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**Characterisation of a mouse cerebral microvascular endothelial cell line (bEnd.3)
after oxygen glucose deprivation and reoxygenation**

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Abstract

Studies have utilised immortalised mouse cerebral endothelial cells (bEnd.3) exposed to oxygen glucose deprivation (OGD) to study blood-brain barrier (BBB) disruption after ischaemia. However, there is a paucity of literature describing the duration of OGD (and reoxygenation [RO]) required to best simulate BBB disruption *in vivo*. In this study we assessed BBB disruption in bEnd.3 cells after exposure to a range of OGD periods, and also after OGD + RO. Exposure of bEnd.3 monolayers to 4, 6, 16, or 24 h of OGD resulted in a significant increase in permeability. The hyperpermeability after 16 or 24 h was associated with decreased expression of tight junction proteins (occludin and claudin-5). Furthermore, there was a decrease in cell viability and increased expression of the pro-apoptotic protein, cleaved caspase-3. Exposure of bEnd.3 monolayers to 1 h OGD + 23 h RO exacerbated hyperpermeability relative to 1 h OGD, which was associated with decreased expression levels of occludin and ZO-1, but no change in cell viability or caspase-3. 4 h OGD + 23 h RO exacerbated hyperpermeability, decreased expression levels of tight junction proteins, decreased cell viability, and increased caspase-3 expression. Thus, bEnd.3 cells exhibit hyperpermeability, a loss of tight junction proteins, and undergo cell death, after exposure to prolonged periods of OGD. Moreover, they exhibit exacerbated hyperpermeability, a loss of tight junction proteins, and increased expression of caspase-3 after OGD + RO. These findings will facilitate the use of this cell line in studies of BBB disruption and for the testing of therapeutics.

Introduction

Cerebral endothelial cells, characterized by the presence of unique (in comparison with peripheral endothelial cells) tight cell–cell junctions and a lack of fenestrations, constitute the structural basis of the blood-brain barrier (BBB). The BBB serves the important role of restricting the entry of molecules and immune cells from the systemic circulation into the central nervous system. During ischaemic stroke the BBB is disrupted (1). Moreover, whereas the BBB may recover from ischaemia if reperfusion occurs early, delayed reperfusion often results in exacerbated BBB disruption (2,3). A key feature of BBB disruption is an increase in paracellular permeability of endothelial cells resulting primarily from the degradation of junctional proteins such as occludin, the claudins, and ZO-1, and subsequent disassembly of junctions (4). Importantly, BBB disruption can contribute to brain injury after ischaemic stroke. Indeed, it can result in interstitial fluid accumulation and

ultimately oedema (termed vasogenic oedema), which in turn may further compromise perfusion of the ischaemic brain leading to an expansion of the brain infarct (5–7). Not surprisingly, therefore, there is a growing appreciation of the importance of protecting the cerebral endothelium for the treatment of ischaemic stroke.

Studying BBB disruption after ischaemia and reperfusion *in vivo* is challenging. A number of *in vitro* models using cerebral microvascular endothelial cells have been employed to study of the effects of ischaemia and reperfusion on the BBB, and to test novel therapeutics. Specifically, the majority of these studies have employed the oxygen glucose deprivation (OGD) method, with or without reoxygenation (RO), using primary or immortalised cerebral microvascular endothelial cells from a number of different species including humans, rats, mice and pigs (8). Primary cerebral capillary endothelial cells have the closest similarity to the BBB phenotype *in vivo* at low passage numbers (8). However, primary cells are difficult to extract, can often be contaminated with other cell types of the neurovascular unit (i.e. pericytes and astrocytes), and are costly to maintain (8). Moreover, they rapidly undergo senescence after several passages and thus, lose their BBB characteristics (8). For these reasons, immortalised cerebral microvascular endothelial cells have become a popular choice. In the present study, our objective was to characterise an *in vitro* OGD model using a mouse immortalised cerebral microvascular endothelial cell line, bEnd.3. This cell line has previously been used in OGD studies (9–12). However, the time of OGD (and RO) exposure used in these studies varies greatly, making it difficult to ascertain which conditions best simulate the key features of BBB disruption *in vivo*. Therefore, the purpose of this study was to assess key indicators of BBB disruption (paracellular hyperpermeability, tight junction protein degradation, and cell death) in bEnd.3 cells after a range of OGD exposure periods, and also after OGD + RO.

Results

Verification of the endothelial phenotype of bEnd.3 cells

Phase-contrast images of bEnd.3 cells confirmed a spindle-shaped morphology that is typical of cerebral microvascular endothelial cells (Figure 1A). We also found that bEnd.3 cells express the endothelial cell markers, eNOS and vWF (Figure 1B). Using immunofluorescence, we also observed a continuous alignment of ZO-1 to the cell membrane of bEnd.3 cells and in some cells, ZO-1 was also found in the cytoplasm (Figure 1C). This is

consistent with the localization of this tight junction protein in endothelial cells of the BBB *in vivo* (10,13). Thus, consistent with the literature, the bEnd.3 cells used in the present study exhibit key features of endothelial cells of the BBB (9,13–15).

Effect of OGD and OGD + RO on endothelial cell permeability

For OGD experiments, there were no significant differences in the permeability to FITC-dextran amongst ‘time-control’ normoxic cells (4 h: 7540 ± 2300 ; 6 h: 8230 ± 2780 ; 16 h: 7590 ± 870 ; 24 h: 9060 ± 1660 , relative fluorescence units, $P > 0.05$, 1-way ANOVA with a Dunnett’s post-hoc test). Thus, exposure of cells to increasing periods of normoxic conditions (4–24 h) does not affect endothelial paracellular permeability. By contrast, exposure of bEnd.3 cells to 4, 6, 16, or 24 h OGD similarly resulted in a 4-fold increase in the passage of FITC-dextran relative to respective ‘time-control’ normoxic cells, indicative of increased endothelial paracellular permeability ($P < 0.05$, Figure 2A).

For OGD + RO experiments, there were no significant differences in the permeability to FITC-dextran amongst ‘time-control’ normoxic cells (1 h: 5760 ± 820 ; 4 h: 5167 ± 1411 , 24 h normoxic: 7500 ± 1410 , 27 h normoxic: 7599 ± 539 , relative fluorescence units, $P > 0.05$, 1-way ANOVA with a Dunnett’s post-hoc test). Exposure of bEnd.3 cells to 1 h OGD alone resulted in a 2-fold increase in FITC-dextran passage relative to normoxic controls, and 1 h OGD + 23 h RO resulted in a further increase in FITC-dextran passage relative to 1 h OGD alone (~1.5-fold; $P < 0.05$, Figure 2B). Similarly, exposure of bEnd.3 cells to 4 h OGD + 23 h RO resulted in a ~1.5-fold increase in FITC-dextran passage relative to 4 h OGD alone ($P < 0.05$, Figure 2C).

Effect of OGD and OGD + RO on tight junction protein expression

Evidence indicates that the increase in BBB permeability during ischaemia is attributed, in part, to the loss and/or re-assembly of tight junction proteins, including occluding, claudin-5, and ZO-1 (4). Therefore, we measured expression levels of these proteins in bEnd.3 cells after exposure to OGD, and OGD + RO. Exposure of bEnd.3 cells to 4 h OGD had no significant effect on expression levels of occludin and claudin-5 ($P > 0.05$, Figure 3A and 3B). There was a trend for expression levels of occludin and claudin-5 to decrease following exposure to 6 h OGD, however, these differences did not reach statistical significance ($P = 0.18$ for occludin, $P = 0.08$ for claudin, Figure 3A and 3B). By contrast, expression levels of occludin and claudin-5 were significantly reduced by ~40–50% in cells exposed to either

16 h or 24 h OGD ($P<0.05$, Figure 3A and 3B). Expression levels of ZO-1 were unchanged at all OGD exposure periods (Figure 3C).

In OGD + RO experiments, exposure of cells to 1 h or 4 h OGD alone had no significant effect on expression levels of all three tight junction proteins ($P>0.05$, Figure 4). Expression levels of occludin and ZO-1 were however significantly decreased in cells exposed to either 1 h OGD + 23 h RO or 4 h OGD + 23 h RO relative to their respective normoxic controls ($P<0.05$, Figure 4A, C, D, and F). Also, there was trend for expression levels of claudin-5 to decrease following 1 h OGD + 23 h RO, however, this failed to reach statistical significance ($P=0.21$) (Figure 4B).

Effect of OGD and OGD + RO on cell viability and cleaved caspase-3 expression

For OGD experiments, there were no significant differences in cell viability amongst ‘time-control’ normoxic cells (4 h: 0.8 ± 0.7 ; 6 h: 1.1 ± 0.1 ; 16 h: 0.97 ± 0.1 ; 24 h: 0.8 ± 0.7 , relative absorbance units, $P>0.05$, 1-way ANOVA with a Dunnett’s post-hoc test). Exposure of cells to either 4 or 6 h OGD had no significant effect on viability relative to respective ‘time-control’ normoxic cells ($P>0.05$, Figure 5A). However, after exposure to 16 h or 24 h of OGD, the viability of bEnd.3 cells was attenuated by ~70% ($P<0.05$, Figure 5A).

For OGD + RO experiments, there were no significant differences in cell viability amongst ‘time-control’ normoxic cells (1 h: 0.95 ± 0.1 ; 4 h: 0.84 ± 0.02 ; 24 h: 0.9 ± 0.1 ; 27 h: 0.91 ± 0.02 , relative absorbance units, $P>0.05$, 1-way ANOVA with a Dunnett’s post-hoc test). There were also no significant differences in cell viability between normoxic, 1h OGD, and 1 h OGD + 23 h RO groups ($P>0.05$, Figure 5B), and between 4 h OGD or 4 h OGD + 23 h RO groups. However, the viability of cells exposed to 4h OGD + 23 h RO was significant lower relative to respective ‘time-control’ normoxic cells ($P<0.05$, Figure 5C).

Next, we assessed protein expression of cleaved caspase-3 in bEnd.3 cells following exposure to OGD, or OGD + RO as an indicator of apoptotic cell death. For all OGD or OGD + RO Western blot experiments, the 19 kDa subunit of cleaved caspase-3 was undetectable. Expression levels of cleaved caspase-3 (17 kDa subunit) were unchanged in cells exposed to 4 h or 6 h OGD relative to normoxic cells ($P>0.05$, Fig 6A). However, expression levels were increased by 1.7-fold and 2.5-fold, respectively, in cells exposed to either 16 or 24 h of OGD ($P<0.05$, Fig 6A).

For OGD + RO experiments, expression levels of cleaved caspase-3 were unchanged in cells exposed to either 1 h OGD or 1 h OGD + 23 h RO when compared to normoxic controls ($P>0.05$, Figure 6B). By contrast, expression levels of cleaved caspase-3 were significantly increased in cells exposed to 4 h OGD + 23 h RO when compared to cells exposed to 4 h OGD alone or normoxic conditions ($P<0.05$, Figure 6C).

Discussion

Studying BBB disruption after ischaemia and reperfusion *in vivo* is challenging. Thus, *in vitro* BBB models, primarily using the OGD (and RO) method, are considered important tools for understanding mechanisms of BBB disruption and for testing novel therapeutics. Although the bEnd.3 cell has been used in OGD studies, there is a paucity of literature describing the duration of OGD (and RO) and culturing conditions required to best simulate BBB disruption *in vivo*. To address this, we examined BBB disruption in bEnd.3 cells after exposure to a range of OGD conditions and after OGD + RO. We anticipate our findings will facilitate researchers who choose to use this cell line in studies of post-stroke BBB disruption and for the testing of novel therapeutics.

The key features of ischaemic-induced BBB disruption include an increase in paracellular permeability, due to the loss of tight junction proteins and subsequent disassembly of tight junctions, as well as endothelial cell death and contraction. In the present study we assessed three of these key features in bEnd.3 cells after OGD, namely hyperpermeability, loss of tight junction proteins, and cell death. We found that there was a rapid and sustained (4-24 hours) increase in endothelial cell permeability after exposure of the cells to OGD, indicating opening of this *in vitro* BBB model. Moreover, from our later OGD + RO experiments (see discussion below), this BBB opening may occur in response to OGD periods as short as 1 hour. These findings are consistent with the kinetics of BBB disruption in rodent models of stroke. Indeed, a number of studies have shown that BBB hyperpermeability occurs as early (2-4 hours) after ischaemia and is sustained up to 72 hours later (9,16,17). Moreover, previous OGD studies using bEnd.3 cells have shown paracellular hyperpermeability to occur in response to 2-3 hours of OGD exposure (9,11).

The tight junction associated proteins occludin, claudin-5, and ZO-1, play important roles in the maintaining the structure and function of tight junctions. *In vivo*, tight junctions become disrupted in the first few hours after ischaemia resulting in increased BBB permeability. Moreover, the majority of these *in vivo* studies infer that this occurs largely as a result of degradation and/or redistribution of the aforementioned tight junction proteins (17). Here, we found a significant decrease in expression levels of occludin and claudin-5 in bEnd.3 cells only at late time points of OGD (≥ 16 hours), although there was a trend for expression levels to also be decreased after 6 hours of OGD exposure. Thus, relatively prolonged OGD exposure periods are required to simulate this characteristic of BBB disruption *in vivo* in this cell line. Notably, re-distribution of tight junction proteins from the cytoskeleton to the cytosol and subsequent tight junction disruption can occur in the absence of tight junction

protein degradation (9). Thus, although not examined in this study, it is conceivable that this mechanism may account for the hypermeability of bEnd.3 cells exposed to relatively shorter OGD periods. In contrast to occludin and claudin-5, we found no change in expression levels of ZO-1 in bEnd.3 cells at any of the OGD exposure time points. Consistent with this, Neuhaus *et al.* found no change in ZO-1 protein expression in bEnd.3 cells after OGD, however they only examined one exposure period (4 hours) (16). However, using immunofluorescence they found that ZO-1 expression was more discontinuous along the cell membrane after OGD, and prominently localised to the cytosol, suggesting re-distribution of ZO-1 in this cell line (16). Similarly, Zehendner *et al* found that ZO-1 was re-distributed from the cell membrane to the cytosol in bEnd.3 cells after only 30 min of OGD (10). Thus, these findings suggest that ZO-1 is susceptible to re-distribution in bEnd.3 cells after OGD rather than protein degradation.

Ischaemia is known to induce both necrotic or apoptotic cell death of BBB endothelial cells, further perpetuating increases in BBB permeability and disruption. Under our experimental conditions, we observed a marked reduction in cell viability; however, only after exposure of cells to more prolonged OGD periods (≥ 16 h). Similarly, increased expression of the key pro-apoptotic effector, cleaved caspase-3 occurred only at these later OGD exposure periods. Thus, in addition to tight junction disruption, hyperpermeability of bEnd.3 cells after more prolonged OGD periods is likely linked to the induction of cell death. These experiments indicate that relatively short periods of OGD are not sufficient to induce cell death of bEnd.3 cells. Consistent with our findings, studies using bEnd.3 cells have found no evidence for cell death following exposure to 2 or 6 hours of OGD (18,19).

The restoration of cerebral blood flow is crucial to limit brain injury. However, delayed reperfusion can exacerbate the ischaemic injury, which is commonly referred to as reperfusion injury (20). Exacerbated BBB disruption plays an important role in reperfusion injury (20). To simulate ischaemia and reperfusion *in vivo*, studies employ the OGD method followed by a period of RO. Here we firstly assessed key features of BBB disruption in bEnd.3 cells exposed to 1 h OGD followed by 23 h RO and found that this experimental paradigm accentuates disruption of this *in vitro* BBB model. Specifically, RO exacerbated hyperpermeability of bEnd.3 cells relative to 1 h OGD alone, an effect that was probably related to the loss of the tight junction proteins such as occludin and ZO-1, and thus presumably tight junctions. However, our cell viability and caspase-3 data suggests that the effect of RO after this short period of 1 h OGD occurs in the absence of overt cellular injury.

Indeed, 1 h OGD + 23 h RO had no significant effect on either the viability of bEnd.3 cells or expression levels of caspase-3 relative to 1 h OGD alone. Other studies using bEnd.3 cells have reported that RO exacerbates cell death after OGD. For example, Hu *et al.* showed that the viability of bEnd.3 cells exposed to 2 h OGD decreased over a 24 h RO period (21). Similarly, Song *et al.*, showed that exposure of bEnd.3 cells to 6 h OGD followed by 18 h RO increased cell death (12). Thus, longer preceding OGD periods may be necessary for the induction of cell death of bEnd.3 cells during RO. Consistent with this, we found here that exposing bEnd.3 cells to 4 hours of OGD followed by 23 hours of RO, markedly increased expression levels of caspase-3 relative to 4 h OGD alone, and there was a modest but significant decrease in cell viability relative to normoxic controls. Importantly, 4 h OGD + 23 h RO also exacerbated hyperpermeability of bEnd.3 cells relative to 4 h OGD in association with a loss of tight junction proteins (occludin and ZO-1). Thus, this experimental paradigm produces three key features of BBB dysfunction in bEnd.3 cells, and thus may be the best approach to simulate ischaemia/reperfusion-induced BBB disruption.

Collectively, we have characterised key indicators of BBB disruption in two *in vitro* BBB models using bEnd.3 cells in combination OGD, or OGD + RO. Under our experimental conditions, bEnd.3 cells exhibit hyperpermeability, a loss of tight junction proteins, and undergo cell death, after exposure to relatively prolonged periods of OGD. Moreover, bEnd.3 cells exhibit exacerbated hyperpermeability and a loss of tight junction proteins after exposure to either a 1 h or 4 h OGD period followed by RO, however longer preceding OGD periods (e.g. 4 h OGD) appear to be necessary to induce cell death of bEnd.3 cells during RO. Although both models have limitations, the findings of this study should facilitate their use in future studies of post-stroke BBB disruption and for the testing of novel therapeutics.

Materials and Methods

Culture of mouse cerebral microvascular endothelial cells (bEnd.3 cells)

bEnd.3 cells are a well-characterised and utilized immortalised cerebral microvascular endothelial cell line (14). bEnd.3 cells form monolayers in culture and possess key characteristics of the BBB phenotype such as the expression of key tight junction proteins, including the zonula occludens (ZO), claudins and occludin, as well as the expression of key

endothelial cell markers such as endothelial NOS (eNOS) and von Willebrand Factor (vWF) (9,14). Monolayers of bEnd.3 cells are known to exhibit moderate paracellular permeability to high molecular weight molecules and have TEER readings ranging from 100-140 Ω cm² (14), which is similar to that of primary cultures from several species (50-180 Ω cm²) (22–25). However, these reading are lower than that of *in vivo* cerebral microvessels, which are estimated to be around 1800 Ω cm² (26).

bEnd.3 cells (ATCC CRL-2299) were grown in culture media (Dulbecco's modified Eagle's medium [DMEM] media containing 10% fetal bovine serum) in tissue culture flasks from Greiner Bio-One (Frickenhausen, Germany) at 37°C in a humidified 5% CO₂ atmosphere. Cells were passaged every 3-4 days. Culture media was changed after 24 h of passaging and every 2 days thereafter. Experiments were performed with cells from passages 24 to 34.

Verification of the endothelial phenotype of bEnd.3 cells

To verify the phenotype of bEnd.3 cells, we firstly determined whether they express the key endothelial cell markers, eNOS and vWF, using Western blotting. Confluent bEnd.3 cells, cultured in T75 cell culture flasks from Greiner Bio-One (Frickenhausen, Germany), washed with 0.01 mol/L PBS and subsequently scraped off in 0.01 mol/L PBS. After centrifugation (800 g, 5 min), the supernatant was aspirated and the remaining cell pellet was homogenized in Laemmli buffer (25% Glycerin, 12.5% β -mercaptoethanol, 7.5% sodium dodecyl sulfate, 25% 1 mol/L Tris/HCl pH 8.0, 0.25 mg/mL bromophenol blue). Protein concentrations were determined using the RCDC assay (BioRad, Australia) as per the manufacturer's instructions. Absorbance of each well was read using the BMG Clariostar plate reader (BMG Labtech, Germany) at 720 nm. Equal amounts of protein (20 μ g) were loaded onto 1 mm thick 5% (vWF) or 10% (eNOS) polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in 5% skim milk in TBS-T for 1 h and then incubated overnight at 4°C with either rabbit polyclonal anti-vWF (1:500) or mouse monoclonal anti-eNOS (1:1000), and a mouse monoclonal anti- β -actin (1:5000), in 5% skim milk. Membranes were then incubated with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (1:10000 for both) for 1 h. Immunoreactive bands were detected by enhanced chemiluminescence.

Immunofluorescence was also used to verify the expression and localisation of the tight junction protein ZO-1, and to confirm the formation of bEnd.3 monolayers under our

culturing conditions. bEnd.3 cells were seeded (density: 7×10^4 cells/cm²) onto glass coverslips (12 mm in diameter, Menzel-Glaser, Braunschweig, Germany) and allowed to grow to confluence. 2 days post-confluence, the cells were immediately fixed for 15 minutes in 4% paraformaldehyde. Cells were then washed with 0.01 mol/L PBS (3 x 10 min), incubated for 30 min with 10% goat serum, and then incubated with a rabbit polyclonal anti-ZO-1 antibody (1:300, Zymed Laboratories, Australia) overnight at 4°C. The following day, cells were washed in 0.01 mol/L PBS (3 x 10 min) and incubated with goat anti-rabbit AlexaFluoro594 antibody (1:500, Invitrogen, Australia) for 2 h at room temperature. Cells were then washed in 0.01 mol/L PBS (3 x 10 min) and nuclei counterstained with Hoescht 33342 solution (1:500, 15 min, Sigma, Australia). After a final washing step with 0.01 mol/L PBS (3 x 10 min), cells were mounted in VectaShield anti-fade mounting medium (Vector Laboratories, California, USA), viewed and photographed at a magnification of x20 with an Olympus confocal laser scanning microscope.

Oxygen glucose deprivation

To simulate ischaemic-like conditions *in vitro*, we exposed bEnd.3 cells to OGD. OGD mimics the abrupt and sustained cessation in glucose and oxygen most likely seen in the infarct core *in vivo* (27). Briefly, bEnd.3 cells were seeded (cell density: 7×10^4 cells/cm²) into 96-well culture plates or T75 tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany) and allowed to grow to confluence. 2 days post-confluence, cells were washed twice and media was replaced with OGD media, composed of DMEM media with no glucose or serum (Invitrogen, Melbourne, Australia), pre-equilibrated with the OGD gas mixture (5 minutes; 100% N₂). Cells were then transferred to a humidified hypoxia chamber (Biospherix, Lacona, NY) flushed with the OGD gas mixture (95% N₂, 5% CO₂). bEnd.3 cells were then left to incubate for 1, 4, 6, 16 or 24 h. The oxygen level was kept constant at approximately 0.3% throughout the duration of the experiment by a digital oxygen controller (Biospherix, New York). For each OGD experiment, time-controlled normoxic controls were run alongside by incubating bEnd.3 cells for the same duration of time as the OGD time point, in glucose-containing, serum-free DMEM media at 37°C (5% CO₂ atmosphere).

Oxygen glucose deprivation with or without reoxygenation

To mimic ischaemia/reperfusion-like conditions *in vitro*, we exposed bEnd.3 cells to OGD followed by RO. RO mimics the rapid restoration of oxygen (and glucose) that would occur *in vivo* after reperfusion (28). bEnd.3 cells were exposed to either 1 h or 4 h OGD by

replacing the culture medium with OGD media (pre-equilibrated for 5 min in 100% N₂) as described above. For RO, cells were subsequently washed twice with DMEM media and allowed to incubate for a further 23 h in glucose-containing, serum-free DMEM media (equilibrated with air) at 37°C (5% CO₂ atmosphere). For time-controlled normoxic controls, bEnd.3 cells were incubated for 1, 4, 24 or 27 h in glucose-containing, serum-free DMEM media at 37°C (5% CO₂ atmosphere).

Assessment of endothelial cell paracellular permeability after OGD or OGD + RO

The paracellular permeability of bEnd.3 cells was assessed by measuring the diffusion of 70 kDa fluorescein isothiocyanate (FITC)-dextran (Sigma, Australia) across monolayers of bEnd.3 cells. bEnd.3 cells were seeded at a density of 4×10^4 cells/well in culture media onto the upper 'apical' chamber of tissue culture inserts (translucent polyethylene terephthalate [PET], 0.4 µm pore size, Greiner Bio-One, Frickenhausen, Germany) fitted into 24-well culture plates (Greiner Bio-One, Frickenhausen, Germany). Culture media was replenished in the upper and lower chambers every 24 h. 2 days post-confluence, bEnd.3 cells were exposed to normoxic, 1, 4, 6, 16, or 24 h OGD, or OGD (1 h or 4 h) followed by RO conditions, as described above. At the end of the experiment, the inserts were transferred into fresh wells containing pre-warmed Krebs-HEPES buffer [composed of (in mmol/l) 99 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.0 KH₂PO₄, 19.6 NaHCO₃, 11.2 glucose, 20 Na-HEPES and 2.5 CaCl₂, pH=7.4]. The inserts were washed twice with pre-warmed Krebs-HEPES buffer, and then FITC-dextran (1 mg/ml) was added to the upper chamber and left to incubate for 60 min at 37°C. Inserts were then removed from the wells and samples of the solution from the lower chamber were collected and transferred into black 96-well plates (PerkinElmer, Melbourne, Australia). Fluorescence intensity was then measured using the BMG Clariostar plate reader (BMG Labtech, Germany) at an excitation and emission wavelength of 492 nm and 518 nm, respectively. Changes in fluorescence intensity were expressed as fold-change relative to its respective 'time-control' normoxic cells.

Tight junction protein expression

Using western blotting, we measured protein expression levels of occludin, claudin-5, and ZO-1 in bEnd.3 cells after exposure to normoxic, 1, 4, 6, 16 or 24 h OGD or, OGD (1 h or 4 h) followed by 23 h RO. bEnd.3 cells were cultured in T75 flasks and allowed to grow to confluence. 2 days post-confluence, cultures were exposed to normoxic, OGD, or OGD + RO conditions, as described above. Samples were collected, homogenized and protein concentrations were determined using the RCDC assay (BioRad, Australia). Equal amounts

of protein (20 µg), along with a dual colour molecular weight marker (Bio-rad, Australia), were loaded onto 1 mm thick 10% (occludin), 12.5% (claudin-5), or 7.5% (ZO-1) polyacrylamide gels and transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked in 5% skim milk for 1 h and then incubated overnight at 4°C with mouse monoclonal anti-occludin (1:1000, Invitrogen, Australia), mouse monoclonal anti-claudin-5 (1:1000, Invitrogen, Australia), or rabbit polyclonal anti-ZO-1 (1:500, Invitrogen, Australia) primary antibodies, and mouse monoclonal anti-β-actin primary antibodies (1:5000 for β-actin, Cell Signaling Technology, USA) in 5% skim milk. Membranes were then incubated with a horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG (1:10000) for 1 h. Immunoreactive bands were detected by enhanced chemiluminescence. Immunoreactive bands were quantified using a ChemiDoc XRS molecular imager (BioRad, Australia). Bands were normalized to intensity of corresponding bands for β-actin and expressed relative to ‘time-control’ normoxic cells.

Measurement of cell viability and apoptosis

Cell viability

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay (Promega, Australia) (29). bEnd.3 cells were seeded into 24-well culture plates at a density of 4×10^4 per well in culture media and allowed to grow to confluence. Once confluent, bEnd.3 cells were exposed to normoxic, 1, 4, 6, 16 or 24 h OGD, or OGD (1 h or 4 h) followed by 23 h RO conditions, as described above. Cells were subsequently washed with pre-warmed Krebs-HEPES buffer. Cells were subsequently incubated with MTS solution (Promega, Melbourne, Australia) for 1 h at 37°C (5% CO₂ atmosphere). Absorbance was then detected at 490 nm using a BMG Clariostar plate reader (BMG, Germany). Mean absorbance of cells exposed to OGD or OGD + RO were expressed as fold-change relative to the absorbance of its respective ‘time-control’ normoxic cells.

Expression of the pro-apoptotic protein caspase-3

Caspase-3 is a key executor enzyme of apoptosis and is known to play an important role in apoptotic cell death of cerebral endothelial cells after ischaemia (10,30). During the early stages of apoptosis, procaspase-3 is proteolytically processed to generate 19 kDa (known as p19) and 12 kDa (known as p12) fragments (31). The 19 kDa fragment is further processed into the 17 kDa (known as p17) fragment, where both p17 and p12 associate to form active

caspase-3 (31). Using western blotting, we measured protein expression levels of the large subunit (p17) of active caspase-3 in bEnd.3 cells after exposure to normoxic, 1, 4, 6, 16 or 24 h OGD, or OGD (1 h or 4 h) followed by 23 h RO. bEnd.3 cells were cultured in T75 flasks and allowed to grow to confluence. 2 days post-confluence, cultures were exposed to normoxic, OGD, or OGD + RO conditions, as described above.. Samples were collected, homogenized and protein concentrations were determined using the RCDC assay (BioRad, Australia). Equal amounts of protein (20 µg) were loaded onto 1 mm thick 12.5% polyacrylamide gels and transferred to a PVDF membrane. Membranes were blocked in 5% skim milk in TBS-T for 1 h and then incubated overnight at 4°C with primary polyclonal rabbit anti-cleaved caspase 3 (1:1000) and primary mouse monoclonal anti-β-actin (1:5000) in 5% skim milk. Membranes were then incubated with a horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG (1:10000) for 1 h. Immunoreactive bands were detected by enhanced chemiluminescence. Immunoreactive bands were quantified using a ChemiDoc XRS molecular imager (BioRad, Australia). Bands were normalized to intensity of corresponding bands for β-actin and expressed relative to ‘time-control’ normoxic cells.

Data and statistical analysis

All results are presented as mean ± SEM and $P < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software Inc., USA). All statistical comparisons between treatment groups were performed using a 1-way ANOVA with a Dunnet’s multiple comparisons post-hoc test.

Antibodies and drugs

Anti-eNOS mouse monoclonal antibody was purchased from BD Biosciences (Sydney, Australia). Anti-occludin and anti-claudin-5 mouse monoclonal and anti-ZO-1 rabbit polyclonal antibodies were purchased from Invitrogen (Australia). Anti-vWF rabbit polyclonal antibodies were purchased from Dako (Australia). All drugs were purchased from Sigma (Australia).

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Figure legends

Figure 1. Phase contrast image showing the morphology of bEnd.3 cells (A). Representative Western blots for von Willebrand factor (vWF) and endothelial nitric oxide synthase (eNOS) in bEnd.3 cells (representative of n=3) with the loading control, β -actin (B). Also shown are representative photomicrograph of immunofluorescence for the tight junction protein, ZO-1 (red), and nuclear staining with Hoescht (blue) in bEnd.3 monolayers (C, n=3). Scale bar = 50 μ m.

Figure 2. Quantification of paracellular permeability of bEnd.3 cells following exposure to normoxic, oxygen glucose deprivation (A, OGD), 1 h OGD + 23 h reoxygenation (B, RO), or 4 h OGD + 23 h RO (C) conditions. Paracellular permeability was assessed using FITC-dextran. Results are expressed as fold-change in FITC-dextran passage relative to respective time-controlled normoxic cells and are given as mean $\pm$ SEM (A, n=4-5, B, n=6-8, **C, n=5-6**). * P <0.05 vs. normoxic cells, # P <0.05 vs. 1 h OGD, ## P <0.05 vs. 4 h OGD (1-way ANOVA with Dunnett's post-hoc test).

Figure 3. Representative Western blots showing expression levels of the tight junction proteins, occludin (A), claudin-5 (B) and ZO-1 (C) in bEnd.3 cells following exposure to normoxic (N) or oxygen glucose deprivation (OGD) conditions, and summary of immunoreactive band intensities. Intensity was normalised to β -actin intensity then expressed relative to its respective normoxic cells. Results are given as mean $\pm$ SEM (A, n=8-11; B, n=6-8; C, n=7-9). * P <0.05 vs normoxic cells (1-way ANOVA with a Dunnett's post-hoc test).

Figure 4. Representative Western blots showing expression levels of the tight junction proteins, occludin (A, D), claudin-5 (B, E) and ZO-1 (C, F) in bEnd.3 cells following exposure to normoxic (N), oxygen glucose deprivation (OGD), or OGD + reoxygenation (RO) conditions, and summary of immunoreactive band intensities. Intensity was normalised to β -actin then expressed relative to its respective normoxic cells. Results are given as mean $\pm$ SEM (A-C, n=7-8, D-F, n=5-8). * P <0.05 vs. normoxic cells, # P <0.05 vs. 1 h OGD (1-way ANOVA with a Dunnett's post-hoc test).

Figure 5. Quantification of cell viability of bEnd.3 cells following exposure to normoxic, oxygen glucose deprivation (OGD, A), 1 h OGD + 23 h reoxygenation (RO, B), or 4 h OGD + 23 h RO (C) conditions. Results are expressed as a percentage of its respective normoxic (N) controls and are given as mean $\pm$ SEM (A, n=4; B, n=5-8; C=4-8). * P <0.05 vs. normoxic cells (1-way ANOVA with a Dunnett's post-hoc test).

Figure 6. Representative Western blots showing protein expression of cleaved caspase-3 in bEnd.3 cells following exposure to normoxic, oxygen glucose deprivation (OGD, A), 1 h OGD + 23 h reoxygenation (RO, B), or 4 h OGD + 23 h RO (C) conditions, and summary of immunoreactive band intensities. Intensity was normalised to β -actin then expressed as fold change relative to normoxic (N) cells. Results are given as mean $\pm$ SEM (A, n=7-8; B, n=6-7;

C, n=4). * $P < 0.05$ vs. normoxic cells, # $P < 0.05$ vs. 4 h OGD (1-way ANOVA with a Dunnett's post-hoc test).

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Figure 1

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Figure 2

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Figure 3

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Figure 4

Tight junction protein expression

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Figure 5

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Figure 6

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