express IL-1α that induce expression of ICAM-1, VCAM-1 and the CXCL-1 chemokine by mouse cerebral endothelial cells. The deleterious effects of IL-1 are demonstrated by in vivo studies that show intracerebral injection of recombinant IL-1RA reduces infarct volume, oedema formation and improves neurological deficits (Touzani et al., 1999; Rothwell, 2003).
1.6.1.2. Tumour necrosis factor-α

Expression of TNF-α in the ischaemic brain of rodents appears to be biphasic. Early expression of neuronal TNF-α within hours of cerebral ischaemia in rats parallels that of IL-1β (Huang et al., 2006). A second increase in TNF-α from microglia and infiltrating immune cells occurs 24 to 48 h later (Amantea et al., 2008). Although cerebral endothelial cells have not been shown to produce TNF-α during ischaemia-reperfusion injury, TNF-α produced by glial cells and infiltrating cells can induce endothelial cell adhesion molecule expression and production of chemokines (Nawroth et al., 1986; Feuerstein et al., 1994). TNF-α appears to have pleiotrophic actions in the ischaemic brain. TNF-α acts on two receptors, TNF receptor-1 (TNFR1)/p55 and TNF receptor-2 (TNFR2)/p75. p55 is associated with transducing the cytotoxic effects of TNF-α through interaction of its death domain with FAS associated death domain FADD and caspase-8 which initiates apoptosis (Vexler et al., 2006). Signalling through p55 increases infarct size and induces apoptosis. Inhibition of TNF-α with soluble TNF receptor or anti-TNF-α antibody reduces the extent of ischaemic brain injury (Allan and Rothwell, 2001). Neuroprotective actions of TNF-α have also been demonstrated that include anti-apoptotic functions and activation of glia to participate in repair processes. Contrary to the role of p55 in apoptosis, p55-deficient mice showed a greater degree of ischaemic injury compared with wildtype (WT) animals (Allan and Rothwell, 2001). It may be that the biphasic expression of TNF-α has differential temporal effects by acting on different cell types through various receptors over the course of ischaemia-reperfusion.

1.6.2. Cerebral chemokine generation

Chemokines are a family of small (8-10 kDa) chemoattractant cytokines classified into four different subfamilies (C, CC, CXC, CX3C) based on positions of cysteine residues (Velxer et al., 2006). Chemokines are released by activated endothelial cells and glia that bind and act through their complementary GPCRs expressed by leukocytes, initiating intracellular signalling cascades to direct leukocyte migration to the endothelium (Cinamon et al., 2001). Murine CXC chemokines include KC/CXCL1 (the murine homolog to rat cytokine-induced neutrophil chemoattractant; CINC and human Gro-α) and macrophage inflammatory protein-2 (MIP-2)/CXCL2 preferentially act as neutrophil chemoattractants by mediating their
actions through the chemokine receptor CXCR2, a GPCR expressed by neutrophils. CC chemokines include MCP-1/CCL2 that act on monocytes and macrophages through the CCR2 receptor expressed by these cell types (Dimitrivijevic et al., 2006).

Within hours of reperfusion (6-12 hours) after MCAo in rodents or oxygen-glucose deprivation (OGD) of endothelial-glial co-cultures, mRNA and protein levels of CINC, MIP-2 and MCP-1 are increased and persist for up to 24 hours. Elevated levels of these chemokines correlate with the migration of neutrophils and monocytes to the endothelium (Bona et al., 1999; Dimitrivijevic et al., 2006; Yamaski et al., 1997). Chemokines and their receptors are also postulated to be involved in regulation of BBB permeability during ischaemic insults. The MCP-1 receptor CCR2 is upregulated on the surface of endothelial cells and astrocytes following ischaemia-reperfusion. In CCR2 KO mice, a greater degree of BBB resistance was maintained with less fragmentation of tight junction proteins and reduced numbers of migrating monocytes compared to wild-type animals (Dimitrivijevic et al., 2006).

1.6.3. Cerebral matrix metalloproteinase production and their actions on the endothelium
MMPs are endopeptidases expressed as zymogens (pro-MMPs) that require activation through proteolytic processing of the pro-domain (Cunningham et al., 2005) Glia, neurones, endothelial cells, pericytes and neutrophils are all capable of upregulating different MMPs in response to inflammatory cytokines TNF-α, IL-1β and oxidative stresses induced during ischaemia (Sandoval and Witt, 2008). Of the MMPs, MMP-2 and MMP-9 have been shown to have prominent roles in breakdown of the BBB during ischaemic injury through degradation of the basal lamina and tight junction proteins. In addition to proteases released by neutrophils, MMPs cause gradual proteolysis of laminin-1, laminin-5, collagen type IV, fibronectin and perlecan of the basal lamina matrix (del Zoppo et al., 2003; Yang et al., 2007). Moreover, the products from degradation of certain matrix components act as chemotactic agents. For example, peptides generated from the breakdown of collagen by MMP-8 act as chemoattractants for granulocytes (del Zoppo, 2008). Pro-MMP-2 is activated by a membrane type-MMP (MT-MMP), both of which are upregulated by astrocytes and neurones within hours of ischemic injury (Yang et al.,
MMP-2 expressed by astrocyte foot processes is associated with fragmentation of claudin-5 and occludin that correlates with early opening of the BBB (Rosenberg et al., 1998; Yang et al., 2007). This early disruption to barrier integrity can be restored with application of MMP inhibitors (Cunningham et al., 2005; Yang et al., 2007). Pro-MMP-9 is expressed by ischaemic neurones, activated astrocytes, endothelial cells and neutrophils in rats 12 to 48 hours after MCAo (Rosenberg et al., 2001). Pro-MMP-9 is activated by MMP-3 expressed by activated microglia as well as NO and the serine proteases plasmin, tissue-type plasminogen activator and urokinase-type plasminogen activator (Rosenberg et al., 2001; Sandoval and Witt, 2008). Elevated expression of MMP-9 within the ischaemic hemisphere correlates with the secondary phase of BBB opening that leads to vasogenic oedema and haemorrhage (Rosenberg et al., 2001). Contrary to the deleterious effect of delayed MMP-9 expression on the BBB, sustained MMP-9 expression days after ischemia is associated with neurovascular remodelling (Sandoval and Witt, 2008; Zhao et al., 2006). Intraventricular injection of a MMP-9 inhibitor into rats reduced expression of genetic and protein markers of neurovascularisation, increased infarct volume and reduced behavioural outcome (Zhao et al., 2006). Thus MMPs may contribute to cerebral recovery following ischaemia.

1.6.4. Cerebral growth factor expression and their actions on the endothelium

During and after experimentally-induced ischaemia in vivo and in vitro, an upregulation in several growth factors including BNDF, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), GDNF, TGF-β and VEGF are documented (Croll et al., 2001). Under such conditions, several of these growth factors have a role in regulating neuronal survival and neurogenesis but also have direct and indirect effects on the cerebral endothelium. In particular, VEGF, angiopoietins and endothelins have vascular specific actions (Croll et al., 2001).

1.6.4.1. Vascular endothelial growth factor

VEGF is an angiogenic peptide upregulated following activation of HIF-1α (Schmid-Brunclik et al., 2008). Within hours of ischaemia, mRNA and protein levels
of VEGF are amplified and released, primarily from astrocytes, but also from microglia, neurones and pericytes (Sinor et al., 1998). VEGF acts on endothelial cells through VEGF receptor-1 (VEGFR-1/flt-1) and VEGF receptor-2 (VEGFR-2/KDR) expressed on the abluminal surface (Sandoval and Witt, 2008). Moreover, neuropilin-1, a receptor for semaphorins mediating outgrowth of neuronal axons has also been identified as a receptor for VEGF, in particular binding to the VEGF165 isoform (Zhang et al., 2001). VEGF appears to have biphasic actions over the course of ischaemia-reperfusion. In animal models of cerebral ischaemia, initial expression of VEGF has detrimental effects; enhancing vascular leakage, increasing infarct size and reducing neurological functional endpoints. Later expression of VEGF (48 h onwards from the onset of ischaemia) is associated cerebral recovery in these animal studies.

A number of experimental studies have demonstrated that upregulation of VEGF contributes to disruption of the BBB, and several mechanisms are proposed for VEGF-induced hyperpermeability. Narasimhan et al., (2009) identified OGD induced murine cerebral endothelial cell VEGF secretion and VEGFR1 expression. VEGF acts in an autocrine manner inducing ERK 1/2 activation in these cells that lead to apoptotic cell death during OGD. Several studies have demonstrated VEGF induces stress fibre assembly of cytoskeletal actin filaments, downregulates expression and disrupts pericellular location of occludin, claudins and ZO-1 in murine endothelial cell cultures and in the brains of mice after MCAo that correlate with increased paracellular permeability and neurological damage (Wang et al., 2001; Fischer et al., 2002; Argaw et al., 2009). Application of anti-VEGF antibody to porcine cerebral endothelial cells during hypoxia prevents alterations to ZO-1 localisation, restoring barrier integrity (Fischer et al., 2002). Whether downregulation of tight junction proteins is due to degradation or reduced synthesis remains to be elucidated. VEGF-induced disruption of tight junction proteins may be via NO that lies downstream of VEGF signalling pathway (Fischer et al., 1999). VEGF induces release of endothelial and astrocyte MMP-2 and MMP-9 in mice studies of cerebral ischaemia (Sandoval and Witt, 2008). VEGF is also associated with a reduction in the number of pericytes in contact with the abluminal surface of the endothelium during the process of angiogenesis (Greenberg et al., 2008) which may contribute to perturbation of BBB integrity.
While early hypoxic induction of VEGF has been shown to be deleterious, delayed induction of VEGF is associated with angiogenesis which is critical for the survival of damaged tissue (Sandoval and Witt, 2008). VEGF may also exert anti-apoptotic actions. Hypoxic induction of VEGF from primary rat astrocytes reduced caspase-8 activity and subsequent apoptosis of the rat endothelial cell line, RBE4 cells (Chow et al., 2001).

Expression of VEGF receptors under ischaemic conditions differs between different cell types. VEGFR1 is upregulated on cerebral endothelial cells whereas VEGFR2 remain at basal levels (Fischer et al., 2002). By contrast, neurones constitutively express VEGFR2 and neuropilin-1 under normal and ischaemic conditions but do not express VEGFR1 (Jin et al., 2000). Such variations in receptor expression may reflect the differential biphasic effects of VEGF over the course of ischaemia-reperfusion. Further to this, neuropilin-1 expression on endothelial cells and astrocytes at the border of ischaemic lesions is enhanced. The role of neuropilin-1 in ischaemia is controversial. Neuropilin-1 expression has been correlated with areas undergoing neovascularisation (Zhang et al., 2001). By contrast, other studies show no neuroprotection following neuropilin-1 activation (Jin et al., 2000).

1.6.4.2. Angiopoietin

Angiopoietins (Ang) are involved in vascular development and maturation. Ang-1 and Ang-2 are agonist and antagonist respectively at the Tie2 receptor expressed at a low level predominantly on the endothelium (Beck et al., 2000). From the onset of cerebral ischaemia, Ang-2 mRNA is upregulated in endothelial cells within the infarct region (Croll et al., 2001). Ang-2, together with the early expression of VEGF are thought to contribute to destabilisation and leakage of the BBB during ischaemia (Croll et al., 2001). Upregulation in Ang-1 mRNA expression in astrocytes appears 48 to 72 hours after cerebral ischaemia that coincides with restoration of BBB integrity, neovascularisation and angiogenesis (Beck et al., 2000; Croll et al., 2001). Amplification in Tie2 expression on the endothelium in peri-infarct regions correlates to that of Ang-1 upregulation and remains elevated for days (Croll et al., 2001). Ang-1 reduces cerebral vascular leakage caused by exogenous VEGF administered to mice following MCAo (Zhang et al., 2002). Similarly, mice treated
with adenovirus expressing Ang-1 show reduced disruption to the BBB and infarct volumes following MCAo (Zhang et al., 2002).

1.6.4.3. Endothelins

Cerebral ischaemia, as well as other cerebrovascular disorders such as subarachnoid haemorrhage, is associated with a rise in plasma endothelin levels (Ziv et al., 1992). Endothelins are endothelium derived vasoconstrictors. The main isoform, ET-1 synthesised by endothelial cells. ET-3 is produced by neurones. Endothelins act on the endothelin receptors ET\textsubscript{A} expressed by endothelial cells and ET\textsubscript{B} is associated with neurones, suggestive of a role of endothelin in modulating neurological functions (Reid et al., 1995). Acute stroke patients have increased plasma and CSF levels of ET-1 (Ziv et al., 1992; Yang et al., 2007). Elevated levels of ET-1 are correlated with increasing severity of neurological impairment in patients after ischaemic stroke (Ziv et al., 1992). Vasoconstriction induced by ET-1 under ischaemic conditions further reduces cerebral blood flow, aggravating the severity of ischaemia. Increased ET-1 levels may be due to a combination of an amplification in synthesis and passive leakage from damaged endothelial cells (Ziv et al., 1992; Cardell et al., 1994). Several stimuli induce ET-1 synthesis including products derived from activated platelets, IL-1, hypoxia/anoxia and low shear stress (Cardell et al., 1994). An in vitro model of the BBB using human brain microvascular endothelial cell-astrocyte co-cultures demonstrated TNF-\alpha stimulation of endothelial cells induced endothelial release of ET-1 which correlated with an increase in endothelial paracellular permeability. Concomitantly, ET-1 induced production of IL-1 by astrocytes which was attributed to the increase in permeability as anti-IL-1\beta attenuated this effect (Didier et al., 2003). Similarly, the role of ET-1 in contributing to perturbations in BBB permeability is shown in mice overexpressing ET-1 on astrocytes. These mice exhibit decreased occludin expression, increased disruption to the BBB concomitant with increased brain water content and infarct volumes and more severe neurological defects (Lo et al., 2005; Yang et al., 2007).
1.7. Experimental models of cerebral ischaemia

Cerebral ischaemia is a multifaceted disorder. Clinical variability in the outcome of injury reflects differences in duration of ischaemic insult, the region of brain affected, differences between individuals and coexisting systemic diseases. Thus there is no single model that is representative of all features of cerebral ischaemia. Nevertheless, pre-clinical *in vivo* animal studies and *in vitro* cellular studies are essential for the effective translation of therapies into the clinic.

1.7.1. *In vivo* models of cerebral ischaemia

*In vivo* studies are usually performed on rodents or non-human primates, although rodents are considered to be more ethically favourable compared to higher mammals, are less expensive and rodents have similar vascular anatomy to that in humans (Macrae, 2011). A common *in vivo* model of ischaemic stroke is occlusion of the middle cerebral artery, MCAo. Permanent focal MCAo in rodents can be achieved by electrocoagulation. However, this method requires a high level of surgical skill to be able to apply the electric current to coagulate the blood without damaging the surrounding tissue. *In vivo* models of temporary focal ischaemia include inserting a filament inside the middle cerebral artery, clipping or tying a ligature around the artery. An advantage of this model is that the occluding filament, ligature or clip can be left in place for permanent interruption of blood flow or removed at any time for temporary occlusion, allowing subsequent reperfusion. Occlusion of the middle cerebral artery can also be achieved by intracerebral injection of long-lasting vasoconstrictors such as ET-1 (Agnati et al., 1991). The vasoconstrictor property of ET-1 irreversibly occludes the middle cerebral artery. This model has been reported to induce similar ischaemic damage to that induced by MCAo using a filament in rats (Sharkey et al., 1993).

1.7.2. *In vitro* models of cerebral ischaemia

Although *in vivo* studies of cerebral ischaemia allow responses of whole tissue and functional and behavioural parameters to be assessed, these studies are time-consuming and it is difficult to study individual cellular responses to ischaemic injury. *In vitro* studies allow detailed insight into cell specific responses and mechanistic processes within a controlled environment. Ideally, these models should
closely mimic ischaemic conditions that occur in humans and reflect the anatomical environment of the brain. A common in vitro model of cerebral ischaemia is hypoxia or combined oxygen and glucose deprivation, OGD to mimic the reduction in oxygen, nutrients and metabolites that occur during cerebral ischaemia. Physiological levels of tissue oxygen ranges between 12-2 % (80-20mmHg) depending upon the tissue type (Sitkovsky and Lukashev, 2005). The brain in particular is a high consumer of oxygen, with the gray matter consuming as much as 94 % of total cerebral oxygen. Pathophysiological levels of oxygen occur when oxygen supply is insufficient to meet the requirement of the tissue, associated with loss of cellular function and adaptation to low oxygen environments through induction of hypoxic-regulated genes. Thus in vitro studies typically expose cultured cells to <2 % oxygen. Reintroduction of cultures to normoxia and glucose and serum-containing media allows investigation of cellular responses to in vitro simulated reperfusion.

In recent years progress has been made towards the development of cell lines and reproducible methods for isolating and culturing primary neurones, glia and cerebral endothelial cells. Hypoxia or OGD with or without reperfusion are typically performed on cell lines or primary cells derived from a variety of different species, including, murine, rat, porcine, bovine and sheep. These studies often use a Transwell system to reproduce the BBB in which brain endothelial cells are grown inside the Transwell insert with glia, pericytes, or neurones grown on the direct underside of the insert or on the culture plate below the insert.

One drawback of the Transwell system is the absence of shear flow. More recently, dynamic in vitro BBB models (DIV-BBB) comprising hollow fibre capillary systems that can be exposed to intraluminal flow have been developed (Cucullo et al. 2002). Endothelial cells cultured under flow conditions exhibit higher TEER values (approximately 1000 Ohm.cm$^2$) compared to endothelial cells grown under static conditions (approximately 600 Ohm.cm$^2$) (Siddharthan et al., 2007; Cucullo et al., 2008). TEER of cultured endothelial monolayers is derived from Ohm’s law ($V = IR$). The electrical resistance of the monolayer is multiplied by the surface area of the Transwell filter and thus independent of the surface area, allowing comparisons between different investigators growing endothelial monolayers on Transwells of different sizes. Further modifications of this dynamic capillary model involve the

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presence of micropores within the capillary tubes making them suitable for immune cell migration studies (Cucullo et al., 2010).

A limitation of the *in vitro* models described above is that the process of isolating and culturing cerebral cells can alter morphological and functional characteristics of the cells. This has led to the use of organotypic slice cultures. Duport et al. (1998) first described organotypic slice cultures as a valid model for studying the BBB from electrophysiological recordings of neuronal activity and electron microscopy for endothelial permeability. More recent *in vitro* studies have exposed organotypic slice cultures to OGD to study the pathophysiology of the cellular components of the neurovascular unit under such conditions (Zehendner et al., 2009).

1.8. Summary

For many years, research into cerebral ischaemia focused on the identification of therapeutic targets that preserved the function of neurones. However, experimental studies have demonstrated multiple and overlapping responses of cerebral endothelial cells, glia and cerebral neurones as well as infiltration of circulating immune cells and blood-borne factors to *in vivo* and *in vitro* models of cerebral ischaemia. Such studies have identified several mediators that can exacerbate injury and/or exert beneficial effects. Thus, the responses and protection of the neurovascular unit as a whole must be considered when identifying potential targets for therapeutic intervention after ischaemic injury. However, the temporal and spatial profile of these responses and interactions between individual cell types of the neurovascular unit during ischaemic injury are still unclear, emphasising the need for further research into this area. Accordingly, this thesis aimed to gain further insight into the inflammatory responses of the cerebral endothelial cells and glial cells to ischaemia *in vitro*.
1.9. Hypothesis
That oxygen-glucose deprivation induces inflammatory activation of the cerebral endothelial cells and glial cells and that cross-talk between these cells regulates their inflammatory responses.

1.10. Objectives
1. To establish a reproducible in vitro oxygen-glucose deprivation model (OGD) involving cerebral endothelial cells and glial cells. This will include identifying the duration of OGD exposure and severity of oxygen deprivation to study endothelial and glial responses.

2. To identify inflammatory responses of mouse brain endothelial cells (MBECs) and glia to OGD by assessment of the expression and secretion of specific cytokines, chemokines, growth factors and cell adhesion molecules and of the regulation of neutrophil transendothelial migration.

3. By the use of MBEC-glial co-cultures to identify whether cross-talk between MBECs and glia regulate their inflammatory responses to OGD.
Chapter 2

Materials and methods
2. Materials and methods

2.1. Animals

Primary mouse brain endothelial cell cultures and murine mixed glial cell cultures were prepared from C57BL/6J mice (Harlan Laboratories, UK). Animals were kept in a 12 h light/dark cycle at 21±1 °C. All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986.

2.2 Cell culture

2.2.1. Primary mouse brain endothelial cells (MBECs)

24 h prior to the isolation of endothelial cells, 24-well plates (Corning) were coated with 50 µg/mL collagen IV (VWR International, UK). Plates were stored at 4 °C until use.

Primary mouse brain endothelial cells (MBECs) were isolated and cultured by adaptation of a protocol by Song and Pachter (2003). Briefly, four to eight week old C57BL/6J mice were euthanised by rising concentration of CO₂ (Schedule one, Animals (Scientific Procedures) Act, 1986). Craniotomy was performed and brains were kept in Dulbecco’s modified Eagle’s medium (DMEM) on ice. Cortices were cleared of surface microvessels. The cerebellum, optical lobes and white matter were removed and cerebral hemispheres were minced with a 10 mm scalpel blade for five min. The minced tissue was homogenised with a 7 mL dounce homogeniser with 30 strokes of the loose pestle (0.04 mm clearance) followed by 25 stokes of the tight pestle (0.07 mm clearance). The homogenate was centrifuged (Sigma 2-16PK centrifuge, Sigma Laborzentrifugen, Germany) 200 g for five min. The pellet was resuspended in 15 mL 18 % (w/v) dextran solution (60,000-90,000 Daltons) and centrifuged 3893 g for ten min. In an adaptation to the preceding protocol, the thick myelin layer was removed and resuspended in 15 mL 18 % (w/v) dextran solution and the centrifugation step repeated. This second centrifugation step retrieved more microvascular fragments. Pellets were resuspended in 1 mL Ca²⁺ and magnesium (Mg²⁺)-free Hank’s balanced salt solution (HBSS, Invitrogen, UK). The suspension was filtered through a 75 µm nylon mesh filter. Microvessels retained on the filter were removed by washing with 0.5 mL HBSS nine times into a glass Petri dish. To degrade extracellular matrix and free endothelial cells from perivascular cells, the
preparation underwent enzymatic digestion by addition of 750 U/mL DNase (Invitrogen, UK), 50 µg/mL collagenase-dispase (Roche, UK) and 50 µg/mL of the protease inhibitor tosyllysine chloromethyl ketone (Sigma Aldrich, USA). In contrast to Song and Pachter’s (2003) 90 min enzyme digestion, to ensure enzyme digestion did not proceed to the single-cell level that would have resulted in poor endothelial cell viability, the digestion step was only allowed to continue for up to 20 min at 37°C in a humidified 5 % carbon dioxide (CO₂) incubator with occasional agitation. The vessel fragments were then centrifuged at 800 g for 7 min. The pellet was resuspended in endothelial cell maintenance medium (DMEM/F-12 supplemented with 10 % plasma-derived horse serum (PDS, First Link Ltd. UK), 10 % foetal calf serum (FCS, PAA Laboratories, UK) 1 % penicillin-streptomycin (P/S, Sigma Aldrich, UK), 1 % glutamine (Sigma Aldrich, UK), 100 µg/mL heparin (Sigma Aldrich, UK) and 100 µg/mL endothelial cell growth supplement (VWR International, UK) supplemented with 3 µg/mL puromycin (Sigma Aldrich, UK). Collagen IV-coated tissue culture plates were washed twice with phosphate buffered saline (PBS) and cells were seeded on these collagen IV-coated plates. After two days in vitro (DIV), medium was replaced with fresh endothelial cell maintenance medium, and every four days thereafter. After about 14 DIV endothelial cells reached confluency.

2.2.2. Primary murine cortical mixed-glial cell cultures
Two to three day old C57BL/6J mice pups were decapitated and heads were pined to a wax Petri dish. The skin and skull were cut to expose the brain. The brains were removed and stored in DMEM on ice. To remove meninges, brains were rolled on sterile filer paper and placed in 10 mL glial maintenance medium (DMEM supplemented with 10 % FCS and 1 % P/S). Tissue was aspirated with a 10 mL and 5 mL stripette and then centrifuged (Sigma 2-16PK centrifuge) at 155 g for 10 min. The pellet was resuspended to desired volume in glial maintenance media. Cells were seeded on uncoated 24-well tissue culture plates (Corning) at a density of 2 cortical hemispheres per 48cm² and grown in a humidified 5 % CO₂ incubator at 37 °C. After five DIV a full medium change was performed and every four days thereafter. After about 12 DIV cells reached confluency.
2.2.3. Primary murine endothelial-glial cell co-cultures

For co-culture experiments, MBECs were cultured on collagen IV coated 0.4 μm pore (1x10^8 pores/cm²) polyethylene terephthalate (PET) Transwell® inserts (VWR International, UK) and glia were cultured in 24-well plastic tissue culture plates and grown to confluency. 24 h prior to experiments, Transwells were transferred the 24-well plates containing glia. Figure 2.1 illustrates this co-culture set-up.

![Figure 2.1. Schematic of MBEC-glial co-cultures. MBECs were grown in collagen IV coated 24-well PET transwell inserts. Glial cells were grown in 24-well tissue culture plates. For co-culture experiments, Transwell inserts were transferred to the wells containing glia.](image)

2.2.4. Murine bone marrow-derived neutrophils

Neutrophils were isolated from the bone marrow of adult C57BL/6J mice. Femurs and tibias were removed and bone marrow was flushed with neutrophil buffer (PBS containing 0.1 % bovine serum albumin (BSA), 1 mM EDTA, 0.22 μm filtered) using a 25G needle. The bone marrow suspension was aspirated with a 19G needle to dissociate larger bone marrow pieces and then centrifuged (Sigma 2-16 PK centrifuge) at 400 g for five min. Red blood cells were lysed with 3 mL 0.2 % NaCl followed by restoration of osmolarity with 7 mL 1.2 % NaCl and then passed through a 30 μm filter to remove debris and red blood cell clots. The filtered suspension was centrifuged at 400 g for five min. The pellet was resuspended in 200 μL neutrophil buffer and 50 μL anti-Ly6G antibody (Miltenyi Biotec, UK) and incubated for 10 min at 4 °C. A further 150 μL neutrophil buffer and 100 μL anti-biotin microbeads (Miltenyi Biotec, UK) were added to the suspension and incubated for a further 15 min. The suspension was washed with 5 mL neutrophil buffer to dissolve the Ly6G-biotin conjugated microbeads and centrifuged at 400 g for five min. The pellet was resuspended in 500 μL neutrophil buffer. A chilled magnetic MACS® LS column (Miltenyi Biotec, UK) was washed with 3 mL neutrophil buffer and resuspended
cells were applied to the column. Unlabelled cells passed through the column. The LS column was removed from the magnet and the retained Ly6G-labelled cells were eluted by adding 5 mL neutrophil buffer and applying a plunger. The number of neutrophils was counted using a haemocytometer and resuspended to 4x10^6 cells/mL in neurobasal medium (Invitrogen, UK) supplemented with 1 % P/S and 1 % glutamine.

To assess purity of neutrophils in the final purified fraction fluorescent activated cell sorting (FACS) analysis was performed. Cells were incubated with Fc block (anti-Ms CD16/32, eBioscience, UK) at 4 °C for 30 min. Cells were washed with FACs buffer (PBS + 0.1 % low endotoxin BSA, 0.05 % sodium azide). Cells were incubated with a combination of the following antibody mixtures: 1. Anti-mouse Ly6G-PE, anti-mouse CD11b-FITC and anti-mouse-CD14-APC. 2. Anti-mouse Ly6G-PE, anti-mouse CD19-FITC and anti-mouse MHC class II-APC. FACS analysis was performed on a Beckman Coulter Cyan (Beckman Coulter, UK) (at least 2000 counts per sample). Granulocytes were identified on a dot plot by forward and side-scatter characteristics. The neutrophil subpopulation was identified within this granulocyte population by gating within a CD11b-FITC vs. Ly6G-PE dot plot.

2.3. In vitro oxygen-glucose-deprivation (OGD)
OGD was used as an in vitro model of cerebral ischaemia. Confluent monolayers of MBECs and glia in monoculture and in co-culture were subjected to OGD by incubation in OGD medium (glucose-free DMEM supplemented with 1 % P/S) in a temperature controlled (37±1 °C) hypoxic chamber (Coy Laboratories, Michigan, USA) for 4, 6, 10, 18 or 24 h. The OGD medium was deoxygenated prior to the addition to cells by purging with N₂ until oxygen levels reached 1 % as monitored using a digital MI-730 O₂ electrode (Microelectrodes Inc., USA). The entire chamber was purged with 94 % nitrogen (N₂), 5 % CO₂ and 1 % O₂. All media changes were performed inside the chamber. Cells were washed twice with OGD medium to remove excess culture medium before incubation. Oxygen levels of the OGD medium was measured at regular time points over the course of OGD using the MI-730 O₂ electrode. Control cultures were kept in glucose-containing DMEM (4.5 g/L glucose) supplemented with 1 % P/S at 37 °C in a humidified 5 % CO₂ incubator. After a specified duration of OGD, conditioned medium and cell lysates were
collected. Reperfused cultures were removed from the hypoxic chamber and the medium was replaced with glucose-containing DMEM (4.5 g/L glucose) supplemented with 10 % FCS and 1 % P/S and placed in a humidified 5 % CO₂ incubator at 37 °C for 24 h. At the end of the reperfusion period, conditioned medium and cell lysates were collected.

2.4. Cell imaging

2.4.1. Light microscopy
To assess growth of MBEC and glial cultures, cells were viewed with an Olympus CKX31 microscope (Olympus, UK). Images were taken using a Moticam 2300 camera (Motic, UK) with Motic Images Plus version 2.0 software. Cultures were used once they reached confluency.

2.4.2. Immunocytochemistry
MBECs and glia were cultured on 24-well glass coverslips. Cells were fixed with 4 % paraformaldehyde-sucrose for 1 h at room temperature. Cells were permeabilised with 0.1 % Triton-X followed by quenching with 0.25 % ammonium chloride. Cells were blocked in 5 % BSA-PBS for 1 h at room temperature. Cells were then incubated with primary antibody (see Table 3, Appendix I) diluted in 1 % BSA-PBS for 1 h at room temperature. The cells were washed three times in PBS and then incubated with corresponding secondary antibody (see Table 3, Appendix I) for 1 h at room temperature. Cells were washed three times with PBS and once in distilled water. Coverslips were mounted onto glass microscope slides using ProLong® Gold mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, UK). Cells were visualised on an Olympus BX51 fluorescence microscope (Olympus, UK) and images were collected using a Coolsnap ES camera (Photometrics, UK) and processed using MetaVue software (Nikon, UK).

2.5. Analysis of MBEC and glial cell death

2.5.1. Lactate dehydrogenase assay
The CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, USA) was used to measure lactate dehydrogenase (LDH) release into the culture medium after OGD and reperfusion. LDH is a stable cytosolic enzyme that is released from cells
upon cell lysis. Released LDH is coupled with a 30 min enzymatic reaction that results in the conversion of a tetrazolium salt (2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyltetrazolium; INT) to a red formazan product. The amount of colour produced is proportional to the number of lysed cells, with absorbance measured at 490 nm using a spectrophotometer (Synergy HT, NorthStar Scientific Ltd., UK). At the end of OGD and reperfusion, 50 µL of conditioned medium was removed from cells and transferred to a 96-well plate. 50 µL of reconstituted substrate mix was added to these wells and incubated in the dark for 30 min. The reaction was stopped with addition of 50 µL 0.1 M acetic acid. LDH release was expressed as a percentage of total release induced by 10x lysis solution that induces total cell death.

### 2.5.2. Trypan blue exclusion

At the end of indicated OGD timepoints and reperfusion, trypan blue solution (0.4 %) (Sigma Aldrich, UK) was added to the cell culture medium in a 1:10 dilution. After two min incubation the medium was removed from cells and cellular uptake of trypan blue was visualised using the Olympus CKX31 microscope. Cells that excluded trypan blue were considered to be viable.

### 2.6. OGD-reperfusion induced MBEC and glial activation

#### 2.6.1. Preparation of whole cell lysates

To obtain whole cell lysates, at indicated periods of OGD and following reperfusion, culture media were discarded and cells were scraped into ice cold triton lysis buffer (50 mM Tris-HCl pH 7.5, 1 % Triton X-100, 1x protease and phosphatase inhibitor cocktail (Calbiochem, Merck, Germany). Protein concentrations in the lysates were quantified using a bicinchoninic acid protein (BCA) protein assay (Thermo Fischer Scientific, UK).

#### 2.6.2. Preparation of nuclear and cytosolic lysates

Nuclear extracts were prepared by scraping cells into ice cold buffer A (see Appendix I) and centrifuged at 18,000 g for 10 min at 4 °C. Pellets were resuspended in 40 µL of buffer A2 (Appendix I) and left on ice for 10 min, after which, suspensions were centrifuged at 18,000 g for 10 min at 4 °C. Cytosolic supernatants were collected and pellets were resuspended in 30 µL buffer B (Appendix I) and left
on ice for 15 min. After centrifugation at 18,000 g for 10 min at 4 °C, 40 µL buffer C (Appendix I) was added to the suspension and stored at -20 °C until use. Protein concentrations in the nuclear and cytosolic lysates were quantified using a BCA assay.

2.6.3. Bicinchoninic acid protein assay
Cell lysates were diluted 1 in 10 in PBS 20 µL of cell lysates were incubated with 200 µL BCA protein assay reagent (Thermo Scientific, UK) for 30 min at 60 °C. Samples were read at 570 nm. Protein concentrations were calculated against a standard curve for BSA.

2.6.4. Western blot
Whole cell, nuclear and cytosolic lysates where mixed 1:5 with 5x sample buffer (see Appendix I) under reducing conditions (sample buffer supplemented with 5 % β-mercaptoethanol). Lysates were separated on 4-12 % SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membrane (PDVF, Scientific Laboratory Supplies Ltd., UK). Membranes were blocked with 10 % non-fat milk in PBS-0.1 % Tween-20 for 1 h at room temperature. Membranes were incubated overnight with primary antibody diluted in 1 % BSA/PBS-0.1 % Tween-20 at 4 °C. The following primary antibodies were used: rabbit anti-cleaved caspase-3 antibody (1:1000 dilution, Cell Signalling Technology, USA), rabbit anti-GLUT-1 antibody (1:500 dilution, Abcam, UK), rabbit-anti-HIF-1α antibody (1:500 dilution, Novus Biologicals, USA), rabbit-anti-NFκB p65 antibody (1:200 dilution, Santa Cruz, USA), goat anti-ICAM-1 antibody (1:300 dilution, R and D Systems, UK) and goat anti-VCAM-1 antibody (1:250 dilution, Rand D Systems, UK). Loading volumes were normalised against anti-β-actin peroxidise (1:1500 dilution, Sigma Aldrich, USA). Membranes were washed four times with PBS-0.1 % Tween-20 and incubated with appropriate horseradish peroxidise-conjugated secondary antibody (Dako Ltd., Denmark) diluted 1:1000 in 5 % non-fat milk/PBS-0.1 % Tween-20 for 1-3 h at room temperature. Membranes were developed by reaction with ECL chemiluminescent substrate (GE Healthcare, UK). Densitometric analysis of blots using Northern Eclipse software (Empix Imaging Inc. Canada) was performed to quantify expression levels expressed as a fold increase over control levels.
2.6.5. Enzyme-linked immunoabsorbent assay (ELISA)

ICAM-1 and VCAM-1 expression by MBECs and KC and VEGF expression and release into the culture medium by MBECs and glia was measured by ELISA (R and D Systems, UK). 96-well plates (Nunc-immunoplate, maxisorp plates, Sigma Aldrich, USA) were coated with capture antibody diluted in PBS overnight at room temperature. Plates were washed three times with wash buffer (0.05 % Tween-20 in PBS). To prevent non-specific binding, plates were blocked with 1 % BSA-PBS for 1 h at room temperature. Plates were washed 3 times with wash buffer and standards and samples were added to plates in duplicate and incubated at room temperature for 2 h. Plates were washed three times with wash buffer and incubated with detection antibody diluted in reagent diluent (1 % BSA-PBS) for 2 h at room temperature. Plates were washed three times with wash buffer and incubated with streptavidin horseradish peroxidase diluted 1:200 in reagent diluent for 20 min in the dark. Following three washes with wash buffer, plates were incubated with substrate reagent for 20 min in the dark. The reaction was stopped with the addition of 1 M H$_2$SO$_4$. Plates were read at 490 nm and 570 nm using a spectrophotometer (Synergy HT, NorthStar Scientific Ltd., UK) and optical densities taken at corrected wavelength (450 nm-570 nm) to account for optical imperfections of the plate.

2.6.6. Cytometric bead array (CBA) assay

A 12-plex mouse Cytometric Bead Array (CBA) (BD Biosciences, UK) was performed on co-cultured MBEC and glia conditioned media and lysates following 4h, 10h and 24 h OGD ± 24 h reperfusion for the simultaneous detection of the following cytokines and chemokines: TNF-α, RANTES, MCP-1, IL-6, IL-1α, IL-1β, IL-17α, IL-10, IFN-γ, G-CSF, CD62L, CD62E. CBA was performed according to manufacturer’s instructions. Briefly, samples were diluted 1:10 in assay diluent. Standard curves for each cytokine and chemokine was prepared. Capture beads were diluted in capture bead diluent and Phycoerythrin (PE) detection reagent was diluted in detection reagent diluent. 50 µL capture beads were added to 96-well plates. 10 µL of standards and samples were added to wells containing capture beads and incubated for 1 h at room temperature. 50 µL PE detection reagent was added to each well and incubated for 1 h at room temperature. 100 µL wash buffer was added to
Each well and shaken at 500 g for 5 min to resuspend beads. Samples were acquired on a BD FACSArray® bioanalyser (BD Biosciences).

2.7. Neutrophil studies

2.7.1. Neutrophil transendothelial migration

MBECs were grown on collagen IV-coated 3 μm pore (2x10^6 pores/cm²). polycarbonate Transwell inserts (Corning). MBEC monocultures and MBEC-glia co-cultures were exposed to 4, 10 and 24 h OGD. Control co-cultures were kept in glucose-containing DMEM (4.5 g/L glucose) supplemented with 1 % P/S at 37 °C in a humidified 5 % CO₂ incubator. At the start of reperfusion immediately at the end of OGD, 2x10^5 neutrophils (see section 2.2.4.) were applied to the MBECs in transwell inserts. At the end of 24 h reperfusion, the media in the abluminal compartment was collected and centrifuged at 18,000 g for 5 min. The pellet was resuspended in 40 μL DMEM and the number of neutrophils were counted using a hemacytometer.

2.7.2. Neutrophil adhesion

To quantify the number of neutrophils adhered to MBECs at the end of reperfusion, Transwells were washed twice with DMEM and fixed with 4% paraformaldehyde-sucrose. Membranes were cut away from the Transwell and processed for immunocytochemistry as outlined in section 2.4.2. The endothelium was stained for ICAM-1 and neutrophils were stained with SJC-4 (kindly provided by Dr. Daniel Anthony, University of Oxford, UK). The number of SJC4-positive cells per field of view were counted.

2.7.3. Mechanisms of neutrophil transendothelial migration

2.7.3.1. Treatment of MBECs, glia and neutrophils

To investigate the mechanisms of neutrophil transendothelial migration during reperfusion, MBECs, glia and neutrophils were treated with blocking antibodies and inhibitors at the start of reperfusion (see table 1). Neutrophil migration was quantified at the end of reperfusion as outlined in section 2.7.1.
<table>
<thead>
<tr>
<th>Cell type treated</th>
<th>Treatment</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBECs</td>
<td>Anti-ICAM antibody</td>
<td>10 µg/mL</td>
<td>R and D Systems</td>
</tr>
<tr>
<td></td>
<td>Anti-VCAM antibody</td>
<td>10 µg/mL</td>
<td>R and D Systems</td>
</tr>
<tr>
<td></td>
<td>Anti-CD31 (PECAM) antibody</td>
<td>25 µg/mL</td>
<td>R and D Systems</td>
</tr>
<tr>
<td></td>
<td>Anti-KC antibody</td>
<td>5 µg/mL</td>
<td>R and D Systems</td>
</tr>
<tr>
<td></td>
<td>IL-1Ra</td>
<td>100 ng/mL</td>
<td>Amgen, USA</td>
</tr>
<tr>
<td></td>
<td>Mouse IgG₁ isotype control</td>
<td>10 µg/mL</td>
<td>R and D Systems</td>
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<tr>
<td></td>
<td>Mouse IgG₂A isotype control</td>
<td>25 µg/mL</td>
<td>R and D Systems</td>
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<tr>
<td></td>
<td>BAPTA-AM</td>
<td>20 µM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Glia</td>
<td>Anti-KC antibody</td>
<td>5 µg/mL</td>
<td>R and D systems</td>
</tr>
<tr>
<td></td>
<td>Anti-VEGF antibody</td>
<td>10 µg/mL</td>
<td>R and D Systems</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Anti-CD18 antibody</td>
<td>10 µg/mL</td>
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</tr>
<tr>
<td></td>
<td>Rat IgG1κ isotype control</td>
<td>10 µg/mL</td>
<td>BD Biosciences</td>
</tr>
</tbody>
</table>

Table 1. Treatment of MBECs, glia and neutrophils at the start of reperfusion to investigate mechanisms of neutrophil migration.

### 2.7.3.2. Heat inactivated glial conditioned media

To determine whether soluble glial-derived mediator(s) contributed to neutrophil migration, reperfused glia conditioned medium was heat inactivated. Reperfused glial conditioned media following 4 h and 24 h OGD were heated to 95°C for 30 min. At the start of reperfusion 600 µL of heat inactivated glial conditioned medium was added to the abluminal side of transwells. 2x10⁵ neutrophils were added to Transwells at the start of reperfusion and the number of migrated neutrophils was quantified as outlined in section 2.7.1.

### 2.7.3.3. Molecular weight cut-off glial conditioned media

To determine the size of the soluble glial-derived mediator(s) contributing to neutrophil migration, fractions of reperfused glial conditioned medium of >50 kilodalton (kDa), 50-10 kDa, 10-3 kDa and <3 kDa were generated. Glia were exposed to 4 h or 24 h OGD with 24h reperfusion. This reperfused conditioned medium was applied to 50 kDa, 10 kDa and 3 kDa Amicon Ultra-0.5 centrifugal filters (Millipore, UK) and centrifuged (Hettich Mikro 200R Microlitre centrifuge,
Wolf Laboratories, UK) at 14000 g for 10-30 min. Filtrates represented molecular weight cut-off (MWCO) fractions of <50kDa, <10kDa and <3kDa. To obtain a fraction of >50 kDa, the 50 kDa filter was inverted and centrifuged for 1000 g for two minutes. MBECs were exposed to OGD and at the start of reperfusion MWCO filtrates were applied abluminally to MBECs in Transwell inserts. 2x10^5 neutrophils were added to Transwells at the start of reperfusion and migration was quantified 24 h later.

2.7.3.4. Neutrophil-induced endothelial paracellular permeability

To investigate whether neutrophil migration was associated with changes in MBEC paracellular permeability, MBEC permeability to 4 kDa fluorescein isothiocyanate (FITC) labelled dextran during neutrophil migration was measured. 2.5 µM FITC-dextran was added to Transwells at the start of reperfusion together with the addition of neutrophils. At the end of 24 h reperfusion, 100 µL of medium from the abluminal compartment was removed and transferred to an opaque 96-well plate. Fluorescence was determined on a spectrofluorometer at 485 nm (emission) and 528 nm (emission) and compared to a standard curve of known concentrations of FITC-dextran. Apparent permeability ($P_{app}$) was calculated using the following equation:

$$P_{app} (\text{cm.sec}^{-1}) = \frac{dc}{dt} \times \frac{V}{A \times C_0}$$

Where:

dc= change in FITC-dextran concentration (µM)
dt= change in time (seconds)
V= volume of receiver (mL)
A= surface area of Transwell insert (cm²)
C₀= initial concentration of FITC-dextran added to donor compartment (µM)

2.8. Statistical analysis

Data were expressed as mean (± standard error of mean (SEM)) and analysed using GraphPad Prism version 5.0. Comparisons between two groups were analysed using Student’s t-test. Groups of three or more with one variable were analysed using one-
way analysis of variance (ANOVA) with Tukey’s multiple comparison test. Groups of three or more with two variables was analysed using two-way ANOVA with Bonferroni post hoc test. P<0.05 was considered to be statistically significant.
Chapter 3
Development of an *in vitro* model of cerebral ischaemia
3. Development of an *in vitro* model of cerebral ischaemia

3.1. Introduction

Understanding cellular responses and molecular mechanisms in ischaemic injury is fundamental for the identification of potential targets for intervention. *In vivo* modelling of cerebral ischaemia is often considered a more favourable option over *in vitro* models owing to their greater physiological relevance. However it is difficult to study responses of individual cell types to such injury. *In vitro* studies allow cell-specific responses and mechanistic processes to be studied in detail within a controlled environment. A combination of oxygen and glucose deprivation (OGD) is used commonly to mimic the reduction in oxygen, nutrient and metabolite supply that occurs during cerebral ischaemia. OGD models differ between the species used, type of ischaemic insult, duration of exposure and occurrence of reperfusion depending on the intended aims of individual studies.

Using *in vitro* BBB models, it is possible to study the developmental, physiological and pathological processes of the BBB. Modelling the BBB *in vitro* commonly utilises a Transwell system in which brain endothelial cells are grown on a semi-permeable membrane of the transwell insert. However brain endothelial cells grown in isolation generally lack cellular and functional characteristics of the BBB including poorly developed tight junctions resulting in high permeability coefficients. Glial cells and pericytes have been shown to induce BBB properties of endothelial cells (Berezowski et al., 2004; Abbott et al., 2006). Therefore co-cultures of endothelial cells with glia and pericytes are now commonly used. Glia and pericytes are cultured either on the direct underside of the transwell membrane or on the culture plate below the transwell insert forming a direct contact co-culture or a non-contact co-culture respectively. Tri-cultures involving endothelial cells and pericytes in direct contact on the transwell insert with glia or neurones on the culture plate are becoming more popular models. More recently, hollow fibre capillary systems that can be exposed to intraluminal flow have been developed (Cucullo et al., 2002; Cucullo et al., 2010). A range of different species are used in *in vitro* BBB models. Primary human cells are desirable but restricted by limited access to material. Rodent models are advantageous due to availability of animals including transgenic animals and the range of specific reagents. However the yield of
endothelial cells is very low. Large quantities of endothelial cells can be obtained from bovine or porcine preparations. Bovine endothelial cells have been shown to exhibit high TEER, comparable to that observed in vivo (Zenker et al., 2003). The cost and labour intensity of primary cultures has made the use of immortalised cells more popular. The rat brain endothelial cell line, RBE4 and the human cell line, hCMEC/D3 maintain many BBB characteristics including expression of tight junction proteins, cell adhesion molecules and multidrug resistance proteins (Regina et al., 1998; Weksler et al., 2005).

By combining in vitro models of cerebral ischaemia and of the BBB it is possible to elucidate cellular responses of the BBB to ischaemic injury. This chapter reports the characterisation and development of an in vitro OGD model.

3.2. Aims

The aim of the first part of this study was to establish a reproducible in vitro OGD model that allowed further investigation into the inflammatory activation of the cerebrovascular endothelium and glia. This was achieved by the following two objectives:

1. Establish and characterise primary cell cultures of mouse brain endothelial cells (MBECs) and murine mixed-glia cell cultures

2. Determine the effect of severity and duration of OGD exposure on MBEC and glial viability that subsequently allowed the activation of MBECs and glia to be studied

3.3. Methods

To determine the purity of MBEC cultures, cells were stained by fluorescence immunocytochemistry (ICC) for PECAM-1 (CD31), ZO-1 and von Willebrand Factor (vWF). Purity of MBEC cultures was expressed as a percentage of DAPI-positive nuclei co-localised with PECAM-1 of the total number of cells present (total number of DAPI) per field of view. To ascertain whether there was any contamination by pericytes, glia or neurones, MBEC cultures were stained for α-smooth muscle actin (α-SMA), GFAP and neuronal nuclei (NeuN) respectively. To characterise cellular composition of primary murine mixed glial cultures, astrocytes
were stained for GFAP, microglia were stained for isolectin B4 (IBA4) and O2A progenitor cells were stained for GT3 ganglioside (A2B5). NeuN staining was performed to determine whether there was any neuronal contamination.

To investigate the severity and duration of OGD on MBEC and glial viability, MBECs and glial monocultures and co-cultures were exposed to OGD at 0.1 % and 1 % oxygen for 4, 6, 10, 18 and 24 h with and without a further 24 h reperfusion period. Cell death was measured by LDH release and uptake of trypan blue (see section 2.5 for full methods).
3.4. Results

3.4.1. Endothelial cell culture characterisation

Primary MBECs were derived from isolated cortical microvessels that attached to collagen IV-coated tissue culture plates (Figure 3.1A). Endothelial cells grew out from the microvessels, initially observed as rounded cells (Figure 3.1B). These regions of growth eventually coalesce and endothelial cells become more aligned (Figure 3.1C). By 14 DIV confluent MBEC monolayers displayed slender spindle-like morphology (Figure 3.1D). MBEC cultures were 99±1 % pure as demonstrated by immunostaining for the endothelial surface marker PECAM-1 (Figure 3.2A and G). MBECs also expressed vWF and the tight junction accessory protein ZO-1 (Figure 3.2B and C). There was no contamination of MBEC cultures by pericytes, astrocytes or neurones as identified by the absence of any staining for α-SMA, GFAP and NeuN (Figure 3.2D – F and G).

![Figure 3.1. Phase contrast images of growth progression of MBECs.](image)

Isolated microvessels attach to collagen IV coated plates (A). Within 5 DIV endothelial cells seen as rounded cells grow out from microvessels (B). By 7-10 DIV, endothelial cells appear more elongated, aligning with neighbouring cells (C). By 14 DIV, MBECs reach confluency displaying slender spindle-like morphology (D). Scale bar = 20 µm.
Figure 3.2. Characterisation of MBEC cultures. MBECs expressed PECAM-1 (A), vWF (B) and ZO-1 (C). There was no staining of α-SMA (D), GFAP (E), or NeuN (F) in these cultures indicating there was no contamination by pericytes, astrocytes or neurones respectively. Images are representative of three separate cultures. Scale bar = 50 µm. (G) Purity of MBECs cultures was quantified as a percentage of PECAM-1 positive cells per total number of cells (total number of DAPI) from three independent cultures using at least ten different fields of view. The percentage of α-SMA, GFAP and NeuN positive cells per field of view was also calculated. Values are mean ± SEM of three separate cultures. Scale bar = 50 µm.
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3.4.2. Mixed-glial cell culture characterisation

Mixed glial cells were isolated from two to three day old pups. By phase contrast microscopy, astrocytes were clearly visible with round shaped microglial cells present on top of the astrocyte monolayer (Figure 3.3). ICC revealed that mixed glial cultures were predominantly composed of astrocytes (79±2%). Two distinct populations of microglia were observed; flat, ramified microglia and rounded ameboid microglia. Together they accounted for 17±3% of the total glial population. Remaining glial cells were O2A progenitor cells (4±1%) (Figure 3.4A - D). No staining for NeuN was detected suggesting that there was no neuronal contamination (image not shown). Astrocytes formed a monolayer on top of the flat ramified microglia and O2A progenitor cells (Figure 3.5iii and ix). Round-shaped microglia were visualised within the same plane or on top of the astrocyte monolayer (Figure 3.5vi).

![Figure 3.3. Phase contrast microscopy of confluent murine mixed glial cultures.](image)

**Figure 3.3. Phase contrast microscopy of confluent murine mixed glial cultures.** Astrocytes form a monolayer with round-shaped microglial cells within and on top of this monolayer (arrows). O2A progenitor cells below the astrocyte monolayer were not visible. x10 magnification (A), scale bar = 150 µm and at x20 magnification (B), scale bar = 75 µm.
Figure 3.4. Characterisation of murine, mixed glial cultures. To identify the cellular composition of mixed glial cultures astrocytes were stained for GFAP (A), microglia were stained for IBA4 (B and C) and O2A progenitor cells were stained for A2B5 (D). The percentage of GFAP, IBA4, A2B5 and NeuN positive cells per total number of cells (number of DAPI) was averaged from three separate cultures using at least ten different fields of view (E). Values are mean ± SEM. Scale bar = 50 µm.
Figure 3.5. Composition of murine mixed glial cultures. Astrocytes were stained for GFAP (i, iv and vii). Microglia were stained for IBA4 (ii and v). O2A progenitor cells were stained for A2B5 (viii). Flat ramified microglia grew on the base of tissue plates and the astrocyte monolayer grew on top of these microglia (iii). Microglia of a round morphology were identified within the plane of or on top of the astrocyte monolayer (vi). Astrocytes also grew on top of O2A progenitor cells (ix). Scale bar= 50 µm.
3.4.3. pH and oxygen analysis of the OGD media

Deoxygenation of the OGD media was achieved by purging with \( N_2 \). This resulted in the pH of the OGD medium increasing to \( 8.8 \pm 0.1 \). After 60 min inside the hypoxic chamber, pH of the OGD medium fell to pH \( 7.7 \pm 0.1 \) (Figure 3.6). Oxygen content within the hypoxic chamber was monitored and maintained at 0.1 % or 1 % by an oxygen controller. Initial experiments found that after degassing the OGD medium, oxygen content of the medium could be maintained at 0.1 % or 1 % while inside the hypoxic chamber over a 24 h period. After 90 min inside the hypoxic chamber, the oxygen content of the OGD medium reached 0.1 % or 1 % oxygen and was subsequently maintained (Figure 3.7A and B). From these results, the OGD medium would be left inside the hypoxic chamber for at least 90 min prior to adding to cell cultures to re-establish pH and reach desired oxygen concentration.
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Figure 3.6. pH of the OGD media. Deoxygenation of the OGD medium caused pH of the OGD medium to increase to 8.8±0.1. After 60 min inside the hypoxic chamber, the presence of 5 % CO₂ within the chamber restored pH to 7.7±0.1. ***P<0.001 Student’s t-test.

Figure 3.7. Monitoring oxygen content of the OGD medium. The hypoxic chamber purged with N₂ to 0.1 % or 1 % oxygen. The OGD medium was deoxygenated with N₂. After 90 min inside the hypoxic chamber, oxygen content of the OGD medium was maintained at 0.1 % (A) and 1 % (B) as monitored using a MI-730 O₂ electrode. Values are mean ± SEM of five individual experiments.
3.4.4. OGD-induced MBEC and glial viability

3.4.4.1. Effect of 0.1 % oxygen on MBEC and glial viability

Having established that both the hypoxic chamber and OGD media could maintain oxygen concentrations of 0.1 % and 1 %, the viability of MBECs and glial monocultures during OGD at 0.1 % and 1 % oxygen was measured by LDH release and trypan blue exclusion over 4, 6, 10, 18 and 24 h with or without a further 24 h reperfusion.

From 4 h OGD at 0.1 % oxygen, MBECs released significant levels of LDH (Figure 3.8A). Changes in morphology were observed from 4 h OGD; MBECs became thin, withered and detached from neighbouring cells resulting in a loss of the continuous monolayer (Figure 3.8B).

Significant LDH release by glia was measured from 6 h OGD (Figure 3.8C). By 6 h, glia became shrunken and almost all cells were stained by trypan blue (Figure 3.8D). For both MBECs and glia, 24 h reperfusion following 4, 6, 10, 18 and 24 h OGD had no further effect on cell viability (Figure 3.8A and C).
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Figure 3.8. MBEC and glial viability during OGD at 0.1 % oxygen. MBECs (A) and glia (C) were exposed to OGD at 0.1 % oxygen for 4, 6, 10, 18 and 24 h without (grey bars) or with a subsequent 24 h reperfusion (checked bars). Control (Ctrl) cultures were kept in glucose-containing media in a humidified 5 % CO₂ incubator for 24 h (open bar) and reperfused with reperfusion media for a further 24 h (dotted bar). LDH release into the culture medium by MBECs and glia was measured at the end of the indicated the OGD timepoint and reperfusion. Data are mean ± SEM of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 OGD vs. Ctrl. *P<0.05, **P<0.01, ***P<0.001 OGD + 24 h reperfusion vs. Ctrl + 24 h reperfusion (two way ANOVA with Tukey’s multiple comparison test). After 4 h OGD exposure of MBECs, cells were incubated with trypan blue (B). Glia were incubated with trypan blue at the end of 6 h OGD (D) Scale bar = 50 μm
3.4.4.2. Effect of 1 % oxygen on MBEC and glial viability

MBEC viability was maintained over a 24 h OGD period (at 1 % oxygen) and reperfusion. However by 48 h and 72 h OGD, 68±6 % and 85±4 % LDH release was measured respectively, and all cells stained positive for trypan blue indicating that all endothelial cells were dead (Figure 3.9A and B).

Exposure of glia to 1 % oxygen induced significant LDH release from 24 h (32±5 %) (Figure 3.9C), although only a few cells were positive for trypan blue at this timepoint (Figure 3.9D). Reperfusion of glia following 24 h OGD caused a significant 3.1-fold reduction in LDH release indicating that reperfusion may partially restore glial viability or induce glial proliferation (Figure 3.9C). After 48 h and 72 h OGD, 73±6 % and 78±4 % LDH release was measured respectively and all glial cells were positive for trypan blue staining by 48 h OGD (Figure 3.9C and D). Glial death induced by 48 h and 72 h OGD could not be restored with subsequent reperfusion (Figure 3.9C).

LDH release is dependent upon membrane rupture which is characteristic of necrosis. To determine whether the low level of MBEC and glial LDH release measured over 24 h OGD and reperfusion was due to cells undergoing apoptosis, MBEC and glia whole cell lysates were probed for cleaved caspase-3 by Western blot. As a positive control for apoptosis, MBECs and glia were treated with 0.5 µM staurosporine for 10 h. There was no expression of cleaved caspase-3 by either MBECs or glia during a 24 h OGD exposure or following reperfusion (Figure 3.10).
Figure 3.9 continued on next page
3.9. MBEC and glial viability during OGD at 1 % oxygen. MBECs (A) and glia (C) were exposed to OGD at 1 % oxygen for 4, 6, 10, 18, 24, 48 and 72 h without or with a subsequent 24 h reperfusion. Control cultures were kept in glucose-containing media in a humidified 5 % CO₂ incubator for 24 h and reperfused with reperfusion media for a further 24 h. Data are mean ± SEM of five independent experiments. \(^*P<0.05, \quad ^{**}P<0.001\) OGD vs. Ctrl. \(^{\#}P<0.05, \quad ^{##}P<0.01, \quad ^{###}P<0.001\) OGD + 24 h reperfusion vs. Ctrl + 24 h reperfusion. \(^{\$}P<0.01\) 24 h OGD vs. 24 h OGD + 24 h reperfusion. (two way ANOVA with Bonferroni’s post hoc test). MBECs (B) and glia (C) were incubated with trypan blue at the end of 24 h and 48 h OGD. Scale bar = 50 µm.
From these results it seems that MBEC and glial viability was maintained during longer exposures to 1 % oxygen compared to OGD at 0.1 % oxygen. Therefore future OGD experiments would be performed at 1 % oxygen. Accordingly, LDH release from MBECs and glia in a non-contact co-culture transwell set-up was measured. Similar to monocultures, viability of co-cultured MBECs and glia was maintained over a 24 h OGD exposure at 1 % oxygen. MBEC and glial death induced by 48 h and 72 h OGD was not restored with reperfusion (Figure 3.11).
Figure 3.11. Co-cultured MBEC and glial LDH release during OGD at 1% oxygen. MBEC-glia co-cultures were exposed to OGD at 1% oxygen for 4, 6, 10, 18, 24, 48 and 72 h without or with a subsequent 24 h reperfusion. Control cultures were kept in glucose-containing medium in a humidified 5% CO₂ incubator for 24 h and reperfused with reperfusion medium for a further 24 h. At the end of the indicated OGD timepoint and reperfusion, LDH release by MBECs (A) was measured from the Transwell insert and LDH release from glia (B) was measured from the abluminal compartment. Data are mean ± SEM of five separate experiments. ***P<0.001 OGD vs. Ctrl. ##P<0.01, ###P<0.001 OGD + 24 h reperfusion vs. Ctrl + 24 h reperfusion (two way ANOVA with Bonferroni’s post hoc test).
3.5. Discussion

The overall aim of the first part of this study was to establish and characterise an \textit{in vitro} OGD model. This was broadly divided into two sections, firstly to successfully culture MBECs and murine mixed glia that would provide a reliable representation of their \textit{in vivo} counterparts. Secondly, to determine the severity and duration of OGD that will allow investigation into the activation of these MBECs and glia in further studies.

3.5.1. Culture of primary murine endothelial and glial cells

The inherent difficulty of maintaining primary endothelial cells in culture is the need to maintain pure cultures that retain cellular and functional characteristics of the BBB. MBECs used here were isolated from young adult mice. They displayed a slender morphology forming a continuous monolayer. These cultures were 99±1\% pure with no contamination by pericytes, glia or neurones (Figure 3.1 and 3.2). MBECs were treated with puromycin for the first two days of culture. Puromycin is a substrate of the P-glycoprotein (P-gp) drug-resistance transporter which is highly expressed by endothelial cells. Thus endothelial cells are able to tolerate relatively high concentrations of puromycin that would otherwise be toxic to contaminating cells (pericytes, glia, fibroblasts and smooth muscle cells) that do not express P-gp. Perriere et al. (2005) demonstrated the absence of any toxic effect of puromycin treatment on rat endothelial cells, yielding pure endothelial cultures as well as increasing TEER of these endothelial monolayers. Additionally, MBECs were maintained in medium containing bovine-derived PDS. Bovine PDS does not contain platelet-derived growth factor (PDGF) known to support the growth of fibroblasts, pericytes, glia and smooth muscle cells (Ziata et al. 1986). MBECs expressed PECAM-1 and the tight junction accessory protein ZO-1, both seen around the periphery of cells. MBECs displayed a punctuated intracellular expression of vWF indicative of storage inside Weibel-Palade bodies. Therefore these cultures were purely of endothelial origin and expressed endothelial specific markers. Furthermore the expression and cellular distribution of these makers were not altered by cultivation \textit{in vitro}. TEER and permeability measurements are often used as an indication of the quality of the culture as these reflect the tightness of the tight junction complexes. It has been reported that primary endothelial cells of murine
origin exhibit very low TEER values. However this can be overcome by co-culturing with astrocytes, astrocyte-conditioned media or supplementation with cAMP (Rubin et al. 1991, Hatherell et al. 2011). Additionally, the majority of in vivo models of ischaemia are established in rodents, therefore for direct comparisons it is conceivable that in vitro models should use rodent cell cultures.

In the murine mixed-glial cultures used in this study, astrocytes were the most abundant glial cell present (79±3%), microglia made up 17±3 % of the glial population and the remaining glia were O2A progenitor cells (4±1 %) (Figure 3.4). Several factors affect the proportion of glial cells in culture including species, animal age, region of brain used and culture conditions. These mixed glial cultures were derived from two to three day old pups as this is the optimal age for yield and purity because it is at the peak of astrogenesis (Saura, 2007). ICC showed that these mixed-glial cultures consisted of two distinct microglial populations; ramified microglia were observed below the astrocyte monolayer while round-shaped ameboid microglia were observed on top of and intermingled within the astroctye monolayer (Figure 3.5). These observations are consistent with other reports of rodent in vitro mixed glial cultures. Astrocytes are known to contribute to the differentiation and proliferation of microglia. Tanaka et al. (1999) identified two types of morphologically distinct microglia in rat mixed glial cultures in which secreted astrocyte-derived insoluble factors played a pivotal role in their differentiation. Granulocyte/macrophage-colony stimulating factor produced by astrocytes can induce microglial proliferation and ramification in vitro (Sawada et al., 1990). In vivo, activation of microglia involves a morphological change from a resting ramified state to ameboid cells with large rounded cell bodies. These ameboid cells are often likened to macrophages based on their ability to phagocytose, secrete cytokines and shared expression of markers (Stoll et al., 1998). The ameboid cells identified in these cultures may represent a population of activated microglia.

3.5.2. Viability of endothelial and glial cells exposed to OGD

While it is generally well documented that neurones are the most sensitive cerebral cell type to ischaemic injury discrepancies exist between different studies regarding susceptibility of the endothelium and glia to ischaemic injury, which likely reflects
variations between models. The second aim of this section of work was to identify the effect of the severity and duration of OGD on MBEC and glial viability. Cells were exposed to OGD at near anoxic (0.1 % oxygen) and hypoxic (1 % oxygen) conditions initially over a 24 h period with or without a further 24 h reperfusion. As expected MBECs and glia were more susceptible to near anoxic conditions. MBEC and glial death was induced by a much shorter exposure to OGD at 0.1 % oxygen compared to that at 1 % (Figure 3.8 and Figure 3.9). MBECs displayed a significant increase in LDH release in a time-dependent manner from 4 h OGD at 0.1 % oxygen. This was associated with a complete loss of monolayer integrity (Figure 3.8A). Similarly, Xu et al. reported OGD at 0.02 – 0.2 % oxygen induced bovine cerebral endothelial cell death within 4 h of exposure (Xu et al., 2000). MBEC viability was maintained over 24 h of OGD at 1 % oxygen. This was observed in both MBEC monocultures and when co-cultured with glia, indicating that glia had no adverse effect on MBECs (Figure 3.9A, B and Figure 3.11A). Prolonged oxygen and glucose withdrawal (from 48 h) did result in a marked increase in MBEC death as observed by total release of LDH and uptake of trypan blue by all cells (Figure 3.9A, B). Some in vivo and in vitro studies suggest endothelial cells are the most resistant cerebral cell type to ischaemic injury, tolerating experimentally-induced ischaemia and in vitro simulated hypoxia for up to 24 h (Hess et al., 1994, del Zoppo, 2009).

Glial death was induced by 6 h OGD at 0.1 % oxygen which was observed as significant LDH release (51±6%) and uptake of trypan blue (Figure 3.8C and D). Glial viability was maintained up to 24 h OGD at 1 % oxygen. After a 24 h exposure glia displayed a significant increase in LDH release (32±5%) compared to normoxic controls (Figure 3.9C). This however is still a relatively low proportion of LDH release and with the finding that almost all glia excluded trypan blue at 24 h OGD (Figure 3.9D) would suggest that the majority of glial cells are still viable at 24 h at 1 % oxygen. Furthermore, a 3.1-fold reduction in LDH release during reperfusion following 24 h OGD indicates that restoration of oxygen and glucose may promote glial viability or induce glial proliferation. Thus reperfusion may have a beneficial effect on glial survival after ischaemic injury in vitro. However while reperfusion involves the restoration of blood flow essential for tissue survival in vivo, it can also exacerbate initial injury through generation of ROS and reactive nitrogen species.
(Van Elzen et al., 2008). During reperfusion following experimentally-induced stroke Gursoy-Özdemir identified the formation of superoxide and peroxynitrite in neurones, astrocytes and endothelial cells. This was associated with activation of MMPs and loss of selective BBB permeability (Gursoy-Ozdemir et al., 2004).

The LDH release assays used in these studies are indicative of disruption to membrane integrity, which is a feature typical of necrosis. To determine whether the low level of LDH release observed by MBECs and glia after exposure to 1 % oxygen was due to cells undergoing apoptosis or cell survival, cleaved caspase-3 expression during OGD and reperfusion was analysed. Staurosporine treatment induced expression of cleaved caspase-3 by both MBECs and glia. However the absence of any cleaved caspase-3 expression over a 24 h OGD exposure and reperfusion indicates that neither OGD nor reperfusion induced MBEC or glial apoptosis (Figure 3.10). This is in contrast to a study which reported OGD-induced bovine endothelial cell death was associated with DNA fragmentation and cytochrome c release (Xu et al., 2000). Differences in experimental procedures may explain such discrepancies and further supports the notion that while apoptosis and necrosis have been observed in in vitro models of ischaemia this is largely dependent on the injury model and cellular species (Newcomb-Fernandez et al., 2001). Some studies suggest that ischaemic cell death by necrosis or apoptosis may not be separate processes but rather a succession of the two events (Ankarcriona et al., 1995). Additionally the same experimentally-induced insults can bring about different mechanisms of cell death in different populations of cells (Wang et al., 2002).

The precise mechanisms of cellular damage during ischaemic injury are not fully understood although oxidative stress has been shown to be a major contributor to cellular injury (Griffin et al., 2005). The close physical and chemical interactions between cerebral cells reflect dynamic intercommunication between these cells during ischaemic-reperfusion injury. OGD-induced activation of microglia has been demonstrated to increase the susceptibility of astrocytes and endothelial cells to OGD. In vivo, inhibition of microglial activation following experimentally-induced stroke reduced infarct size and reduced the occurrence of haemorrhagic transformation and BBB disruption (Yenari et al., 2006). Evidence from conditioned
media transfer experiments demonstrate autocrine and paracrine signalling may occur under pathological conditions (Li et al., 2009). Conditioned media from endothelial cells has been shown to protect neurones from OGD-induced injury attributed to endothelial production of brain-derived neutrophic factor (Guo et al., 2008). Lin et al. (2006) showed that astrocyte-mediated protection of ischaemic microglia, neurones and astrocytes in vitro was dependent upon secretion of soluble mediators by astrocytes and the concomitant expression of their receptors on target cells (Lin et al., 2006).

From the results presented in this chapter and other studies, it can be summarised that the extent of cellular injury or death to in vitro stimulated ischaemia is dependent upon the experimental model including cellular species and severity of insult.

3.5.3. Summary
This section of work has established and characterised the cellular components and injury conditions of an in vitro OGD model that will be used for further experiments to investigate the activation of the cerebral endothelium and glia to this insult. In these studies MBEC and glial viability was maintained over 24 h under conditions where oxygen is reduced to 1 %. Therefore it was decided that for future experiments, MBEC and glial monocultures and co-cultures would be exposed to OGD at 1 % oxygen over 24 h with or without a subsequent 24 h reperfusion. Additionally, taking into account that deoxygenation of the OGD media caused a rise in pH, prior to start of any OGD timepoint, the OGD medium would be kept inside the hypoxic chamber for at least 60 minutes to restore pH and ensure oxygen content was at correct levels.
Chapter 4
Oxygen-glucose deprivation-induced endothelial and glial cell activation
4. Oxygen-glucose deprivation-induced endothelial and glial cell activation

4.1. Introduction
Cerebral ischaemia induces a robust inflammatory response from resident cerebral cells that further exacerbates injury as well as having beneficial actions for tissue repair and recovery. The depletion of energy that occurs in response to a reduction or cessation of cerebral blood flow is considered to underlie the pathophysiology of cerebral ischaemia. Energy failure, the loss of ionic homeostasis and cellular accumulation of intracellular Ca$^{2+}$ results in neuronal death and triggers the inflammatory activation of other cerebral cells (Wang et al., 2007). Reperfusion of the ischaemic tissue is essential for tissue survival but also augments complex inflammatory responses that evolve within minutes and last for days (Van Elzen et al., 2008). These long term responses that can last hours to days involve the activation of the vasculature and glia. This activation is associated with a modulation in gene expression leading to the production of cytokines and chemokines, changes in endothelial cell adhesion molecule expression, enzymatic activation, further oxidative stress and accumulation of inflammatory cells (Vexler et al., 2006).

In this chapter, the inflammatory activation of MBECs and glia in response to OGD-reperfusion in vitro will be presented.

4.2. Aims
- To identify the temporal profile of OGD-reperfusion induced inflammatory changes in MBECs and glia by assessment of cytokine, chemokine and growth factor production and expression of cell adhesion molecules.
- To determine whether MBECs and glia regulate the activation of each other by the use of MBEC-glia co-cultures.

4.3. Methods
MBEC monocultures, glia monocultures and MBEC-glia co-cultures were exposed to OGD (1 % oxygen) for 4, 6, 10, 18 or 24 h with and without subsequent 24 h reperfusion with normoxic and glucose-containing media. At the end of OGD and
reperfusion, MBEC and glial lysates and conditioned media were collected for ELISA, Western blot and CBA (see section 2.6 for full methods).

4.4. Results

4.4.1. OGD-reperfusion induced MBEC activation

4.4.1.1. OGD-induced MBEC GLUT-1 expression

To determine if MBECs could respond to the reduction in oxygen and glucose, expression of HIF-1α and GLUT-1 were analysed during OGD and reperfusion. OGD induced a time-dependent increase in GLUT-1 expression with a maximal 6.5±2.1 fold increase in expression induced by 24 h OGD (Figure 4.1). Reperfusion significantly reduced OGD-induced GLUT-1 to basal levels. No expression of HIF-1α or HIF-2α by MBECs was detected during OGD or reperfusion (data not shown).
Figure 4.1. OGD-induced MBEC GLUT-1 expression. (A) Western blot analysis for MBEC GLUT-1 (~55 kDa) expression during OGD and following reperfusion. Loading volumes were normalised to β-actin (42 kDa). (B) Densitometric analysis of GLUT-1 western blots was performed to quantify fold change in GLUT-1 expression during OGD and reperfusion compared to normoxic controls. Data are mean ± SEM of five separate experiments. *P<0.05, **P<0.01 OGD vs. Ctrl. *P<0.05, ###P<0.001 OGD vs. OGD + 24 h reperfusion (two way AVOVA with Bonferroni post hoc test).
4.4.1.2 OGD-reperfusion induced MBEC NFκB expression

Normoxic MBECs expressed the NFκB p65 subunit predominantly in the cytosolic fraction. This cytosolic localisation of NFκB was unaffected by OGD (Figure 4.2A and B) indicating that NFκB remains sequestered in the cytoplasm during OGD. However OGD did induce a time-dependent increase in nuclear NFκB p65 during the reperfusion phase with a maximal 27.2 ± 4.3 fold increase in expression induced by reperfusion following 24 h OGD (Figure 4.2C and D).
Figure 4.2 continued on next page
Figure 4.2. OGD-reperfusion induced MBEC NFκB expression. Western blot analysis of MBEC nuclear and cytosolic expression of NFκB p65 (65 kDa) during OGD (A) and following reperfusion (C). Loading volumes were normalised to β-actin. Densitometric analysis of Western blots identified change in nuclear and cytosolic NFκB p65 expression during OGD (B) and following reperfusion (D) over normoxic controls. Data are mean SEM of at least three separate experiments. *P<0.05, **P<0.01 OGD vs. Ctrl (one way ANOVA with Tukey’s multiple comparison test)
4.4.1.3. OGD-reperfusion induced MBEC ICAM-1 and VCAM-1 expression

There was no change in basal ICAM-1 and VCAM-1 expression at the end of 24 h reperfusion following 4, 6, 10, 18 or 24 h OGD of MBEC monocultures (Figure 4.3A, B and Figure 4.4A, B). MBECs co-cultured with glia displayed a significant 3.0±0.4, 4.1±1.3 and 5.0±1.3–fold increase in ICAM-1 expression over normoxic controls at the end of reperfusion following 4, 18 or 24 h OGD respectively (Figure 4.3C and D). Co-cultured MBECs also displayed significant 3.8±1.0 and 3.0±0.5-fold increases in VCAM-1 expression over normoxic controls at the end of reperfusion following 4 h and 24 h OGD respectively (Figure 4.4C and D).
Figure 4.3. OGD-reperfusion induced MBEC ICAM expression. Western blots for ICAM-1 (110 kDa) were performed on whole cell lysates collected from MBEC monocultures (A) and co-cultured MBECs (C) at the end of 24 h reperfusion following 4, 6, 10, 18 or 24 h OGD. Loading volumes were normalised to β-actin. Densitometric analysis of Western blots was performed to determine fold change in ICAM-1 expression by MBEC monocultures (B) and co-cultured MBECs (D) over normoxic controls. *P<0.05 OGD + 24 h reperfusion vs. Ctrl + 24 h reperfusion (one way ANOVA with Tukey’s multiple comparison test).
Figure 4.4. OGD-reperfusion induced MBEC VCAM-1 expression. Western blots for VCAM-1 (110 kDa) were performed on whole cell lysates collected from MBEC monocultures (A) and co-cultured MBECs (C) at the end of 24 h reperfusion following 4, 6, 10, 18 or 24 h OGD. Loading volumes were normalised to β-actin. Desitometric analysis was performed to determine fold change in VCAM-1 expression by MBEC monocultures (B) and co-cultured MBECs (D) over normoxic controls. *P<0.05, ***P<0.001 OGD + 24 h reperfusion vs. Ctrl + 24 h reperfusion (one way ANOVA with Tukey’s multiple comparison test).
4.4.1.4. OGD-reperfusion induced MBEC cytokine and chemokine production

MBEC expression and secretion of KC were measured by ELISA during 24 h OGD with or without 24 h reperfusion. To obtain a temporal profile of a wider range of cytokine and chemokine responses by MBECs, CBA analysis for TNF-α, RANTES, MCP-1, IL-6, IL-1α, IL-1β, IL-17α, IL-10, IFN-γ, GM-CSF, CD62L and CD62E were performed on lysates and conditioned media of co-cultured MBECs.

There was no change in basal expression of KC by MBEC monocultures or cocultured MBECs over 24 h OGD or after reperfusion (Figure 4.5A and C). Basal KC secretion by MBEC monocultures did not change over 24 h OGD. However a significant 3.0-fold and 2.8-fold increase in KC secretion by these monocultures was induced during reperfusion following 18 h (172.7±57.0 pg/mL) and 24 h OGD (165.1±28.5 pg/mL) respectively (Figure 4.5B). Similar to MBEC monocultures, OGD did not induce any change in basal KC secretion by co-cultured MBECs. However reperfusion following 4 h OGD induced a significant 3.1-fold increase (220.3±57.0 pg/mL) in KC secretion by these co-cultured MBECs (Figure 4.5D).

Further experiments showed that KC cannot pass across the 0.4 µm PET transwell membrane or the MBEC monolayer in an abluminal to luminal direction (Figure 4.6). Therefore in these co-cultures, the amount of KC measured in the conditioned medium in the luminal side of the transwell was secreted by MBECs and not KC secreted by glia in the abluminal compartment that traversed the Transwell membrane in an abluminal to luminal direction.

CBA analysis of co-cultured MBEC lysates and conditioned media revealed significant changes in MCP-1 and RANTES during OGD and reperfusion. Normoxic MBECs expressed and secreted high levels of MCP-1 (340.1±25.9 pg/mL and 6769.0±388.3 pg/mL respectively). MCP-1 expression and secretion significantly declined over a 24 h OGD period. 24 h reperfusion following 4 or 10 OGD partially restored MCP-1 expression and secretion to baseline levels, although this did not reach statistical significance (Figure 4.7A and B). There was no change in basal expression or secretion of RANTES during OGD. Reperfusion following 4 h OGD induced a significant increase in RANTES secretion (Figure 4.8A and B). MBEC
expression and secretion of all other cytokines, chemokines and cell adhesion molecules measured by CBA were not detected or measured at very low levels that did not change during OGD or reperfusion (Table 4, Appendix II)
Figure 4.5. OGD-reperfusion induced MBEC keratinocyte-derived chemokine expression and secretion. KC expression (A) and secretion (B) by MBEC monocultures and KC expression (C) and secretion (D) by co-cultured MBECs over the course of OGD and reperfusion. Control cultures were kept in normoxic conditions for 24 h and reperfused with reperfusion media for 24 h. Data are mean ± SEM of at least five independent experiments. **P<0.01, ***P<0.001 OGD + 24 h reperfusion vs. Ctrl + 24 h reperfusion. $P<0.05, $$$P<0.01$ OGD vs. OGD + 24 h reperfusion (two way ANOVA with Bonferroni post hoc test).
Figure 4.6. Abluminal to luminal permeability of keratinocyte-derived chemokine across the Transwell membrane and MBEC monolayer. 400 ng/mL exogenous KC was added to the abluminal compartment of co-cultures. 24 h later media was collected from the abluminal and luminal compartments and analysed for KC by ELISA. Data are mean ± SEM of four separate experiments.

Figure 4.7. OGD reperfusion-induced MBEC monocyte chemoattractant protein-1 production. MCP-1 expression (A) and secretion (B) by co-cultured MBECs during OGD and following 24 h reperfusion. Control cultures were kept in normoxic conditions for 24 h and reperfused for 24 h. Data are mean ± SEM of at least four independent experiments. *P<0.05, **P<0.01, ***P<0.001 OGD vs. Ctrl. #P<0.05 OGD + 24 h reperfusion vs. Ctrl + 24 h reperfusion (two way ANOVA with Bonferroni post hoc test).
Figure 4.8. OGD-reperfusion induced MBEC RANTES production. RANTES expression (A) and secretion (B) by co-cultured MBECs during OGD and following 24 h reperfusion. Control cultures were kept in normoxic conditions for 24 h and reperfused for 24 h. Data are mean ± SEM of at least four independent experiments. ###P<0.001 4 h OGD + 24 h reperfusion vs. Ctrl + 24 h reperfusion. $$$P<0.001 4 h OGD vs. 4 h OGD + 24 h reperfusion (two way ANOVA with Bonferroni post hoc test).
4.4.1.5 OGD-reperfusion induced changes in MBEC permeability.

There was no difference in the permeability of MBEC monocultures or co-cultured MBECs to 4 kDa FITC dextran under normoxic conditions (0.24±0.01 cm/min⁻¹ x10⁻³ and 0.24±0.10 cm/min⁻¹ x10⁻³ respectively). 4 h OGD induced a significant transient 3.2-fold increase (0.76±0.14 cm/min⁻¹ x10⁻³) in permeability of MBEC monocultures to FITC-dextran that subsequently returned to basal levels with longer exposures to OGD (from 10 h) (Figure 4.9A). Co-culturing MBECs with glia reduced this increase in permeability at 4 h to basal levels. Reperfusion had no effect on basal permeability of either MBEC monocultures or co-cultured MBECs (Figure 4.9A). The increased permeability of MBEC monocultures to FITC-dextran at 4 h OGD was not associated with any disruption to ZO-1 localisation (Figure 4.9B). Exposure of MBEC monocultures to 10 or 24 h OGD with and without reperfusion and co-cultured MBECs to 4, 10 or 24 h OGD, with or without reperfusion also had no effect on ZO-1 localisation (data not shown). As a positive control for paracellular permeability increase, 30 min treatment of MBEC monolayers with 1.4 M mannitol induced a 25-fold increase in basal permeability of MBECs to FITC-dextran (6.01±1.47 cm/min⁻¹ x10⁻³). Although MBECs still retained peripheral ZO-1 immunostaining after mannitol treatment, large gaps appeared between endothelial cells (Figure 4.9B).
Figure 4.9. OGD-reperfusion induced changes in MBEC permeability. (A) Permeability of MBEC monocultures and co-cultured MBECs to 4 kDa FITC-dextran during 4, 10 or 24 h OGD and following reperfusion. Data are mean ± SEM of at least four independent experiments. ***P<0.001 4 h OGD monoculture vs. Ctrl monoculture. $^5$P<0.001 4 h OGD co-culture vs. 4 h OGD monoculture (two way ANOVA with Bonferroni post hoc test). (B) Immunofluorescence staining of MBECs for ZO-1 after normoxia (i), 4 h OGD (ii) or after treatment with 1.4 M mannitol. Mannitol caused gaps to appear between endothelial cells (arrows) (iii). Scale bar = 75 μm.
4.4.1.6. Summary of OGD-reperfusion induced MBEC activation

These experiments show dynamic and overlapping responses of MBECs during OGD and reperfusion which are summarised in Figure 4.10.

Figure 4.10. Summary of OGD-reperfusion induced MBEC responses. Summary of co-cultured MBEC responses to exposure to 4, 6, 10, 18 and 24h OGD with and without 24 h reperfusion. Differences in the responses by MBEC monocultures to those when co-cultured with glia are highlighted in blue.
4.4.2. OGD-reperfusion induced glial activation

4.4.2.1. OGD-reperfusion induced glial HIF-1α and GLUT-1 expression

To determine if glia could respond to the reduction in oxygen and glucose, HIF-1α and GLUT-1 expression during OGD and reperfusion were analysed. OGD induced a time-dependent increase in nuclear HIF-1α expression with a maximal 7.0±0.6-fold increase in expression by 18 h while cytosolic expression was maintained at basal levels (Figure 4.11A and B). After reperfusion, nuclear HIF-1α returned to basal levels (Figure 4.11C and D). Only 24 h OGD exposure of glia induced a significant 4.6±1.2-fold increase in GLUT-1 expression over normoxic controls that were significantly reduced to basal levels with subsequent reperfusion (Figure 4.12).
Chapter 4  OGD-reperfusion-induced MBEC and glial activation

Figure 4.11 continued on next page
Figure 4.11. OGD-reperfusion induced glial HIF-1α expression. Western blots for HIF-1α (112 kDa) were performed on nuclear and cytosolic extracts at the end of 4, 6, 10, 18 or 24 h OGD (A) and following 24 h reperfusion (C). Loading volumes were normalised to β-actin. Densitometric analysis of HIF-1α western blots was used to assess the increase in nuclear and cytosolic HIF-1α over normoxic controls following OGD (B) and reperfusion (D). Data are mean ± SEM of four independent experiments. *P<0.05 OGD vs. Ctrl (one way ANOVA with Tukey’s multiple comparison test).
Figure 4.12. OGD-reperfusion induced glial GLUT-1 expression. (A) Western blot for glial GLUT-1 (~ 45 kDa) expression at the end of 4, 6, 10, 18 or 24 h OGD with or without 24 h reperfusion. Loading volumes were normalised to β-actin. (B) Densitometric analysis of GLUT-1 western blots was used to assess the increase in GLUT-1 over normoxic controls following OGD and reperfusion. Data are mean ± SEM of five independent experiments. \( P < 0.05 \) OGD vs. Ctrl. \( 3^{5}P < 0.01 \) 24 h OGD vs. 24 h OGD + 24 h reperfusion (two way ANOVA with Bonferroni post hoc test).
4.4.2.2. OGD reperfusion-induced glial NFκB expression

Normoxic glia expressed NFκB p65 in the cytosolic fraction, but very low levels of expression were detected in the nucleus (Figure 4.13A). Exposure of glia to 10 h OGD induced a significant fold increase in nuclear (4.8±1.9 fold) and cytoplasmic (3.0±0.4 fold) NFκB p65 expression compared to normoxic glia (Figure 4.13A and B). Reperfusion following 4 h OGD exposure induced a significant 6.0±2.3 fold upregulation in nuclear NFκB p65 expression (Figure 4.13C and D).
Figure 4.13 continued on next page
Figure 4.13. OGD reperfusion-induced glial NFκB expression. Western blot analysis of glial nuclear and cytosolic expression of NFκB p65 (65 kDa) during OGD (A) and following reperfusion (C). Loading volumes were normalised to β-actin. Densitometric analysis of NFκB p65 Western blots was performed to assess fold change in nuclear and cytosolic NFκB p65 expression during OGD (B) and following reperfusion (D). Data are mean SEM of at least three separate experiments. *P<0.05 OGD vs. Ctrl. #P<0.05 OGD + 24 h reperfusion vs. Ctrl + 24 h reperfusion. (one way ANOVA with Tukey’s multiple comparison test).
4.4.2.3. OGD reperfusion-induced glial cytokine, chemokine and growth factor production

There was no change in basal expression or secretion of KC by glial monocultures during 24 h OGD or reperfusion (Figure 4.14A and B). OGD and reperfusion also had no effect on KC expression by co-cultured glia (Figure 4.14C). During the reperfusion period, normoxic co-cultured glia secreted low concentrations of KC (68.3±14.9 pg/mL). Reperfusion induced a significant increase in KC secretion following 4 h (288.6 ± 80.9 pg/mL), 6 h (368.3±127.3 pg/mL) and 10 h OGD (301.2±61.1 pg/mL) by co-cultured glia (Figure 4.14D). Fluorescent ICC showed that KC was strongly co-localised with IBA4 indicating that microglia were the source of KC. These microglia were of an ameboid morphology indicating that they were activated (Figure 4.14E).

OGD and reperfusion had no effect on VEGF expression by either glial monocultures or co-cultured glia (Figure 4.15A and C). Glial monocultures and co-cultured glia secreted low concentrations of VEGF under normoxic conditions (7.9±3.6 pg/ml and 32.7±18.9 pg/mL respectively) but displayed a time-dependent increase in VEGF secretion over a 24 h period of OGD and following reperfusion (Figure 4.16B and D). Maximal VEGF secretion was measured after 24 h OGD from both glia monocultures (278.9±10.6 pg/mL) and co-cultured glia (225.6±56.0 pg/mL). VEGF strongly co-localised with GFAP, indicating that astrocytes were the source of VEGF (Figure 4.15E). Further experiments showed that exogenous KC and VEGF could not pass across the MBEC monolayer or the 0.4 µm PET transwell membrane in a luminal to abluminal direction (Figure 4.16). Therefore KC and VEGF measured in the abluminal compartment of these co-cultures were secreted by glia and not the result of KC and VEGF secretion by MBEC transversing the transwell membrane from the luminal to abluminal compartment.

To identify a wider profile of cytokine and chemokine responses in glia, CBA analysis for TNF-α, MIP-1, RANTES, MCP-1, KC, IL-6, IL-1α, IL-1β, IL-17α, IL-10, IFN-γ, G-CSF, CD62L, CD62E were performed on lysates and conditioned media of co-cultured glia. Within 4 h OGD and following reperfusion there was a significant increase in both the expression and secretion of RANTES from co-
cultured glia. Prolonged exposure of glia to OGD (> 4 h) with or without reperfusion reduced expression and secretion of RANTES to basal levels (Figure 4.17). Glia showed very low levels of expression and secretion of all other cytokines and chemokines measured by CBA that did not change during OGD or reperfusion (Table 5, Appendix II)
Figure 4.14 continued on next page
Figure 4.14. OGD-reperfusion induced glial KC expression and secretion. KC expression (A) and secretion (B) by glial monocultures and KC expression (C) and secretion (D) by co-cultured glia over the course of OGD and reperfusion. Control cultures were kept in normoxic conditions for 24 h and reperfused with reperfusion media for 24 h. Data are mean ± SEM of at least five independent experiments. *P<0.05 OGD + 24 h reperfusion vs. Ctrl + 24 h reperfusion. **P<0.001 OGD vs. OGD + 24 h reperfusion (two way ANOVA with Bonferroni post hoc test). (E) Co-cultured glia were exposed to 18 h OGD + 24h reperfusion. At the end of reperfusion double labelling for GFAP (i) or IBA4 (iv) with KC (ii and v) was performed. No co-localisation of KC with GFAP was detected (iii). KC strongly co-localised with IBA4 (vi) indicating microglia produced KC in these mixed glia cultures in response to OGD-reperfusion. Scale bar = 50 µm.
Figure 4.15 continued on next page
Figure 4.15. OGD-reperfusion induced glial vascular endothelial growth factor expression. VEGF expression (A) and secretion (B) by glial monocultures and VEGF expression (C) and secretion (D) by co-cultured glia over the course of OGD and reperfusion. Control cultures were kept in normoxic conditions for 24 h and reperfused with reperfusion media for 24 h. Data are mean ± SEM of at least five independent experiments. *P<0.05, **P<0.01 OGD vs. Ctrl. #P<0.05, ##P<0.01, ###P<0.001 OGD + 24 h reperfusion vs. Ctrl + 24 h reperfusion (two way ANOVA with Bonferroni post hoc test). (E) Co-cultured glia were exposed to 18 h OGD + 24 h reperfusion. At the end of reperfusion double labelling for GFAP (i) or IBA4 (iv) with VEGF (ii and v) was performed. No co-localisation of VEGF with IBA4 was detected (vi). VEGF strongly co-localised with GFAP (iii) indicating astrocytes produced VEGF in these mixed glial cultures in response to OGD-reperfusion. Scale bar = 50 µm.
Figure 4.16. Luminal to abluminal permeability of keratinocyte-derived chemokine and vascular endothelial growth factor across the Transwell membrane and MBEC monolayer. 400ng/mL exogenous KC (A) and VEGF (B) was added to the luminal compartment of co-cultures. 24 h later media was collected from the luminal and abluminal compartments and analysed for KC and VEGF by ELISA. Data are mean ± SEM of four separate experiments. ND= none detected.
Figure 4.17. OGD-reperfusion induced glial RANTES production. RANTES expression (A) and secretion (B) by co-cultured glia during OGD and following 24 h reperfusion. Control cultures were kept in normoxic conditions for 24 h and reperfused for 24 h. Data are mean ± SEM of at least four independent experiments. *P<0.05, ***P<0.001 OGD vs. Ctrl. *P<0.05, **P<0.01 OGD + 24 h reperfusion vs. Ctrl + 24 h reperfusion (two way ANOVA with Bonferroni post hoc test)
4.4.2.4. Summary of OGD-reperfusion induced glial activation

From these experiments, exposure of glia to OGD over 24 h and reperfusion induces activation of transcription factors and production of certain cytokines, chemokines and growth factors. These responses are summarised in Figure 4.18.

![Figure 4.18](image-url)

**Figure 4.18. Summary of OGD-reperfusion induced activation of mixed glial cells.** Summary of responses by mixed glial cells during 4, 6, 10, 18 and 24 h OGD and during 24 h reperfusion.
4.5. Discussion
The aim of the experiments described in this chapter was to determine whether OGD and reperfusion induced inflammatory activation of MBECs and glia by investigating the activation of transcription factors, the production of specific cytokines, chemokines and growth factors and expression of endothelial cell adhesion molecules. In these studies, MBEC monocultures, glial monocultures and MBEC-glial co-cultures were exposed to various durations of OGD with or without 24 h reperfusion.

4.5.1. OGD-reperfusion induced MBEC and glial oxygen sensing
In these experiments oxygen was reduced to 1 % over a 24 h period as the viability of both MBECs and glia were maintained over this duration of OGD exposure (Chapter 3). Oxygen sensing is crucial for cell survival and the ability to adapt to changes in cellular oxygen tensions (Sharp et al., 2004). A well characterised cellular oxygen sensing pathway occurs through the induction of HIF. HIF-1 is a heterodimeric transcription factor composed of the constitutively expressed HIF-1β subunit and the oxygen-regulated HIF-1α subunit (Graven et al., 2003). OGD induced a time-dependent increase in nuclear HIF-1α expression in glial cells (Figure 4.10A and B). Therefore reduction of oxygen to 1 % was sufficient to cause glial HIF-1α stabilisation and translocation to the nucleus. The absence of glial HIF-1α expression in the nuclear and cytosolic fractions during reperfusion indicates that reoxygenation probably caused HIF-1α degradation in the cytoplasm (Figure 4.10C and D). One of the downstream gene targets of HIF is the glucose transporters. 24 h OGD induced an increase in glial expression of a 40-50 kDa GLUT-1 (Figure 4.11). This is consistent with findings that 45 kDa GLUT-1 is associated with non-vascular regions of the brain and is upregulated after experimentally-induced ischaemia in rodents (Maher et al., 1994).

The absence of HIF-1α or the endothelial specific HIF-2α expression by MBECs was unexpected. Both HIF-1α and HIF-2α have been shown to be stabilised and expressed in endothelial cells during hypoxia (Sharp and Bernaudin 2004). However in many of these other studies endothelial cells were exposed to anoxic or near anoxic (< 0.2 % oxygen) conditions (Graven et al., 2003). Therefore it may be that
the severity of oxygen deprivation determines the ability of endothelial cells to stabilise HIF. In the current study, the absence of HIF-1α expression by MBECs during OGD does not mean MBECs are insensitive to changes in oxygen. Recent studies have investigated the kinetics of hypoxic induction of HIF (Chamboredon et al., 2011). HIF mRNA and protein levels have been shown to increase with acute hypoxia (typically within 3 h) but progressively decline with prolonged hypoxia. This downregulation has been shown to be mediated by tristetrapolin (TTP) family members binding to the (AU)-rich elements (ARE) in the 3’-untranslated region (3’-UTR) of HIF mRNA, promoting mRNA decay. By acting in a negative feedback loop this regulatory system prevents excessive accumulation of HIF-1α during prolonged hypoxia (Chamboredon et al., 2011). In the experiments presented in this thesis, 4 h OGD was the earliest timepoint at which HIF-1α expression was analysed. Therefore by this timepoint, HIF-1α expression may have already peaked and subsequently declined. OGD induced a time-dependent increase in MBEC GLUT-1 expression, with peak expression at 24 h OGD exposure (Figure 4.1). Therefore alterations in endothelial glucose metabolism may also contribute to MBECs adaptation to hypoxia as well as to aglycaemia. GLUT-1 expressed by MBECs was observed as the more heavily glycosylated 55 kDa isoform which has been identified in other studies to be highly expressed by cerebral microvessels and is upregulated following experimentally-induced ischaemia in rodents (Maher et al., 1994; Vannucci et al., 1998b).

**4.5.2. OGD-reperfusion induced MBEC and glial transcriptional activation**

Another transcription factor induced by ischaemia is the redox-sensitive NFκB. The most common form of NFκB is a heterodimer composed of the p50 and p65 subunits (Wang et al., 2007). In the studies presented here, glial NFκB activation was dependent on the duration of OGD. Significant increases in glial nuclear and cytosolic expression of NFκB p65 were observed at 10 h OGD and at the end of reperfusion following 4 h OGD (Figure 4.13). OGD did not induce MBEC NFκB activation. MBECs expressed NFκB p65 predominantly in the cytosolic fraction during OGD indicating that the p65 subunit remains sequestered in the cytoplasm, presumably in association with IκB. However OGD did induce a time-dependent
increase in MBEC nuclear NFκB during reperfusion (Figure 4.2). A similar induction of NFκB activation has been observed by others in cultured human brain endothelial cell lines and primary human brain endothelial cells during reperfusion following a previous hypoxic exposure but not during hypoxia alone (Howard et al., 1998; Flamant et al., 2009). The activation of NFκB is a tightly regulated processes involving the inhibitory protein IκB, IκB kinases and NFκB-inducing kinases (Culver et al., 2010). Although the precise mechanism of NFκB activation during ischaemia-reperfusion injury is not fully understood, the generation of ROS during reperfusion has been associated NFκB activation (Wang et al., 2007). From these results it could be concluded that the cell type and experimental conditions determine NFκB activation.

### 4.5.3. OGD-reperfusion induced MBEC cell adhesion molecule expression

Cell adhesion molecules contribute to secondary post-ischaemic injury by facilitating leukocyte recruitment across the BBB. Elevated plasma and cerebrospinal fluid concentrations of soluble cell adhesion molecules have been identified in patients after ischaemic stroke making them potentially useful markers to study the pathology of cerebral ischaemia (Simundic et al., 2004). MBECs expressed very low levels of E-selectin (CD62E) that did not change during OGD or reperfusion as measured by CBA (Table 2, Appendix II). Human brain endothelial cells exposed to 4 h ischaemia in vitro transiently upregulate E-selectin expression during early reperfusion, returning to baseline levels by 24 h (Stanimirovic et al., 1997). Therefore in this current study, E-selection may have already been upregulated within the first few hours of reperfusion and subsequently reduced to basal levels by the end of 24 h reperfusion. In these studies, ICAM-1 and VCAM-1 were measured at the end of 24 h reperfusion following OGD. MBEC monocultures displayed no change in basal expression of ICAM-1 or VCAM-1 (Figure 4.3 and Figure 4.4). Expression of ICAM-1 and VCAM-1 by co-cultured MBECs was dependent upon the duration of the preceding OGD exposure. Figure 4.3 and 4.4 showed that both short (4 h) and prolonged (18 to 24 h) OGD exposure of co-cultured MBECs resulted in significant increases in ICAM-1 and VCAM-1 expression. These different durations of OGD may reflect clinical and experimentally induced-transient and permanent cerebral ischaemia. Elevated expression of ICAM-1 and VCAM-1 by the cerebral
endothelium has been observed after experimentally-induced transient and permanent cerebral ischaemia in rodents (Zhang et al., 1995; Ge et al., 2000). The non-contact co-cultures used in these experiments suggest that soluble mediators secreted by glia during reperfusion act on the abluminal surface of MBECs to induce ICAM-1 and VCAM-1 expression. Although several animal studies of experimentally-induced cerebral ischaemia and \textit{in vitro} stimulated ischaemia of endothelial cultures have identified an upregulation of cell adhesion molecule expression, the role of glia in regulating endothelial cell adhesion molecule expression has not been studied extensively. Zhang et al., (2000a) reported that IL-1β secreted by hypoxic astrocytes induced expression of many inflammatory mediators by cerebrovascular endothelial cells, including ICAM-1. Other studies have shown that \textit{in vitro} OGD induces transcription of cell adhesion molecules by human microvascular endothelial cells (Stanimirovic et al., 1997). In particular, NFκB activation in human brain endothelial cells during reperfusion after hypoxia \textit{in vitro} has been associated with ICAM-1 gene expression (Howard et al., 1998). Whether the elevated nuclear expression of NFκB observed in MBECs during reperfusion (Figure 4.2) contributes to ICAM-1 and VCAM-1 expression of these co-cultured MBECs requires further investigation. Future studies could investigate the mechanisms regulating ICAM-1 and VCAM-1 expression in response to different durations of OGD and identify what the glial-derived mediator(s) are regulating the expression of these cell adhesion molecules.

4.5.4. OGD-reperfusion induced MBEC and glial production of cytokine, chemokines and growth factors

In the experiments presented in this thesis, the production of a range of inflammatory mediators by MBECs and glia in response to OGD-reperfusion injury was investigated. Prominent changes in production of KC, VEGF, RANTES and MCP-1 by MBECs and glia during OGD and reperfusion were detected.

The results described in this study indicate that there was an interplay between MBECs and glia to regulate KC secretion. Over 24 h OGD there was no change in basal KC expression or secretion by either MBEC (Figure 4.5A and B) or glial monocultures (Figure 4.14A and B). Increased KC secretion by MBEC monocultures
during reperfusion after 18 or 24 h OGD indicates that prolonged OGD exposure of MBECs induces a significant upregulation in KC secretion during the subsequent reperfusion phase. However the presence of glia on the abluminal side of MBECs induced a significant increase in MBEC KC secretion during reperfusion after a much shorter OGD exposure (4 h) (Figure 4.5D), suggesting that glia have a role in regulating MBEC KC secretion after short exposures to OGD. This may have important implications in vivo, in that the duration of ischaemia may have an effect on glial-induced regulation of endothelial KC production. Reperfusion of glial monocultures had no effect on basal KC secretion. However when co-cultured with MBECs, a significant increase in glial KC secretion was measured during reperfusion following 4, 6 or 10 h OGD (Figure 4.14D). This suggests that MBECs also have a role in regulating glial KC secretion during reperfusion. Furthermore, although microglia constitute a relatively small proportion of the glial population (17 ± 3 %) (see chapter 3) they were identified to be the source of KC in the mixed glial cultures (Figure 4.14E). Indeed, it is well documented that microglia are activated by ischaemia and are likely to contribute to ischaemic injury (Mabuchi et al., 2000). KC, belonging to the CXC family of chemokines is associated with neutrophil accumulation into the ischaemic brain (Wang et al., 2007). Transient upregulation of KC and CINC, the rat homolog of KC, during reperfusion after transient ischaemia in mice and rats have been shown to precede neutrophil infiltration (Liu et al., 1993; Yamasaki et al., 1997). The role of MBEC and glial-derived KC in neutrophil transendothelial migration will be discussed in the next chapter.

The results of these experiments suggest that although OGD did not induce MBEC or glial KC production, restoration of oxygen and glucose supply augmented KC secretion and this was dependent on the duration of earlier OGD exposure. As already discussed, cerebral ischaemia induces a hypoxic response by cells, primarily mediated through HIF which is essential for cell survival in low oxygen environments. Cellular oxygen is crucial for ATP production that, in turn is required for protein synthesis. Therefore under ischaemic conditions when energy resources are limiting, suppression of non-essential energy-consuming processes including protein synthesis at both the translational and transcriptional level may also be essential for the cellular adaptation to a low oxygen environment (Koumenis et al.,
The absence of any increase in KC expression by MBECs or glia during reperfusion suggests that the upregulation in KC secretion during reperfusion may not have been under transcriptional control but rather was secretion of preformed KC. Gro-α, the human homolog of KC, has been identified to be stored in small granules throughout the cytoplasm of endothelial cells that are secreted rapidly upon cytokine stimulation (Øynerbråten et al., 2005).

OGD and reperfusion caused a time-dependent increase in VEGF secretion by both glial monocultures and co-cultured glia. The concentrations of VEGF secreted by co-cultured glia were similar to that secreted by glial monocultures, indicating MBECs had no effect on glial VEGF secretion (Figure 4.15). Additionally, the ELISA used to detect VEGF was specific for VEGF$_{121}$ and VEGF$_{165}$, which have been identified to be the main secreted isoforms of VEGF (Neufeld et al., 1999). VEGF is one of the targets of HIF and in these studies VEGF secretion correlated with OGD-induced glial nuclear HIF-1α expression. Astrocytes were found to be the source of VEGF in these mixed glial cultures (Figure 4.15E). This is consistent with other reports that have identified an upregulation in VEGF production by rat astrocytes within hours of OGD exposure (Sinor et al., 1998; Schmid-Brunclik et al., 2008) and after experimentally-induced ischaemia in rats in vivo (Kaur et al., 2006). The endothelium and neurones have also been shown to be sources of VEGF under hypoxic conditions in vivo and in vitro (Sun and Guo, 2005). Cultured porcine brain endothelial cells have also been shown to upregulate mRNA and abluminal expression of VEGFR1 and VEGFR2 under hypoxia conditions (Fischer et al., 1999). In the experiments reported in this thesis, MBECs did not express or secrete VEGF during OGD or reperfusion (data not shown) and the expression of the VEGF receptors was not explored. Elevated VEGF levels in the ischaemic brain of rodents and in in vitro cultures has shown to have both beneficial and detrimental effects on the outcome of ischaemic injury. VEGF has been shown to have a direct neuroprotective effect on cultured neurones after in vitro simulated ischaemia (Jin et al., 2000). Topical administration of VEGF to the surface of rat brains at the start of reperfusion after experimentally-induced ischaemia has been shown to reduce brain oedema and number of apoptotic cells within the ischaemic territory (Hayashi et al., 1998). Conversely, Zhang et al. (2000b) showed that early (1 h) administration of
VEGF to rat brains after ischaemia caused increased leakage of the BBB and haemorrhagic transformation, whereas late (48 h) administration of VEGF reduced brain oedema and the number of apoptotic cells within the ischaemic territory. Thus their study identified a dual role for VEGF in mediating ischaemic damage and recovery. In the present study, the effect of glial-derived VEGF during OGD and reperfusion on MBECs and the glia themselves could be investigated further. VEGF-mediated ischaemic damage is associated with enhancing vascular leakage of the BBB (Schoch et al., 2002). VEGF-mediated increases in BBB permeability are associated with disruption and down-regulation of tight junctions and tight junction accessory proteins (Wang et al., 2000; Fischer et al., 2002). In the present study there was no change MBEC ZO-1 localisation during OGD or reperfusion suggesting that VEGF secreted by glia during OGD and reperfusion had no effect on the tight junction accessory proteins.

MBECs expressed and secreted high concentrations of MCP-1 that were downregulated over 24 h OGD and after reperfusion (Figure 4.6). MCP-1 secretion by MBECs and astrocytes in co-culture has been shown to peak 24 to 48 h after OGD in vitro (Dimitrijevic et al., 2006). Therefore in the studies presented here, 24 h reperfusion may not have been long enough to observe an upregulation in MCP-1. Yamagami et al., (1999) reported a differential temporal production of MCP-1 and CINC regulated infiltration of different leukocytes into the ischaemic brains of rats following experimentally-induced ischaemia. They showed CINC concentration peaked at 6 h reperfusion, corresponding to early infiltration of neutrophils into the ischaemic brain. MCP-1 peaked at 48 h post-ischaemia resulting in later infiltration of macrophages.

RANTES expression and secretion by MBECs during reperfusion appeared to be dependent on the duration of OGD. An increase in RANTES secretion by MBECs was observed during reperfusion after 4 h OGD but not after longer OGD exposures (Figure 4.7). Glia displayed a transient increase in RANTES expression and secretion within 4 h OGD and during reperfusion that subsequently declined with longer durations of OGD (Figure 4.17). The absence of RANTES production by MBECs during a 24 h OGD exposure and by glia with longer durations of OGD (from 4 h)
may be because prolonged oxygen and glucose withdrawal results in a downregulation of RANTES protein synthesis by the endothelium and glia. Prolonged expression of RANTES has been implicated in reperfusion injury following experimentally-induced cerebral ischaemia in rodents, contributing to the delayed recruitment of macrophages and lymphocytes into the ischaemic brain (Bona et al., 1999). Furthermore, platelets and T-lymphocytes have been identified as a source of RANTES. Secretion of RANTES from these circulating blood cells has been shown to contribute to reperfusion injury following experimentally-induced cerebral ischaemia in mice (Terao et al., 2008). Therefore, to obtain a more detailed cellular and temporal profile of RANTES production during reperfusion in this OGD model, production of RANTES by MBECs and glia as well as by other cerebral and blood cells over a longer duration of reperfusion could be investigated.

CBA analysis revealed that MBECs and glia expressed and secreted low baseline levels of TNF-α, MIP-1, IL-6, IL-1α, IL-1β, IL-17α, IL-10, IFN-γ, G-CSF, CD62L and CD62E that did not change during OGD or reperfusion (Table 3, Appendix II). Many of these mediators are under the transcription control of NFκB. These experiments showed that MBECs displayed no NFκB activation during OGD and NFκB activation by glia during OGD and reperfusion was dependent on the duration of OGD exposure, which may explain why these NFκB-regulated cytokines were not upregulated. Flamant et al., (2008) showed that hypoxic exposure (1 % oxygen) of the immortalised human brain endothelial cell line, EA.hy926 cells, over a 24 h period with or without reperfusion reduced DNA binding activity of NFκB whereas HIF-1 binding activity was increased. This was concomitant with a reduction in the expression of a range of NFκB target genes and increased expression of genes transcriptionally regulated by HIF. They concluded that hypoxia-reperfusion injury does not induce an inflammatory phenotype in cultured endothelial cells, but rather endothelial responses to ischaemic injury are regulated predominantly by cellular oxygen-sensing pathways. The results presented in this thesis show that OGD and reperfusion challenge of MBECs as well as glial cells induces both hypoxic and inflammatory mediated responses. Discrepancies between the studies presented here and other published work may reflect differences in the in vitro ischaemic conditions and cell types used.
4.5.5. Effect of OGD-reperfusion on MBEC permeability

OGD-induced permeability changes were measured by the passage of 4 kDa FITC-dextran which crosses the MBEC monolayer via a paracellular route. MBEC monolayers displayed a transient increase in permeability to 4 kDa FITC-dextran by 4 h OGD that was subsequently reduced to basal levels with longer durations of OGD. The presence of glia on the abluminal side of MBECs restored the increased in MBEC permeability at 4 h OGD to basal levels (Figure 4.9A). This suggests that under conditions of OGD, glia contribute to maintaining low permeability of the MBEC monolayer. This is consistent with other *in vitro* studies that have identified secreted glial factor(s) prevent hypoxia-induced permeability changes in cultured brain endothelial cells (Fischer et al., 2000). Other *in vitro* co-culture studies have suggested that glia play a role in maintaining the barrier properties of the endothelium (Reinhart and Gloor., 1997). Moreover, the data in Figure 4.9 show that reperfusion induced no further alterations in permeability to FITC-dextran. Maintenance of an intact endothelium may be beneficial in an *in vivo* environment, reducing the extent of vascular leakage and subsequent damage after ischaemia. Several studies have identified increased permeability and leakage of the cerebral vasculature *in vivo* and of cultured endothelial monolayers *in vitro* during the ischaemic insult and the reperfusion phase. Increased paracellular permeability is associated the formation of intercellular gaps in the endothelium due to alterations in the localisation of tight junction proteins and the cytoskeleton (Mark and Davis, 2002; Witt et al., 2003). In this present study, the increase in permeability of MBEC monocultures at 4 h OGD was not associated with any disruption to the peripheral localisation of ZO-1 (Figure 4.8B). The functional importance of ZO-1 in the regulation of paracellular permeability has been demonstrated by studies that show increased ZO-1 expression correlates with increased TEER of cultured porcine brain endothelial cells (Krause et al., 1991). To further the studies presented here, the effect of OGD on the localisation of other tight junction proteins should be investigated. Animal models of cerebral ischaemia also show increased transcellular transport across the BBB caused by increased vesicular transport of the endothelial cells (Plateel et al., 1997). However this has not been very well characterised in *in vitro* endothelial cultures (Kvietys and Sandig, 2001). The precise mechanism of increased vascular permeability during ischaemia is still largely unknown and may
be a combination of paracellular and transcellular routes depending upon the severity and duration of injury.

4.5.6. Summary and conclusions

In the present study, MBECs and glia were exposed to OGD to mimic an ischaemic episode either with or without a return to normoxia and glucose containing media simulating the reperfusion phase. The results presented here show that OGD and reperfusion induced inflammatory activation of MBECs and glia. These inflammatory responses included the activation of transcription factors, production of cytokines, chemokine and growth factors and expression cell adhesion molecules. Re-establishment of oxygen and glucose was critical for regulating some of these responses. Furthermore, communication between MBECs and glia was important in the regulation of some of these responses. Thus, this study has identified specific endothelial and glial responses to ischaemic-like conditions that may have important consequences in the pathophysiology of ischaemia. Further studies could now proceed in vivo to determine whether if these in vitro findings are replicated in an in vivo animal model of cerebral ischaemia. Future studies should also identify the mechanisms underlying these endothelial and glial responses as detailed understanding of the mechanisms involved in cellular responses to ischaemia is crucial for the pharmacological development of therapeutics.
Chapter 5

Oxygen-glucose deprivation-reperfusion induced neutrophil-endothelial interactions
5. OGD-reperfusion induced neutrophil-endothelial interactions

5.1. Introduction

In the previous chapter, the inflammatory profile of MBECs and glia in response to OGD and reperfusion was established. In vivo, inflammatory activation of the endothelium and glial cells in response to ischaemia can lead to secondary post-ischaemic injury which involves the recruitment of circulating immune cells into the brain parenchyma. Neutrophils are the first type of leukocyte to infiltrate the brain followed by monocytes/macrophages and T lymphocytes (Hallenbeck et al., 1996). Leukocyte adhesion and diapedesis across the cerebral vasculature is mediated by chemoattractants, interactions between leukocyte integrins and endothelial cell adhesion molecules and secreted leukocyte products. Although the initial adhesive interactions between leukocytes and the endothelium have been well characterised, the mechanisms involved in leukocyte migration across the endothelium are still not fully understood. Studies of leukocyte diapedesis across both the cerebral and other vasculatures have shown that leukocytes migrate across regions of the endothelium of least resistance (DiStasi and Ley, 2009). The interendothelial junctions of endothelial monolayers in vitro are generally less well defined than their in vivo counterparts. Thus, leukocyte migration across the cultured endothelium was thought to occur predominantly via paracellular routes at these cell-cell junctions (Kvietys and Sandig, 2001). However the frequency of transcellular migration in vitro may be underestimated as transcellular events have been shown to occur within close proximity to endothelial junctions and can consequently be mistaken as paracellular migration (Wolburg et al., 2005).

In this chapter, the inflammatory activation of MBECs and glia induced by OGD-reperfusion regulating neutrophil adhesion and transendothelial migration during reperfusion was investigated.
5.2. Aims

- To identify if OGD-reperfusion induces neutrophil adhesion to and/or migration across MBEC monolayers.
- To determine the mechanisms regulating OGD-reperfusion induced neutrophil migration.

5.3. Methods

Neutrophils were isolated from the bone marrow of C57BL/6J mice by positive immunomagnetic separation for Ly6G-labelled cells (see section 2.2.4. for full description). Flow cytometry was performed to establish purity of neutrophils. Granulocytes were identified on a dot plot by forward and side-scatter characteristics. The neutrophil subpopulation was identified within this granulocyte population on a CD11b-FITC vs. Ly6G-PE dot plot. The proportion of neutrophils was expressed as a percentage of acquired events.

MBEC monocultures and MBEC-glia co-cultures were exposed to OGD for 4, 10 or 24h OGD. At the start of reperfusion, $2 \times 10^5$ neutrophils were added to MBECs in the luminal compartment of Transwells (polycarbonate membrane, 3 µm pore). At the end of 24 h reperfusion neutrophil adhesion and migration was quantified (see section 2.7 for full methods).

To investigate the mechanisms of neutrophil migration, MBECs, glia and neutrophils were treated with specific blocking or neutralising antibodies or inhibitors for 30 min at the start of reperfusion prior to the addition of neutrophils. The concentration of antibodies and inhibitors used were in accordance with manufacturer’s instruction (see Table 1, section 2.7.3.1, Chapter 2). Preliminary experiments showed that OGD-reperfusion induced MBEC KC secretion, ICAM-1 and VCAM-1 expression and glial KC and VEGF secretion was significantly reduced upon treatment with their specific blocking antibodies (Figure 7.1 – 7.5, Appendix III). Neutrophil migration at the end of reperfusion was quantified as described previously (see section 2.7 for full methods).
To investigate the effect of migrated neutrophils on glial viability, at the end of 24 h reperfusion, media in the abluminal compartment containing neutrophils that had migrated across the MBEC monolayer was removed. Glial cells were washed twice with DMEM and 600 µL reperfusion medium was added to the glial cells. 0.5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the glia and incubated for 2 h at 37 °C in a humidified CO\textsubscript{2} incubator. Medium was removed from the glial cells and MTT crystals were dissolved by addition of 500 µL propan-2-ol. Absorbance was read at 570 nm using a spectrophotometer (Synergy HT, NorthStar Scientific Ltd., UK).
5.4. Results

5.4.1. Purity of isolated neutrophils

Prior to purification of the bone marrow suspension on the MACS® LS column, FACs analysis revealed that neutrophils (CD11b-Ly6G double positive, side-scatter high (SS\textsubscript{high}) cells made up 54.3±3.7 % of the mixed-cell bone marrow suspension (Figure 5.1A and B). B lymphocytes (CD19-MHC class II double positive, SS\textsubscript{low} cells) were present in low amounts (16.1±0.4 %). Negligible amounts of monocytes were present (< 1 %) (Figure 5.1B). Other cells within the bone marrow suspension may have been T lymphocytes, NK cells, dendritic cells or mast cells. Positive selection for Ly6G-labelled cells yielded a population containing 90.5 ± 0.8 % neutrophils and the proportion of B lymphocytes was further reduced (Figure 5.1A and B).
Figure 5.1. Purification neutrophils from bone marrow. (A) Murine bone marrow suspension was analysed by flow cytometry for Ly6G-positive cells before and after purification on a MACS® LS column. Granulocytes in the unpurified whole bone marrow suspension (i) and the purified neutrophil fraction (iii) were identified from side-scatter (SS) vs. forward-scatter (FS) dot pots. Within these granulocyte populations, the neutrophil subpopulation of the whole bone marrow fraction (ii) and the purified neutrophil fraction (iv) were identified by gating within Ly6G-PE vs. CD11b-FITC dot plots. (B) Quantification of the proportion of neutrophils (CD11b-Ly6G double positive), monocytes (CD11b-CD14 double positive) and B lymphocytes (CD19-MHC class II double positive) in the bone marrow suspension before purification and in the final purified sample were expressed as a percentage of the number of acquired leukocyte events. Data are mean ± SEM of three separate cultures.
5.4.2. OGD-reperfusion induced neutrophil adhesion to MBECs.

Fluorescent ICC revealed a trend for increased neutrophil adhesion to MBEC monocultures and co-cultured MBECs during reperfusion after previous exposure to 4 or 24 h OGD compared to normoxic controls or 10 h OGD, however this did not reach statistical significance (Figure 5.2).
Figure 5.2. OGD-reperfusion induced neutrophil adhesion to MBECs. (A) Neutrophils were applied to MBECs monocultures and co-cultures at the start of reperfusion following 4, 10, 24 h OGD or 24 h normoxia (Ctrl). At the end of 24 h reperfusion, transwells were processed for ICC. Neutrophils were stained for SJC-4 and MBECs were stained for ICAM-1. Scale bar= 50 µm. Quantification of the number of SJC-4 positive cells attached MBEC monocultures (B) and Co-cultured MBECs (C) was expressed as a percentage of the total number of neutrophils added to MBECs, averaged from four separate experiments using at least five different fields of view. One way ANOVA revealed that this was not statistically significant.
5.4.3. OGD-reperfusion induced neutrophil transendothelial migration

Under normoxic conditions, a very low number of neutrophils migrated across MBEC monocultures (1.1±0.3 %). Exposure of MBEC monocultures to 4, 10 or 24 h OGD had no effect on this basal level of neutrophil migration during reperfusion (Figure 5.3). Co-culturing MBECs with glia increased neutrophil migration to 2.6 ± 0.4 % under normoxic conditions. Addition of neutrophils upon reperfusion to co-cultured MBECs previously exposed to 4 or 24 h OGD resulted in a significant increase in neutrophil migration (5.2±0.8 % and 4.8±0.7 % respectively) compared to normoxic co-cultured MBECs and MBEC monocultures (Figure 5.3).

![Figure 5.3. OGD-reperfusion induced neutrophil transendothelial migration. MBEC monocultures and co-cultures were exposed to 4, 10 or 24 h OGD. Control monocultures and control co-cultures were kept in normoxic conditions. Neutrophils were added to MBECs at the start of reperfusion. Neutrophil migration was quantified by counting the number of neutrophils in the abluminal compartment and expressed as a percentage of total number of neutrophils added to transwells (2x10^5 neutrophils). Data are mean ± SEM of eight separate experiments. *P<0.05 Co-culture OGD vs. Ctrl Co-culture. **P<0.05 monoculture OGD vs. co-culture OGD (two way ANOVA with Bonferroni post hoc test).]
5.4.4. Mechanisms of OGD-reperfusion induced neutrophil migration

The increase in neutrophil migration across co-cultured MBECs during reperfusion following 4 h OGD was significantly reduced (62.5 %) by an antibody directed against ICAM-1 (Figure 5.4A). Simultaneous application of anti-ICAM-1 and anti-VCAM-1 antibody to MBECs also caused a reduction in neutrophil migration, but this was not significantly different to blocking ICAM-1 alone indicating VCAM-1 did not contribute to neutrophil migration (Figure 5.6A). Additionally, treatment of neutrophils with an anti-CD18 antibody and MBECs with an anti-ICAM-1 antibody resulted in a synergistic (84.0 %) reduction in neutrophil migration (Figure 5.6A). Chelation of MBEC calcium by pre-treatment with BAPTA-AM resulted in a 72.8 % reduction in neutrophil migration during reperfusion (Figure 5.4A). The viability of MBECs was not affected by pre-treatment with BAPTA-AM as assessed by LDH release (data not shown). Figure 5.5A shows that glia had a role in neutrophil migration. Heat-inactivated glial conditioned media and reperfused glial-conditioned medium of less than 50 kDa caused a significant reduction in neutrophil migration after 4 h OGD.

The increase in neutrophil migration across co-cultured MBECs during reperfusion following 24 h OGD was significantly reduced by an antibody directed against ICAM-1 when used simultaneously with an antibody directed against VCAM-1 (68.4% reduction) (Figure 5.4B) or with CD18 antibody treatment of neutrophils (57.9 % reduction) (Figure 5.6B). Chelation of MBEC calcium by pre-treatment with BAPTA-AM resulted in a 72.0 % decrease in neutrophil migration during reperfusion (Figure 5.4B). Abluminal application of heat inactivated reperfused glial conditioned medium (following 24 h OGD) caused a 79.1 % reduction in neutrophils migration. This glia-derived mediator(s) may have been less than 50 kDa as indicated by a decrease in neutrophil migration with glial conditioned medium of less than 50 kDa (Figure 5.5B). Furthermore, glial-derived VEGF may have been one of these mediators as shown by a 68.8 % reduction in neutrophil migration across the MBECs when glia were treated with an anti-VEGF antibody (Figure 5.5B).
Figure 5.4. Role of MBECs in neutrophil transendothelial migration following 4 and 24 h OGD.

At the start of reperfusion following 4 h OGD (A) and 24 h OGD (B) of co-cultures, MBECs were treated with antibodies directed against ICAM-1, VCAM-1, PECAM and KC. IgG1 and IgG2A served as the isotype controls. MBECs were pretreated with the IL-1 receptor antagonist IL-1Ra and BAPTA-AM for 30 min and then washed twice with DMEM prior to the addition of neutrophils. 2x10⁵ Neutrophils were added to the transwells and migration was quantified at the end of 24 h reperfusion. The blue line indicates neutrophil migration during reperfusion after 4 h OGD (A) or 24 h OGD (B) without any additional treatment of MBECs. Data are mean ± SEM of at least six separate experiments. *P<0.05 neutrophil migration across treated MBECs after OGD vs. untreated MBECs after OGD (one way ANOVA with Tukey’s multiple comparison test).
Figure 5.5. Role of glial-derived secreted mediators in neutrophil transendothelial migration following 4 and 24 h OGD. At the start of reperfusion following 4 h OGD (A) and 24 h OGD (B) of co-cultures, glial cells were treated with antibodies directed against KC and VEGF. IgG1 served as the isotype control. Heat-inactivated reperfused glial conditioned media following 4 or 24 h normoxia (HI Nx CM) or OGD (HI OGD CM) was applied to the abluminal compartment of the co-cultures. Reperfused glial MWCO conditioned medium of >50 kDa, <50kDa, <10 kDa, <3 kDa generated after 4 or 24 h OGD was added to the abluminal compartment of the co-cultures. Neutrophils were added to the transwells and migration was quantified at end of 24 h reperfusion. The blue line indicates neutrophil migration during reperfusion after 4 h (A) OGD or 24 h OGD (B) only. Data are mean ± SEM of at least six separate experiments. *P<0.05 treated glia vs. untreated glia (one way ANOVA with Tukey’s multiple comparison test).
A

![Graph A](image1.png)

Treatment of neutrophils / MBECs

B

![Graph B](image2.png)

Treatment of neutrophils / MBECs

Figure 5.6. Role of neutrophil integrins in neutrophil migration following 4 and 24 h OGD. At the end of 4 h OGD (A) or 24 h OGD (B) neutrophils were pre-treated for 30 min with anti-CD18 antibody or the IgG1 isotype control. Neutrophils were washed twice with DMEM and added to MBECs in the presence or absence of anti-ICAM-1 and anti-VCAM-1 antibody. The blue line indicates neutrophil migration during reperfusion after 4 h (A) OGD or 24 h OGD (B) only. Data are mean ± SEM of at least seven separate experiments. *P<0.05 (one way ANOVA with Tukey’s multiple comparison test).
5.4.5. Effect of neutrophil migration on MBEC permeability

Addition of neutrophils to co-cultured MBECs previously exposed to 4, 10 or 24 h OGD had no effect on permeability of MBECs to 4 kDa FITC-dextran during reperfusion (Figure 5.7A). As reported in Chapter 4, OGD and reperfusion had no effect on ZO-1 localisation (Figure 4.9B). Neutrophils had no further effect on the cellular localisation of ZO-1 (Figure 5.7B).

**Figure 5.7. Effect of neutrophils on MBEC permeability.** (A) Permeability of co-cultured MBECs to 4 kDa FITC-dextran was measured during 24 h reperfusion following 4, 10 or 24 h OGD exposure in the absence or presence of neutrophils. Data are mean ± SEM of six separate experiments. (B) Effect of neutrophils on MBEC ZO-1 localisation under normoxic conditions (i) or following previous exposure of MBECs to 4 h (ii) or 24 h OGD (iii). Scale bar = 75 μm.
5.4.6. Effect of transmigrated neutrophils on glial viability or proliferation

OGD induced a time-dependent decrease in the viability of glia (assessed from the reduction of MTT to formazan) during subsequent reperfusion. Migration of neutrophils across the MBEC monolayer onto glia in the abluminal compartment restored the ability of glia to reduce MTT to formazan (Figure 5.8). Application of naïve neutrophils directly onto glia in the abluminal compartment had no effect on OGD-induced glial reduction of MTT during reperfusion. (Figure 5.8)

**Figure 5.8. Effect of migrated neutrophils on glial MTT reduction.** At the start of reperfusion, naïve neutrophils were added directly to glia in the abluminal compartment and neutrophils that were to transmigrate were added to the inside of the Transwell insert. At the end of 24 h reperfusion, glial cells were washed twice to remove neutrophils and 0.5 mM MTT was added to the glia. Data are mean ± SEM of six separate experiments. ""P<0.05, """"P>0.001 OGD + 24 h reperfusion vs. Ctrl + 24 h reperfusion. """"""""P<0.01 Glia (no neutrophils) vs. glia + transmigrated neutrophils (two way ANOVA with Bonferroni post hoc test).
5.5. Discussion

The results presented in this chapter show that exposure of MBECs and glia to OGD induces neutrophil migration across the MBEC monolayer during the subsequent reperfusion phase. The number of neutrophils that migrated was dependent upon the duration of exposure to OGD and was regulated by a combination of MBEC, glial and neutrophil-derived mediators.

A simple method of obtaining neutrophils is directly from the blood, however due to the low blood volume in mice, the number of neutrophils obtained this way are very limited. In this study, neutrophils were derived from the bone marrow of mice. The bone marrow contains a heterogeneous population of cells and here it was established that neutrophils made up over 50% of the bone marrow population. To obtain a pure neutrophil population, neutrophils were isolated by positive selection for Ly6G-positive cells. Ly6G is a surface antigen highly expressed by neutrophils with low expression by eosinophils, dendritic cells and developing monocytes making this a good marker for neutrophils (Daley et al., 2008). Positive selection for Ly6G-labelled cells increased neutrophil content in the final purified fraction to 90.5±0.8%. These neutrophils were likely to be mature neutrophils as the bone marrow of mice stores mature neutrophils prior to their release into the circulation (Boxio et al., 2004). Furthermore, several studies have showed that bone marrow derived neutrophils isolated by positive immunomagnetic separation do not cause activation of the neutrophils and they retain morphological and functional characteristics of mature neutrophils (Cotter et al., 2001; Hasenberg et al., 2011). Additionally, compared to neutrophils isolated from the blood, bone marrow derived PMN neutrophils survive significantly longer in culture, suggesting that they receive anti-apoptotic signal(s) that are absent in the blood (Boxio et al., 2004).

5.5.1. OGD-reperfusion induced neutrophil adhesion and migration

Fluorescent ICC revealed a trend towards an increased number of neutrophils attached to MBECs during reperfusion following 4 or 24 h OGD, however this was not statistically significant (Figure 5.2). Although ICC gave an indication of the number of neutrophils attached to MBECs during reperfusion it was not possible to determine whether leukocytes were rolling, firmly adhered or starting to
transmigrate. Using more sophisticated methods such as scanning and transmission electron microscopy it would be possible to observe the positioning of leukocytes on endothelial cells and formation of leukocyte pseudopodia, thus giving a more detailed analysis of the stage of adhesion or migration of the leukocytes. Using these methods, other studies have shown that neutrophils adhere to cerebral microvessels within 4 h of reperfusion after experimentally-induced ischaemia in mice (Ishikawa et al., 2004). Ichikawa et al. (1997) showed that a biphasic increase in neutrophil adhesion occurred after 30 min and 4 h of reperfusion following previous exposure of endothelial cultures to hypoxia in vitro. The results presented in this chapter quantified neutrophil adhesion only at the end of the 24 h reperfusion period. Therefore, maximal neutrophil adhesion may have also already occurred earlier in reperfusion. Engagement of neutrophil integrins with the Ig superfamily of cell adhesion molecules expressed by endothelial cells induces activation of neutrophils. In addition to neutrophil degranulation, neutrophil activation also involves clustering of neutrophil integrins and rearrangement of the cytoskeleton. This results in the strengthening of adhesion between neutrophils and the endothelium (Labrador et al., 2002; Woodfin et al., 2010). Stanimorovic et al. (1997) identified a greater proportion of activated neutrophils adhered to hypoxic human cerebral endothelial cells during reperfusion compared to the adhesion of unstimulated neutrophils. Therefore, in the experiments presented in this chapter the absence of any significant neutrophil adhesion to MBECs during reperfusion may have also been due to the fact neutrophils were not activated prior to their addition to MBECs.

Despite the simplicity of the Transwell system for studying leukocyte transmigration, very few studies have investigated leukocyte migration across ischaemic cerebral endothelial cells in vitro. Neutrophils are the first type of leukocyte to infiltrate the ischaemic brain and therefore it is necessary to consider their contribution in the pathophysiology of ischaemic injury. Co-culturing MBECs with glia resulted in a significant 2.3 fold increase in neutrophil migration compared to normoxic monocultures (Figure 5.3). Exposure of co-cultures to 4 or 24 h OGD induced a further increase in neutrophil migration compared to normoxic co-cultures or after 10 h OGD. These results suggest that the presence of glia induces a basal level of neutrophil migration which is augmented following exposure of MBECs and glial cells to short or prolonged OGD. These findings may have important clinical
implications because the duration of ischaemia may determine the extent of subsequent neutrophil migration during reperfusion and glia may also have a key role in neutrophil migration.

One limitation of the model used in this thesis is the absence of shear flow. Physiological shear flow has been shown to influence leukocyte interactions with the endothelium in which shear stress induces conformational changes in leukocyte integrins that increases the avidity of these adhesion molecules (Toetsch et al., 2009). *In vitro* flow systems and *in vivo* intravital microscopy allows visualisation of leukocyte trafficking under physiological conditions of flow and in real-time. Using intravital microscopy, Abbassi et al. (1993) showed that E-selectin supported the rolling of neutrophils on IL-1β activated HUVECs under conditions of flow that preceded ICAM-1 dependent firm adhesion to the endothelial cells. Studies have shown transmigration of T lymphocytes *in vitro* is enhanced with shear flow compared to static conditions (Cinamon et al., 2001). The current study only focused on neutrophil migration after *in vitro* stimulated ischaemia. *In vivo* studies of experimentally-induced ischaemia in rodents have shown that monocytes/macrophages and T lymphocytes infiltrate the ischaemic brain during later stages of reperfusion, typically 2 to 7 days after the ischaemic episode (Yamagami et al., 1999; Prabhakar et al., 2010). To further the studies presented in this chapter, future experiments should also investigate if OGD induces transendothelial migration of monocytes or T lymphocytes across MBECs during reperfusion.

### 5.5.2. Mechanisms of OGD induced neutrophil migration

The results presented in this chapter show that different mechanisms regulate neutrophil transendothelial migration following 4 h and 24 h OGD exposure of co-cultures (Figure 5.4 to 5.6). The increase in neutrophil transendothelial migration during reperfusion correlated with the upregulation in ICAM-1 and VCAM-1 expression by MBECs induced by 4 and 24 h OGD (Figure 4.3 and Figure 4.4, Chapter 4). ICAM-1 appeared to have a more prominent role in neutrophil migration following short (4 h) OGD compared to prolonged (24 h) exposure as blocking ICAM-1 alone after 4 h OGD caused a significant reduction in neutrophil migration.
ICAM-1 has been identified as a major contributor to neutrophil migration across the BBB in vivo and cultured endothelial cells after experimentally induced ischaemia (Stanimirovic et al., 1997). Blocking both ICAM-1 and VCAM-1 caused a significant reduction in neutrophil migration after 24 and 4 h OGD (Figure 5.4). However after 4 h OGD, this reduction in migration was no different to blocking ICAM-1 alone indicating that VCAM-1 was not involved in neutrophil migration. The contribution of VCAM-1 to neutrophil migration following 24 h OGD was interesting as VCAM-1 is implicated in adhesion and migration of lymphocytes through binding VLA-4 expressed by lymphocytes (Ley et al., 2007). However recent studies have reported that cross-linking of VCAM-1 causes the disruption and loss of VE-cadherin resulting in the formation of gaps between endothelial cells that allows the migration of leukocytes (van Wetering et al., 2003). In the present study, the antibody directed against VCAM-1 may have induced cross-linking of MBEC VCAM-1. Additionally, simultaneously blocking of endothelial ICAM-1 and neutrophil CD18 resulted in a synergistic reduction in neutrophil migration after 4 and 24 h OGD. This suggests that both ICAM-1 and neutrophil integrins contribute for neutrophil migration. However, in these experiments, by blocking the common β₂-integrin on neutrophils it was not possible to identify which integrin αβ heterodimer(s) was involved in neutrophil migration.

This present study also showed that intracellular MBEC calcium regulated neutrophil migration after 4 and 24 h OGD, as demonstrated by the reduction in neutrophil migration after with chelation of MBEC calcium with BAPTA-AM. Although the precise role(s) of calcium in the regulation of leukocyte trafficking following cerebral ischaemia remains to be elucidated, intracellular endothelial calcium has been implicated in several distinct steps of leukocyte adhesion and migration across cultured endothelial cells. A rise in endothelial calcium causes release of P-selectin from Weibel-Palade bodies. Interaction of leukocyte integrins with the endothelial Ig superfamily of cell adhesion molecules results in a rise in endothelial calcium that leads to firm adhesion of leukocytes. Clustering of ICAM-1 and cytoskeletal rearrangements also result in rises in endothelial calcium. (Kielbassa-Schnepp et al., 2001; Etienne-Manneville et al., 2000). Chelation of endothelial calcium has been shown to diminish migration of T lymphocytes and PMN cells across cultured brain endothelial cells without affecting their adhesion (Etienne-Manneville et al., 2000).
This highlights the difference in importance of intraendothelial calcium in leukocyte adhesion and migration. The precise role of MBEC calcium in the migration of neutrophils shown in this chapter should be explored further.

Heat inactivation of reperfused glial conditioned media and removal of molecules with a molecular weight less than 50 kDa caused a significant reduction in neutrophil migration following 4 and 24 h OGD (Figure 5.5). This suggests a proteinaceous glial factor of less than 50 kDa contributes to neutrophil migration across MBECs during reperfusion. One of these glial-derived mediators may be the 42 kDa VEGF as indicated by a significant reduction in neutrophil migration after 24 h OGD by treatment of glia with an anti-VEGF antibody (Figure 5.5B). As reported in Chapter 4, astrocytes were a prominent source of VEGF during reperfusion after 24 h OGD. Therefore in this model, OGD and reperfusion induced glia-derived VEGF secretion may have a role in regulating neutrophil migration across MBECs through acting on the abluminal membrane of MBECs as well as acting in an autocrine manner on the astrocytes and other glial cells. Published studies have shown that VEGF indirectly mediates leukocyte migration by inducing the production of chemokines by endothelial and glia cells and upregulating endothelial expression of ICAM-1, VCAM-1 and E-selectin (Kim et al., 2000; Lee et al., 2002). However VEGF regulation of endothelial cell adhesion molecules has been observed only in non-cerebral vasculature (Kim et al., 2001). Although VEGF increases the permeability of the endothelium via the disruption of tight junction proteins after cerebral ischaemia, no study to date has identified if this is associated with leukocyte migration. In rat traumatic brain injury, Chodobski et al. (2003) showed that within hours of brain injury, deposition of neutrophil-derived VEGF within the extracellular matrix correlated with early opening of the BBB and subsequent neutrophil infiltration into the brain parenchyma. The precise mechanism of VEGF-induced neutrophil migration in this OGD model should be explored further.

The significant upregulation in secretion of the murine neutrophil chemokine KC by co-cultured MBECs and glia after 4 h OGD (Figure 4.5 and Figure 4.14, Chapter 4) apparently did not contribute to neutrophil migration (Figure 5.4 and Figure 5.5). The neutralising KC antibody used in this study caused an 18 and 43-fold decrease in KC secretion by MBECs and glia respectively (Figure 7.1. and Figure 7.4,
Therefore the inability to reduce neutrophil migration was not due to a failure to prevent KC activity. Other neutrophil chemoattractants such as MIP-1α (CCL3) may have been upregulated by MBECs and glia and induced neutrophil migration even in the absence of KC.

This section of work demonstrated that neutrophil migration across MBECs was dependent upon the duration of the preceding OGD exposure. Importantly, different mediators regulated neutrophil migration after short and prolonged OGD. These mediators contributing to neutrophil migration are summarised in Table 2. This complexity in neutrophil migration may have significant implications in vivo when targeting neutrophil infiltration as a potential therapeutic strategy.

<table>
<thead>
<tr>
<th>4h OGD + reperfusion</th>
<th>24h OGD + reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBEC ICAM-1</td>
<td>MBEC ICAM-1 + VCAM-1</td>
</tr>
<tr>
<td>MBEC ICAM-1 + neutrophil CD18</td>
<td>MBEC ICAM-1 + neutrophil CD18</td>
</tr>
<tr>
<td>Soluble glial mediator (&lt;50kDa)</td>
<td>Soluble glial mediator (&lt;50kDa)- VEGF?</td>
</tr>
<tr>
<td>MBEC intracellular Ca^{2+}</td>
<td>MBEC intracellular Ca^{2+}</td>
</tr>
</tbody>
</table>

Table 2. Cellular mediators contributing to neutrophil migration after 4 and 24 h OGD.

### 5.5.3. Effect of migrated neutrophils on MBEC permeability

The results discussed above identified the cellular mediators that may contribute to neutrophil migration after OGD, however it was not possible to determine the route of neutrophil migration across the MBEC monolayer. As shown in chapter 4, OGD or reperfusion did not alter permeability of co-cultured MBECs to FITC-dextran. Neutrophils migrating across co-cultured MBECs had no further effect on MBEC permeability to FITC-dextran and there were no changes in the localisation of ZO-1. This suggests that neutrophil migration was not associated with changes in MBEC paracellular permeability. The route of neutrophil migration across the BBB is still not fully understood, in part because of discrepancies between different experimental approaches. Activation of neutrophils induced by engagement of integrins with endothelial cell adhesion molecules includes neutrophil degranulation. Upon
degranulation, neutrophils release a range of cytotoxic and inflammatory mediators, enzymes and plasma proteins. The serine proteases elastase and cathepsin G target cadherins for digestion resulting in increased vascular permeability (DiStasi and Ley, 2009). Collard et al. (2002) showed that neutrophil-derived glutamate increased permeability of the BBB after *in vivo* hypoxia in mice. This is in contrast to other studies that have shown that neutrophils restore permeability of the endothelial monolayer to FITC-dextran after a short (1 h) exposure to OGD (Cowan and Easton, 2010). There is substantial evidence that neutrophils traverse the peripheral endothelium via a transcellular route (Carman et al., 2004). Although neutrophil migration across the BBB after cerebral ischaemia has not been very well characterised, von Wedel-Parlow et al. (2010) gave evidence for a transcellular migratory pathway of neutrophils across an *in vitro* BBB model. They showed that TNF-α activated porcine cerebral endothelial cells extended surface membrane protrusions that contacted neutrophils that were penetrating the centre of the endothelial cell. This indicates that neutrophils can migrate across an *in vitro* BBB via a transcellular route.

### 5.5.4. Effect of migrated neutrophils on glia

Neutrophils contribute to secondary injury through the production of inflammatory cytokines, release of serine proteases and MMPs (DiStasi and Ley, 2009). Studies have shown that blocking neutrophil infiltration into ischaemic brain reduces infarct volume and improves behavioural deficits following transient ischaemia in rodents, indicating neutrophils have a deleterious role in cerebral ischaemia (Garau et al., 2005). To date, no other *in vitro* cerebral ischaemia studies have identified the effect of transmigrated neutrophils on glia. The results presented in Figure 5.8 show that OGD induced a time-dependent decrease in the ability of glia to reduce MTT to formazan during reperfusion, indicative of a loss of viability. After 24 h OGD, migrated neutrophils but not freshly isolated naïve neutrophils restored the ability of glia to reduce MTT. This suggests that migrated neutrophils restored viability of glia or induced glial proliferation. The consequences of this *in vivo* may result in further exacerbation of the inflammatory response by glia. Further studies should distinguish whether this is a result of increased glial viability or proliferation by using specific assays. For example uptake of neutral red or exclusion of trypan blue will give an indication of cell viability, whereas, incorporation of the synthetic
nucleoside, bromodeoxyuridine into the DNA of dividing cells will give a direct readout of cell proliferation. Reactive astrogliosis is a key feature of cerebral ischaemia exerting both harmful and beneficial effects (Stoll et al., 1998). Moreno-Flores et al. (1993) demonstrated that application of PMN neutrophils to astrocyte cultures initially caused astrocyte death characterised by aggregation, detachment from the substrata and association with granules secreted by neutrophils. After 6 days in vitro, stellate type 2 astrocytes grew out from these aggregates indicative of a reactive astrocyte response to this injury.

**5.5.5. Summary and conclusions**

The studies presented in this chapter show that neutrophil migration was dependent upon the duration of the preceding OGD exposure of MBECs and glia. Different mechanisms regulated neutrophil migration following short and prolonged OGD which is likely to be a reflection of the temporal activation of the MBECs and glia to OGD. This may have important clinical implications, in that the duration of an ischaemic episode may determine the extent of neutrophil infiltration during subsequent reperfusion. Furthermore, neutrophil infiltration into the ischaemic brain may be regulated by different mechanisms dependent upon the type and duration of ischaemia. This poses more complications for attempts to target the neutrophils as a potential therapeutic strategy. Additionally, in this study, migrated neutrophils were shown to have a beneficial effect on glia, either restoring glial viability or inducing glial proliferation. Again, the clinical implications of this may result in further exacerbation of the inflammatory response after an ischaemic episode.
Chapter 6
General discussion and conclusions
6. Discussion

6.1. Aims of this thesis

Inflammatory mediators play a pivotal role in the pathophysiology of cerebral ischaemia. Given that cerebral ischaemia is largely referred to as a vascular disease, an understanding of inflammatory responses at the level of the BBB is critical for the development of anti-inflammatory strategies that may have a significant role in ameliorating ischaemic brain damage. Therefore the overall aim of this thesis was to determine whether conditions mimicking cerebral ischaemia in vitro induce inflammatory activation of brain endothelial cells and glial cells and whether cross-talk between endothelium and glial cells regulate their inflammatory responses. To this end, murine derived cerebral endothelial cells and glial cells monocultures and co-cultures were exposed to OGD with or without reperfusion.

6.2. Summary of the findings described in this thesis

An in vitro OGD model was optimised to study the inflammatory activation of primary murine brain derived endothelial cells and primary murine mixed glial cells. The BBB was reconstructed in vitro based on co-culture of MBECs and glia, thus providing a tool to study cellular responses to oxygen and glucose withdrawal at the BBB level. The viability of MBCs and glia to OGD was dependent upon the duration and severity of oxygen withdrawal (Chapter 3). MBEC and glia viability were maintained during a 24h exposure to OGD at 1 % oxygen, making this a suitable duration and oxygen concentration to study their inflammatory responses. The inflammatory activation of MBECs and glial cells, summarised in Figure 6.1, was established by the expression of transcription factors, expression and secretion of certain cytokines, chemokines and growth factors and endothelial expression of cell adhesion molecules (Chapter 4). In particular, communication between MBECs and glia apparently regulated their secretion of KC. Secreted glial mediator(s) also regulated MBEC expression of ICAM-1 and VCAM-1 during reperfusion and this was dependent upon the duration of previous OGD exposure. MBEC and glial activation after short or prolonged exposure to OGD was associated with an increase in neutrophil transendothelial migration (Figure 5.3, Chapter 5). In particular, co-cultured MBEC upregulation in ICAM-1 and VCAM-1 and glial-derived VEGF secretion contributed to neutrophil migration. Additionally, other secreted glial-
derived mediators and MBEC intracellular calcium may also have contributed to neutrophil migration (Figure 5.4-5.6, Chapter 5). Transmigrated neutrophils restored glial viability or promoted glia cell proliferation after 24 h OGD (Figure 5.8, Chapter 5). Thus, it was concluded that MBEC and glial inflammatory activation induced by OGD may play a significant role in mediating neutrophil transendothelial migration. Neutrophils that have migrated across the BBB may then further exacerbate the post-ischaemic inflammatory response by acting upon the glia.

Figure 6.1. Summary of MBEC and glial responses to OGD and reperfusion. (1) OGD induces MBEC and glial nuclear stabilisation of HIF-1α and cellular expression of GLUT-1. Glial cells also express the NFκB p65 subunit in the nucleus.
(2) OGD induces secretion of VEGF by astrocytes. VEGF may act in an autocrine or paracrine manner on neighbouring microglial cells, oligodendrocytes or at the abluminal surface of MBECs.
(3) Upon reperfusion, MBECs increase nuclear expression of the NFκB p65 subunit. Expression of nuclear NFκB p65 by glia is dependent upon the duration of the preceding OGD exposure.
(4) Reperfusion induces MBECs to increase secretion of KC, expression and secretion of RANTES and expression of ICAM-1 and VCAM-1. Microglia increase secretion of KC and astrocytes continue to secrete VEGF.
(5) The presence of neutrophils at the luminal membrane of MBECs during reperfusion leads to increased neutrophil transendothelial migration after 4 and 24 h OGD. Neutrophil migration may be regulated by neutrophil integrins (CD18) engaging with ICAM-1 and VCAM-1 and secreted soluble glial derived mediators, including VEGF. MBEC intracellular Ca\textsuperscript{2+} may also have a role in regulating neutrophil migration.
6.3. Inflammation in cerebral ischaemia
As illustrated in Figure 1.3 (Chapter 1), post-ischaemic inflammation is a complex and multifaceted process that involves vascular, glial and neuronal components of the CNS. The extent of inflammatory-mediated tissue damage may depend upon the severity of ischaemia and the timing of cellular activation. Therefore, understanding the temporal profile of cellular responses and interactions that drive these inflammatory responses is essential for determining the timeframe and targets for effective pharmacotherapy.

6.3.1. Endothelial-glial cell inflammatory interactions in cerebral ischaemia
The close proximity between endothelial cells of the BBB, astrocyte end-feet and perivascular glial cells is proposed to play a significant role in regulating inflammatory responses at the site of the BBB. Whether cell-cell contact or secreted factors drive these inflammatory responses may be dependent upon the severity or duration of ischaemia or the brain region affected. Zhang et al. (2000a) proposed that inflammatory activation of endothelial and glial cells may be induced initially via signals within the endothelial and glial cell themselves, for example transcription factor activation and the expression of early response genes. Sustained activation is then likely to be mediated by pro-inflammatory mediators secreted by endothelial cells and glia that act in an autocrine and paracrine fashion (Zhang et al., 2000b). Consistent with this notion, the results described in Chapter 4 of this thesis identified MBEC and glial cell responses to OGD involved the activation of both hypoxic (HIF-1α) and inflammatory (NFκB) regulated transcription factors and their downstream target genes such as VEGF, GLUT-1, ICAM-1, VCAM-1, KC and RANTES. Cross-talk between the MBECs and glia may have contributed to regulation of their responses to OGD. This communication was likely to be mediated by secreted soluble factors because of the non-contact nature of the co-cultures. This is in agreement with other in vitro studies that have shown that secreted endothelial and glial mediators act in a paracrine manner during OGD injury (Brillualt et al., 2002; Yenari et al., 2006).
Association of perivascular glial cells and contact of astrocyte endfeet with the abluminal surface of the endothelium have been recognised to play a role in regulating phenotypic properties of the endothelium (Abbott et al., 2006), permeability of the BBB and functional properties such as drug efflux (Reinhardt and Gloor, 1997). Ischaemic disruption to the BBB is associated partly with loss of integrin expression by both the endothelial cells and glial cells and upregulation of astrocytic AQP4 leading to astrocytic swelling (Panickar and Norenberg, 2005; Abbott et al., 2006). Consequently, detachment of glia from the vasculature results in the loss of glial regulation of the endothelium, contributing to secondary injury including oedema and haemorrhage. This highlights the significance of maintaining endothelial-glial contact for preservation of the integrity of the BBB.

6.3.2. Endothelial-neuronal inflammatory interactions in cerebral ischaemia

Although endothelial cells of the cerebral microvessels and neurones are not in direct physical contact, astrocytes provide both a structural and functional link between them. Thus, it was once considered that the relationship between the endothelium and neurones was mediated indirectly via these intervening astrocytes. However, homeostatic interactions between the endothelium and neurones are essential for the regulation of angiogenesis and neurogenesis. Secreted soluble factors from brain endothelial cells stimulate proliferation and neurogenesis of neuronal stem cells (Shen et al., 2004). Inhibition of vascular-derived angiogenic signals suppresses neuronal regeneration within the ischaemic tissue after experimentally-induced cerebral ischaemia in mice (Taguchi et al., 2004). Furthermore, neurovascular coupling reflects a stringent and ordered relationship between the neurones and microvessels that is essential for normal functioning of the healthy brain. Thus any perturbation to the relationship between neuronal activity and cerebral blood flow will have a substantial impact on brain function (Girouard et al., 2006). Ischaemic challenged endothelium loses the ability to provide functional support for neurones. Changes in endothelial paracrine signalling after ischaemia come from in vitro studies that have shown conditioned media from healthy brain endothelial cells preserves neuronal function whereas conditioned media from hypoxic endothelial cells have a neurotoxic effect (Li et al., 2009). Loss of endothelial expression of α1
and β1 integrins after ischaemia in rodents is associated with greater neuronal injury (Tagaya et al., 2001). Therefore preserving endothelial function may also be critical for neuronal survival to improve outcome of ischaemic injury.

### 6.3.3. Glial cell-neuronal inflammatory interactions in cerebral ischaemia

Glial cells have been shown to exacerbate neuronal injury as well as confer neuroprotection after cerebral ischaemia. Astrocytes have a crucial role in regulating the extracellular ionic cerebral environment, storage of energy substrates and providing structural support for neurones. Consequently, astrocyte dysfunction as well as microglial activation after ischaemic injury can lead to further neuronal injury and death (Liu et al., 1999; Takano et al., 2009). *In vitro*, ischaemic astrocytes and microglia produce a range of neurotrophic factors such as neurotropin-3, glial-cell-line-derived neutrophic factor (Lin et al., 2006), brain derived neutrophic factor and growth factors such as insulin like growth factor which promote neuronal survival (Lai and Todd, 2006). The balance between neurotoxic and neuroprotective actions of glia is likely to be dependent upon the severity and duration of ischaemia.

### 6.4. Critique of this study

A general limitation of *in vitro* approaches is that cells are in a simplified artificial environment which may not accurately mimic the situation *in vivo*. Furthermore, the isolation and culture conditions can alter morphological and functional characteristics of cells. Despite these limitations, *in vitro* methods are essential for the progress of scientific research. Cellular responses to ischaemic injury can first be evaluated leading to the development of compounds that can be tested for their ability to reduce cell damage or target a specific cellular pathway or mechanism. *In vitro* observations can then be translated *in vivo* for validation of whole tissue responses and parameters such as dosing, safety and efficacy which may then eventually progress to the clinical setting.

The cultures used in this thesis were non-contact co-cultures. *In vivo*, the astrocyte end-feet completely surround the abluminal surface. Therefore using a contact co-culture with glial cells grown on the direct underside of the Transwell membrane may have been a better representation of the *in vivo* environment. A non-contact co-
culture was chosen to be used in this thesis as it would allow discrimination of specific endothelial and glial secreted mediators. Furthermore, as shown in this thesis, contact between the endothelium and glia was not necessary for eliciting their responses to OGD (Chapter 4 and Chapter 5).

These co-cultures lacked a neuronal component. Studies in our own lab have found that cultured murine and rat neurones survive better when co-cultured with mixed glial cells (Britton, F. (2010), personal communication). Therefore using tri-cultures with endothelial cells grown on the luminal membrane of Transwells and glial-neuronal co-cultures grown in the abluminal compartment would add a greater physiological dimension to this current model and would also allow interactions between all three cell types to be studied. However, when using multiple cell types within an in vitro system it becomes increasing difficult to establish individual cellular responses. It is for this reason that co-cultures of only two cell types were used in this study.

Another limitation of this model was the lack of shear flow. Cerebral endothelial cells of different vascular beds are exposed to different magnitudes of shear stress which has an important role in the structure and function of the blood vessel (Chiu et al., 2004). Human cerebral endothelial cells cultured with shear flow display reduced permeability, increased TEER and increased expression of the tight junction proteins and tight junction accessory proteins compared to endothelial cells cultured in static conditions (Siddharthan et al., 2007). Flow systems have been deployed more frequently in studying the pathology of cerebral ischaemia in vitro to mimic the loss and reintroduction of flow that occurs during cerebral ischaemia and reperfusion. By manipulating changes in the force of flow, such in vitro flow systems can closely resemble the events of cerebral ischaemia, allowing the precise analysis of cellular responses to such stimuli. Additionally the presence of leukocytes and other blood-borne cells such as platelets with the reintroduction of flow would yield greater knowledge of the interactions between these cells and the endothelium during the reperfusion phase.

Despite the limitations of this study, it should be noted that no single model either in vitro or in vivo can fully mimic all aspects of human cerebral ischaemia due to the
heterogeneity of the pathology. As long as the limitations of any study are recognised then their findings will continue to provide information on the pathophysiology of cerebral ischaemia.

6.5. Clinical relevance of this study

Cerebral ischaemia is a heterogeneous disorder, with differences in the duration of ischaemia, the region of brain affected and coexisting risk factors resulting in a wide variation in the type and extent of injury. Despite this, combined oxygen and glucose deprivation is the only in vitro model that reflects the loss of oxygen and nutrients as well as the accumulation of cellular products that occurs in cerebral ischaemia. As shown in this study and in other in vitro OGD studies, similar cellular and biochemical responses are induced in response to OGD compared to experimentally-induced ischaemia in vivo in rodents and in clinical cases of patients after ischaemic stroke (Issa et al., 1999). An example is the stabilisation of HIF-1α and expression of HIF-1 regulated genes such as GLUT-1 and VEGF.

In this study, MBEC and glial inflammatory responses to different durations of OGD up to 24 h with or without 24 h reperfusion were established. Short durations of OGD may be representative of transient ischaemic injury, whereas longer OGD durations reflect that of permanent cerebral ischaemia. Alternatively, this 24 h timecourse of OGD may also reflect that of the hypoperfused penumbra that develops after focal cerebral ischaemia. The moderately ischaemic penumbral tissue is potentially salvageable with timely intervention, giving rise to a therapeutic timeframe. However, the irreversibly damaged core infarction evolves in time and space as the penumbral tissue begins to die, thus expanding the core infarct. The extent and rate at which this tissue dies varies according to many factors such as duration and severity of ischaemic insult, ischaemic threshold of tissue, collateral circulation, age and co-existing abnormalities (Ma et al., 2009). Magnetic resonance imaging and computerised tomography analysis of patients after acute ischaemic stroke have shown that penumbral tissue with a minimal cerebral blood flow rate of 22 mL/100 g/min can remain viable for up to 12 h after ischaemia (Heiss et al., 2001) whereas with other patients, the penumbral region may be lost as early as 2 h after onset of ischaemic symptoms (Kaufmann et al., 1999). Thus, the therapeutic window for intervention is highly variable between individuals. Therefore understanding the
temporal cellular and molecular pathways in reduced oxygen and glucose conditions would pave the way for pharmacological preservation of this tissue.

Reoxygenation and reintroduction of glucose-containing media to simulate reperfusion was an important aspect of this model. Clinically, reperfusion either due to a natural restoration of cerebral blood flow or through therapeutic intervention such as the administration of tPA, is essential for tissue survival but also potentiates further tissue damage (Van Elzen et al., 2008). As with all cases of cerebral ischaemia, rapid assessment and treatment are essential for the best outcome. tPA is currently the only licensed therapeutic for ischaemia and it only has a therapeutic window of three hours (Kwiatkowski et al., 1999). Often, delays in hospital administration mean that diagnosis is made several hours after the ischaemic episode and subsequently many patients are unable to receive this thrombolytic therapy. Therefore there is urgent need for the development of more therapeutics. Using an in vitro approach to elucidate temporal biochemical and cellular responses to ischaemia and reperfusion underpins the identification of targets for the development of pharmacological therapeutic agents.

6.6. Conclusion

Inflammation is the key driver of ischaemic cerebral damage. This study demonstrated that oxygen and glucose withdrawal induces inflammatory activation of murine-derived cerebral endothelial cells and mixed glial cells at both the transcriptional and protein level. The differential timing and regulation of these inflammatory responses via cross-talk between the MBECs and glia further reveals the complexity of these post-ischaemic responses. Consequences of endothelial and glial inflammatory activation may involve regulation of neutrophil transendothelial migration that may lead to secondary injury. This study has therefore brought attention to the significance of the endothelium and glial cells in ischaemic injury, identifying many potential targets for therapeutic intervention.
6.7. Future directions

The key question arising from this thesis is what are the effects of these inflammatory mediators on the outcome of ischaemic injury? Further studies should firstly establish whether these in vitro observations translate to the in vivo situation and then identify the consequences of these mediators within the ischaemic brain including exacerbation of initial injury and/or tissue recovery.

Further studies should identify the soluble glial-derived mediator(s) that induces upregulation in MBEC expression of ICAM-1 and VCAM-1 following short and prolonged exposure to OGD. This should include characterisation of receptors on the abluminal MBEC membrane that these glial-derived mediator(s) may act through. This may lead to the identification of potential targets for therapeutic intervention that reduce endothelial activation under ischaemic conditions.

The data presented in this thesis revealed mediators that may regulate neutrophil migration. Further studies should consider whether OGD and reperfusion induces the adhesion and migration of other circulating cells such as monocytes and lymphocytes. Additionally, accumulation of platelets in the cerebral microvessels after experimentally induced ischaemia in mice has been shown to play a role in activation of the endothelium (Thornton et al., 2010). Therefore the interactions between platelets and endothelial cells during reperfusion should also be considered.

As discussed in Chapter 5 this study did not investigate the effect of shear flow on MBEC activation or the interaction between neutrophils and the endothelium. Genomic and proteomic studies have shown that shear stress in vitro induces BBB properties and function including promoting barrier tightness through the induction in expression of tight junction proteins, adherens junctions and matrix-binding integrins (Cucullo et al., 2011). The use of a flow chamber in vitro would also allow delineation of the discrete steps of neutrophil tethering, rolling and transendothelial migration. This may lead to the development of therapeutics that target specific steps in leukocyte infiltration across the BBB.

Research into cerebral ischaemia has largely overlooked the role of the pericytes. To elucidate the role of pericytes in ischaemic injury, initial studies should be conducted
in vitro. The use of Transwells would provide a simple model to investigate pericyte responses to ischaemic conditions and would provide another physiological dimension to the current study. Additionally, the effect of OGD and reperfusion on neuronal injury could be investigated. This would provide a greater insight into the role of endothelial and glial cells in OGD-induced neuronal injury.

Remodelling of the ECM plays a key role in the pathophysiology of cerebral ischaemia and is also essential for tissue recovery (Fukuda et al., 2004). In these studies, MBECs were grown on tissue culture plastic or on the membrane of Transwells coated with collagen IV. Brain endothelial cells have been shown to synthesise and deposit subcellulary their own collagen IV, fibronectin and laminin (Tilling et al., 2002). Thus, in these studies, MBECs may have been growing on a mixture of exogenous synthetic collagen IV and endogenously-produced extracellular matrix components. Further experiments could be conducted to verify whether MBECs did indeed produce their own ECM and if this was modified after exposure to OGD. Further to this, previous work carried out in our lab has shown attachment of endothelial cells to different ECM components regulated the proinflammatory IL-1β-induced signalling pathways. Thus it would be interesting to establish whether endothelial responses to OGD and reperfusion are also regulated by the ECM.
Appendices
<table>
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<tr>
<th>Primary Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
<th>Secondary antibody</th>
</tr>
</thead>
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<td>BD Biosciences</td>
<td>Anti-rat Alexa Fluor® 594</td>
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<tr>
<td>ICAM</td>
<td>1:200</td>
<td>R and D Systems</td>
<td>Anti-goat Alexa Fluor® 594</td>
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<td>R and D Systems</td>
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<td>Gift from Dr Daniel Anthony (Oxford, UK)</td>
<td>Anti-rabbit Alexa Fluor® 488</td>
</tr>
</tbody>
</table>

Table 3. Antibodies used for immunocytochemistry

All secondary antibodies were purchased from Invitrogen (UK)
Appendix I

Nuclear and cytosolic lysate buffers

**Buffer A:**
9.78 mL Water + 10 mM HEPES, pH 7.9
- 1.5 mM MgCl₂
- 10 mM KCl
- 0.5 mM Dithiothreitol (DTT)
- 1x protease and phosphatase inhibitor cocktail (Calbiochem)

**Buffer A2:**
1 mL buffer A + 1 µL NP40

**Buffer B:**
639 µL Water + 20 mM HEPES, pH 7.9
- 200 mM NaCl
- 0.15 mM MgCl₂
- 0.2mM EDTA
- 25 % Glycerol
- 1x protease and phosphatase inhibitor cocktail

**Buffer C:**
587 µL Water + 10mM HEPES, pH 7.9
- 50 mM KCl
- 20mM EDTA
- 20 % Glycerol
- 0.5 mM DTT
- 1x protease and phosphatase inhibitor cocktail

**5x Sample buffer:**
400 mM Tris, pH 6.8
- 10 % sodium dodecyl sulphate (SDS),
- 50 % glycerol,
- 0.025 % bromophenol blue
### Table 4. CBA analysis of co-cultured MBEC lysates and conditioned media during OGD and after 24 h reperfusion. Data are mean concentration (pg/mL) ± SEM of at least four separate experiments. ND = not detected.

<table>
<thead>
<tr>
<th>Conditioned media</th>
<th>Ctrl</th>
<th>Ctrl + reperfusion</th>
<th>4 h OGD</th>
<th>4h OGD + reperfusion</th>
<th>10h OGD</th>
<th>10h OGD + reperfusion</th>
<th>24 h OGD</th>
<th>24 h OGD + reperfusion</th>
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</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>22.2 ± 2.3</td>
<td>16.4 ± 2.6</td>
<td>16.2 ± 2.0</td>
<td>36.0 ± 14.8</td>
<td>16.6 ± 2.7</td>
<td>17.1 ± 4.5</td>
<td>16.1 ± 3.0</td>
<td>22.0 ± 4.6</td>
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<tr>
<td>IL-6</td>
<td>6.9 ± 0.9</td>
<td>16.5 ± 8.37</td>
<td>7.6 ± 0.8</td>
<td>19.0 ± 6.8</td>
<td>6.0 ± 0.7</td>
<td>9.1 ± 1.8</td>
<td>7.3 ± 0.8</td>
<td>12.0 ± 3.4</td>
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<tr>
<td>IL-1α</td>
<td>7.0 ± 1.3</td>
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<td>10.4 ± 0.6</td>
<td>8.7 ± 1.1</td>
<td>9.7 ± 1.1</td>
<td>8.1 ± 1.2</td>
<td>8.0 ± 1.4</td>
<td>8.7 ± 1.4</td>
</tr>
<tr>
<td>IL-1β</td>
<td>17.2 ± 3.8</td>
<td>143 ± 1.5</td>
<td>139 ± 0.7</td>
<td>12.3 ± 2.0</td>
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<td>9.9 ± 1.3</td>
<td>15.1 ± 3.4</td>
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<td>IL-17α</td>
<td>5.1 ± 0.4</td>
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<td>5.0 ± 0.24</td>
<td>3.8 ± 0.8</td>
<td>4.6 ± 0.6</td>
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<td>IL-10</td>
<td>28.5 ± 6.0</td>
<td>245 ± 2.6</td>
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<td>32.2 ± 4.7</td>
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<td>IFN-γ</td>
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<td>ND</td>
<td>3.2 ± 0.2</td>
<td>4.54 ± 0.2</td>
<td>3.4 ± 0.7</td>
<td>3.5 ± 0.2</td>
<td>2.3 ± 0.1</td>
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<td>GM-CSF</td>
<td>22.8 ± 3.1</td>
<td>18.9 ± 1.8</td>
<td>19.8 ± 1.1</td>
<td>22.6 ± 4.1</td>
<td>17.8 ± 1.8</td>
<td>20.1 ± 1.7</td>
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<td>22.8 ± 3.2</td>
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<tr>
<td>CD62L</td>
<td>16.8 ± 3.9</td>
<td>8.5 ± 1.2</td>
<td>9.3 ± 1.6</td>
<td>11.6 ± 1.3</td>
<td>13.1 ± 2.8</td>
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<td>ND</td>
<td>12.8 ± 0.6</td>
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<td>CD62E</td>
<td>44.1 ± 16.4</td>
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<td>35.8 ± 0.4</td>
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<td>33.3 ± 6.4</td>
<td>27.9 ± 3.2</td>
<td>33.7 ± 10.8</td>
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</table>

**Lysates**

| TNF-α             | 14.22 ± 0.8 | 16.2 ± 1.4 | 8.6 ± 1.3  | 18.5 ± 4.1 | 14.4 ± 1.1 | 13.9 ± 1.6 | 9.9 ± 1.0  | 9.7 ± 1.9  |
| IL-6              | 5.8 ± 0.5  | 8.0 ± 1.5  | 6.1 ± 0.9  | 5.3 ± 0.9  | 5.9 ± 1.1  | 6.4 ± 1.2  | 5.8 ± 1.6  | 5.9 ± 0.9  |
| IL-1α             | 7.5 ± 0.6  | 8.2 ± 2.2  | 6.6 ± 2.4  | 6.5 ± 1.2  | 9.2 ± 1.2  | 5.4 ± 1.1  | 6.5 ± 1.9  | 7.0 ± 1.6  |
| IL-1β             | 17.0 ± 4.6 | 12.0 ± 1.1 | 8.3 ± 1.0  | 14.1 ± 1.4 | 13.1 ± 2.2 | 142 ± 3.1  | 129 ± 2.0  | 11.8 ± 3.4 |
| IL-17α            | ND          | ND        | ND        | ND        | ND        | ND         | ND        | ND         |
| IFN-γ             | ND          | ND        | ND        | ND        | ND        | ND         | ND        | ND         |
| GM-CSF            | 18.3 ± 4.8 | 8.2 ± 2.8  | 9.9 ± 3.3  | 18.1 ± 0.1 | 12.8 ± 5.1 | 9.1 ± 1.6  | 5.7 ± 1.2  | 16.6 ± 3.3 |
| CD62L             | 19.2 ± 1.2 | 12.1 ± 4.1 | 8.6 ± 3.0  | 8.9 ± 2.4  | 13.7 ± 1.5 | 11.8 ± 0.9 | ND        | 11.7 ± 6.6 |
| CD62E             | 37.5 ± 10.3| 30.1 ± 3.2 | 26.2 ± 4.8 | 19.2 ± 3.3 | 38.6 ± 14.3| 26.2 ± 0.2 | 18.6 ± 8.6 | 36.8 ± 13.4|
## Appendix II

<table>
<thead>
<tr>
<th>Conditioned media</th>
<th>Ctrl</th>
<th>Ctrl + reperfusion</th>
<th>4 h OGD</th>
<th>4 h OGD + reperfusion</th>
<th>10 h OGD</th>
<th>10 h OGD + reperfusion</th>
<th>24 h OGD</th>
<th>24 h OGD + reperfusion</th>
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<td>IL-6</td>
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**Table 5.** CBA analysis of co-cultured glial lysates and conditioned media during OGD and after 24 h reperfusion. Data are mean concentration (pg/mL) ± SEM of at least four separate experiments. ND = not detected.
Figure 7.1. Reduction in OGD-reperfusion induced MBEC KC secretion with anti-KC antibody treatment. Co-cultured MBECs were treated with 5μg/mL anti-KC antibody or IgG1 isotype control at the start of reperfusion after 4 h or 24 h OGD to establish whether the anti-KC antibody caused a reduction in MBEC KC secretion. Data are mean ± SEM of three separate experiments. *P<0.05 OGD-reperfusion vs. OGD-reperfusion + anti-KC ab. #P<0.05, ##P<0.01 OGD-reperfusion + IgG1 vs. OGD-reperfusion + anti-KC ab.
Appendix III

A

![Image of Western Blot](image-url)

**Figure 7.2. Reduction in OGD-reperfusion induced MBEC ICAM-1 expression with anti-ICAM-1 antibody treatment.** Co-cultured MBECs were treated with 10µg/mL anti-ICAM-1 antibody or IgG1 isotype control at the start of reperfusion after 4 h or 24 h OGD to establish whether the anti-ICAM-1 antibody caused a reduction in ICAM-1 expression. Data are mean ± SEM of three separate experiments. *P<0.05, **P<0.01 OGD-reperfusion vs. OGD-reperfusion + anti-ICAM-1 ab. *P<0.05 OGD-reperfusion + IgG1 vs. OGD-reperfusion + anti-ICAM-1 ab.
Appendix III

A

![Image of Western Blot](image-url)

**Figure 7.3. Reduction in OGD-reperfusion induced MBEC VCAM-1 expression with anti-VCAM-1 antibody treatment.** Co-cultured MBECs were treated with 10µg/mL anti-VCAM-1 antibody or IgG isotype control at the start of reperfusion after 4 h or 24 h OGD to establish whether the anti-VCAM-1 antibody caused a reduction in VCAM-1 expression. Data are mean ± SEM of three separate experiments. *P<0.01 OGD-reperfusion vs. OGD-reperfusion + anti-VCAM-1 ab. **P<0.05, ***P<0.01 OGD-reperfusion + IgG1 vs. OGD-reperfusion + anti-VCAM-1 ab.
Figure 7.4. Reduction in OGD-reperfusion induced glial KC secretion with anti-KC antibody treatment. Co-cultured glial cells were treated with 5µg/mL anti-KC antibody or IgG1 isotype control at the start of reperfusion after 4 h or 24 h OGD to establish whether the anti-KC antibody caused a reduction in glia KC secretion. Data are mean ± SEM of three separate experiments. *P<0.05 OGD-reperfusion vs. OGD-reperfusion + anti-KC ab. #P<0.05, ##P<0.01 OGD-reperfusion + IgG1 vs. OGD-reperfusion + anti-KC ab.

Figure 7.5. Reduction in OGD-reperfusion induced glial VEGF secretion with anti-VEGF antibody treatment. Co-cultured glia were treated with 5µg/mL anti-VEGF antibody or IgG1 isotype control at the start of reperfusion after 4 h or 24 h OGD to establish whether the anti-VEGF antibody caused a reduction in glia VEGF secretion. Data are mean ± SEM of three separate experiments. ND= not detected. *P<0.05 OGD-reperfusion vs. OGD-reperfusion + anti-VEGF ab. #P<0.05 OGD-reperfusion + IgG1 vs. OGD-reperfusion + anti-VEGF ab.
References


References


Defilippi, P., Silengo, L. and Tarone, G. (1992). $\alpha_6\beta_1$ integrin (laminin receptor) is down-regulated by tumour necrosis factor-$\alpha$ and interleukin-1$\beta$ in human endothelial cells. J. Bio. Chem. 267, 18303-18307.


References


