Attenuation of urokinase activity during experimental ischaemia protects the cerebral barrier from damage through regulation of matrix metalloproteinase-2 and NAD(P)H oxidase

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Abstract
Ischaemic injury impairs the integrity of the blood–brain barrier (BBB). In this study, we investigated the molecular causes of this defect with regard to the putative correlations among NAD(P)H oxidase, plasminogen–plasmin system components, and matrix metalloproteinases. Hence, the activities of NAD(P)H oxidase, matrix metalloproteinase-2, urokinase-type plasminogen activator (uPA), and tissue-type plasminogen activator (tPA), and superoxide anion levels, were assessed in human brain microvascular endothelial cells (HBMECs) exposed to oxygen–glucose deprivation (OGD) alone or OGD followed by reperfusion (OGD + R). The integrity of an in vitro model of BBB comprising HBMECs and astrocytes was studied by measuring transendothelial electrical resistance and the paracellular flux of albumin. OGD with or without reperfusion (OGD ± R) radically perturbed barrier function while concurrently enhancing uPA, tPA and NAD(P)H oxidase activities and superoxide anion release in HBMECs. Pharmacological inactivation of NAD(P)H oxidase attenuated OGD ± R-mediated BBB damage through modulation of matrix metalloproteinase-2 and tPA, but not uPA activity. Overactivation of NAD(P)H oxidase in HBMECs via cDNA electroporation of its p22-phox subunit confirmed the involvement of IPA in oxidase-mediated BBB disruption. Interestingly, blockade of uPA or uPA receptor preserved normal BBB function by neutralizing both NAD(P)H oxidase and matrix metalloproteinase-2 activities. Hence, selective targeting of uPA after ischaemic strokes may protect cerebral barrier integrity and function by concomitantly attenuating basement membrane degradation and oxidative stress.

Introduction
Composed of brain microvascular endothelial cells surrounded by a basement membrane and astrocytic endfeet, the blood–brain barrier (BBB) regulates the passage of circulating substances into the brain parenchyma (Hayashi et al., 2004). Disruption of the blood supply to the brain during ischaemic stroke evokes a cascade of deleterious events, such as depletion of ATP and acidosis, leading to disruption of the BBB (Hayashi et al., 2004; Brouns & De Deyn, 2009). Ensuring reperfusion of ischaemic tissue accompanied by excessive release of superoxide anion (O$_2^-$), a prominent reactive oxygen species, may exacerbate the damage to the BBB (Walder et al., 1996; Margali et al., 2005; Selemdis et al., 2008).

Endothelial NAD(P)H oxidase represents the main source of O$_2^-$ in the ischaemic vasculature. It contains several cytosolic subunits and a membrane-bound cytochrome b$_{558}$ consisting of gp91-phox and p22-phox, which are key subunits required for enzymatic activity and stability as a whole (Kleinschmit et al., 2010; Fleikers et al., 2012). Although many studies have shown the significance of this enzyme complex in ischaemia–reperfusion injury (Eltzschig & Collard, 2004; Hong et al., 2006), the p22-phox subunit has not been studied significantly in this context.

In addition to O$_2^-$, the enhanced activation of serine proteases, namely tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), along with their catalytic product plasmin, may also compromise BBB integrity under ischaemic conditions. The link between these serine proteases and ischaemic stroke is well established, with studies showing both beneficial and detrimental roles (Hosomi et al., 2001; Cho et al., 2012). The activities of plasminogen activators (PAs) are, among other mechanisms, controlled by the local availability of their inhibitors, namely plasminogen activator inhibitor-1 (PAI-1) and, in the case of uPA, also by its specific receptor, uPAR (Irigoyen et al., 1999).

Plasminogen activators (PAs) have been linked to the levels and activities of matrix metalloproteinases (MMPs), namely MMP-2 and MMP-9, during ischaemic stroke (Rosenberg et al., 1994; Tsuji et al., 2005; Thornton et al., 2008; Barr et al., 2010). MMPs can be activated during different stages of a stroke, with emerging evidence indicating that MMP-9 may have a more severe impact, especially during the later stages (Asahi et al., 2000, 2001a,b; Lucivero et al., 2007). Recent studies have shown that deletion of both MMP-2 and MMP-9...
protects against cerebral infarction in mouse models (Suofu et al., 2012), and that these MMPs can contribute to BBB damage through degradation of the tight junction protein occludin (Liu et al., 2012).

In light of the above, the current study examined whether NAD(P)H oxidase-dependent release of O$_2^-$ might compromise the integrity and function of an in vitro model of human BBB under ischaemic conditions through interactions with PAs and/or MMPs.

It was found that inhibition of uPA, NAD(P)H oxidase or MMP-2 has barrier-protective effects. It was also found that uPA acts upstream of both NAD(P)H oxidase and MMP-2, meaning that this enzyme has significant therapeutic potential regarding the maintenance of BBB integrity and function.

Materials and methods

Cell culture

Human brain microvascular endothelial cells (HBMECs) were purchased from TCS CellWorks Ltd (Buckingham, UK), and grown to subconfluence in their specialized media before exposure to oxygen–glucose deprivation (OGD) (94.5% N$_2$, 0.5% O$_2$, and 5% CO$_2$) or normoxia (N, 75% N$_2$, 20% O$_2$, and 5% CO$_2$) for 4 h. In some experiments, OGD was followed by 20 h of reperfusion, in that the ischaemic culture medium lacking glucose and fetal bovine serum (FBS) was replaced with fresh medium containing 5.5 mM glucose and 10% FBS before cells were exposed to normoxic conditions. Similar experiments were carried out with the addition of amiloride (uPA inhibitor, 2.5 μM), apocynin [NAD(P)H oxidase inhibitor, 1 mM] or 2-[(isopropoxy)-(1,1-dimethylethyl)-amino]-H-hydroxycacetamide (MMP-2 inhibitor III, 100 μM; Calbiochem, Nottingham, UK) during the OGD or reperfusion stages.

In vitro model of human BBB

Human astrocytes (ScienCell) were seeded onto the outer surface of Transwell inserts (pore size, 0.4 μm; Corning Costar, High Wycombe, UK) seated upside down, and, on the following day, HBMECs were seeded onto the inner surface of the same inserts. Both sets of cells were grown to confluence before exposure and/or treatment.

Assessment of BBB permeability

Blood–brain barrier (BBB) integrity and function were studied, as previously described, by measurements of transendothelial electrical resistance (TEER) and the flux of Evan’s blue-labelled albumin (EBA) (67 kDa), respectively, across co-cultures (Allen & Bayraktutan, 2009a). TEER was measured with STX electrodes and an EVOM resistance meter (World Precision Instruments, Hertfordshire, UK). To measure EBA flux, inserts were washed twice with Hank’s Balanced Salt Solution (HBSS), and then transferred to new 12-well plates containing 2 mL of HBSS. EBA (500 μL, 165 μg/mL) was added to the luminal compartments and, after 60 min, samples were taken from both the abluminal and luminal chambers. The concentration of dye in each chamber was determined by measuring the absorbance of the sample at 610 nm, and the flux was calculated [(absorbance abluminal × 2000)/absorbance luminal].

Immunoblotting

Equal amounts (40–60 μg) of protein were run on 10–15% sodium dodecyl sulphate–polyacrylamide gels before being transferred onto a Hybond-P poly(vinylidene difluoride) membrane (GE Healthcare, Buckinghamshire, UK). The proteins were successively detected with primary antibodies specific for β-actin (mouse; synthetic β-cytoplasmic actin N-terminal peptide; Sigma (Dorset, UK); cat. no. A5414; internal control; 1: 10 000), p22-phox [rabbit (FL-195); full-length human p22-phox; Santa Cruz (CA, USA); cat. no. sc-20781; 1: 200], PAI-1 [rabbit (H-135); amino acids 24–158 of the N-terminus of human PAI-1; Santa Cruz; cat. no. sc-8979; 1: 750], tPA [rabbit (H-90); amino acids 1–90 of human tPA; Santa Cruz; cat. no. sc-15346; 1: 300], uPA [rabbit (H-140); amino acids 136–275 of human uPA; Santa Cruz; cat. no. sc-14019; 1: 500], or uPAR [rabbit (FL-290); full-length human uPAR; Santa Cruz; cat. no. sc-10815; 1: 500], and infrared dye-tagged appropriate secondary antibodies (goat anti-mouse and anti-rabbit; LI-COR Biosciences (Cambridge, UK); cat. nos. 926-68020 and 926-32211, respectively; 1: 30 000). The bands were scanned and analysed with the Odyssey Infrared Imaging System (LI-COR Biosciences).

Cell viability

An aliquot of cells mixed with 0.1% Trypan blue was visualized under a light microscope. To calculate percentage viability, 100 cells were counted.

Transfection experiments

HBMECs (~5 × 10^6) were re-suspended in 500 μL of medium without FBS and antibiotics, and stored on ice in a pre-chilled sterile cuvette. Anti-PAI-1 or anti-uPAR antibody (each 3 μg) or double-stranded p22-phox cDNA (50 ng) was added to the cell suspension, and the mixture was electroporated at 1.8 kV with an Easyjet Prima Electroporator (Equibio, Ashford, UK). Cells electroporated with equal volumes of vehicle (distilled H$_2$O), control cDNA (28S rRNA) or a rabbit IgG served as controls. As electroporation reduced the viability rates by ~40%, the cell numbers were adjusted accordingly before cells were seeded in reasonably high numbers in flasks or on inserts to ensure that all experimental groups reached confluence simultaneously. Cells were exposed to OGD, and harvested within 3 days of transfection.

Pyrogallol experiments

In some experiments, HBMECs alone or in co-culture with human astrocytes were exposed to pyrogallol (O$_2^-$ generator, 2 μM) for a period of 3 days, and fed twice daily with fresh medium containing pyrogallol.

Measurements of total O$_2^-$ levels and NAD(P)H oxidase activity

Total O$_2^-$ levels were detected with the cytochrome c reduction assay, as previously described (Allen & Bayraktutan, 2009a). Briefly, cell pellets were sonicated in cold lysis buffer (20 mM Hepes, pH 7.2, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose). Equal amounts of homogenate (100 μg) were incubated with cytochrome c (50 μM) for 60 min at 37 °C before absorbances were measured at 550 nm.

NAD(P)H oxidase activity was measured with the lucigenin chemiluminescence assay. Briefly, samples of homogenates (~100 μg) were incubated at 37 °C in assay buffer [50 mM potassium phosphate buffer (pH 7.0), 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin] containing the specific inhibitors for other reactive oxygen species-generating enzymes, i.e. nitric oxide synthase (NG-nitro-l-arginine methyl ester,
100 μM), mitochondrial complex I (rotenone, 50 μM), xanthine oxidase (allopurinol, 100 μM), and cyclooxygenase (indomethacin, 50 μM). After 15 min, NADH or NADPH (100 μM; Calbiochem) was added to initiate the reaction. The reaction was monitored every minute for 2 h, and the rate of the reaction was calculated. Buffer blanks were also run for both assays and subtracted from the data.

**Determination of PA activities**

Urokinase-type plasminogen activator (uPA) and tPA activities were measured with an enzyme-linked immunosorbent assay. Streptavidin-coated plates (Thermo Scientific, Loughborough, UK) were coated with biotinylated PAI-1 (300 ng/mL; Abcam, Cambridge, UK) prior to addition of equal volumes of cell culture medium or uPA and tPA standards (Calbiochem). Bound active uPA or tPA was then detected to addition of equal volumes of cell culture medium or uPA and tPA standards (Calbiochem). Bound active uPA or tPA was then detected by successive incubations with the respective primary antibody (see above; 1 : 50) and horseradish peroxidase-linked secondary antibodies (donkey anti-rabbit; Santa Cruz; cat. no. sc-2313; 1 : 5000). A colorimetric substrate, 3,3',5,5'-tetramethylbenzidine (Thermo Scientific), was added to the reaction before it was stopped with H₂SO₄. Absorbances were read at 450 nm, and normalized against the respective standards and total protein concentrations.

**Gelatin zymography**

Total cell protein (20 μg) or culture medium devoid of FBS used to grow HBMECs was run on 9% sodium dodecyl sulphate–polyacrylamide gels containing 0.1% gelatin. MMP-2-positive and MMP-9-positive controls were also run on each gel. The gels were washed three times for 15 min each in 2.5% Triton X-100 before being incubated overnight at 37 °C in buffer consisting of 50 mM Tris (pH 7.5), 10 mM CaCl₂, 150 mM NaCl, and 0.05% Brij-35 (Calbiochem). Gels were then successively stained for 2 h with 0.1% Coomassie blue dye in 25% methanol and 5% acetic acid and then overnight in massie blue dye in 25% methanol and 5% acetic acid, and destained for 1 h in 50% methanol and 5% acetic acid, and destained for 1 h in 50% methanol and 10% acetic acid and then overnight in water. MMP activity was detected as clear bands, and gels were scanned and analyzed with the Odyssey Imaging System.

**Reagents**

All reagents, unless specifically stated, were from Sigma.

**Statistical analyses**

Data are presented as means ± standard errors of the mean from a minimum of three independent experiments. Statistical analyses were performed with a one-way ANOVA followed by Tukey post hoc analysis or, where appropriate, with an independent Student’s t-test or a two-way ANOVA followed by Tukey post hoc analysis. A P-value of <0.05 was considered to be significant.

**Results**

**Effects of OGD and reperfusion on BBB integrity and function**

Oxygen–glucose deprivation (OGD) alone and OGD followed by reperfusion (OGD + R) impaired BBB integrity and function as confirmed by decreases in TEER and concurrent increases in EBA flux, respectively. The magnitudes of these changes were greater during the reperfusion phase. Whereas inhibition of uPA and NAD (P)H oxidase restored barrier function after OGD ± reperfusion (OGD ± R), the inhibition of MMP-2 appeared to be only effective during the reperfusion stage [two-way ANOVA followed by Tukey post hoc analysis: n ≥ 3, F₄,₄₇₁ = 2.79, P < 0.05 (Fig. 1A); n ≥ 3, F₄,₃₆ = 5.37, P < 0.03 (Fig. 1B)].

**Effects of OGD ± R on NAD(P)H oxidase**

Exposure of HBMECs to OGD ± R led to substantial increases in NAD(P)H oxidase activity and O₂⁻ levels that were effectively suppressed by attenuation of uPA and NAD(P)H oxidase activities but not by attenuation of MMP-2 activity [two-way ANOVA with Tukey post hoc analysis: n ≥ 3, F₄,₁₁₂ = 1.68, P < 0.05 (Fig. 2A); n ≥ 3, F₄,₁₁₆ = 1.84, P < 0.05 (Fig. 2B)]. These findings imply that uPA and MMP-2 may act upstream and downstream of the oxidase, respectively. The increases in oxidase activity were mimicked by those observed in p22-phox protein expression, confirming the regulatory role of this particular subunit in determining overall enzyme activity (one-way ANOVA followed by Tukey post hoc analysis: n ≥ 3, F₄,₂₆ = 9.65, P < 0.05; Fig. 2C). The difference in p22-phox expression between the OGD and OGD + R groups cannot be attributed to reintroduction of glucose during the reperfusion phase, as longer exposures to hyperglycaemia (≥3 days) are required to affect NADPH oxidase isoform protein expression in HBMECs (Allen & Bayraktutan, 2009a; Shao & Bayraktutan, 2013).

O₂⁻ impairs BBB integrity and increases tPA protein expression

To ascertain the contribution of O₂⁻ to BBB dysfunction, the co-cultures were treated with an O₂⁻ generator, pyrogallol, which impaired...
After establishment of the pivotal role of O$_2^-$ in barrier damage, it was critical to unravel the involvement of NAD(P)H oxidase in this phenomenon. Hence, NAD(P)H oxidase activity was augmented in some HBMECs through electroporation of double-stranded p22-phox cDNA, which significantly increased p22-phox protein levels and produced similar quantities of O$_2^-$ as pyrogallol, whereas electroporation of control cDNA had no impact on NADPH oxidase activity or O$_2^-$ release. Inhibition of uPA activity with amiloride or with an anti-uPAR antibody normalized O$_2^-$ levels and NAD(P)H oxidase activity. Electroporation with an IgG antibody did not reduce O$_2^-$ levels or NAD(P)H oxidase activity (one-way ANOVA followed by Tukey post hoc analysis: n ≥ 3, F$^{2,12}_{5.14} = 19.0$, P < 0.05 (Fig. 4A); n ≥ 3, F$^{2,15}_{3.13} = 14.3$, P < 0.05; Fig. 4B) (Student’s t-test: n ≥ 3, P < 0.05; Fig. 4C). Specific increases in NAD(P)H oxidase activity also substantially increased tPA protein expression (Student’s t-test: n ≥ 3, P < 0.05; Fig. 4D). Inhibition of uPA and uPA–uPAR binding with amiloride and an anti-uPAR antibody, respectively, abolished all barrier-disruptive effects induced by p22-phox overexpression and ensuing NAD(P)H oxidase overactivity, whereas electroporation of control cDNA or non-specific isotype-matched antibody had no effect on barrier integrity or function (one-way ANOVA followed by Tukey post hoc analysis: n ≥ 3, F$^{2,15}_{2.18} = 68.5$, P < 0.05 (Fig. 4E); n ≥ 3, F$^{2,6}_{2.0} = 0.92$, not different (NS) (Fig. 4F); n ≥ 3, F$^{2,1}_{2.7} = 85.77$, P < 0.05 (Fig. 4H); n ≥ 3, F$^{2,6}_{0.2} = 0.27$, P not different (NS) (Fig. 4J) (Student’s t-test: n ≥ 3, P < 0.05; Fig. 4F and I).

**Effects of OGD ± R on MMP-2 and MMP-9 activities**

Although dramatic increases in intracellular and secreted MMP-2 activities were obtained selectively during the reperfusion phase of the ischemic insult, MMP-9 activity stayed below the level of detection after OGD ± R. As expected, MMP-2 inhibitor III normalized the increases observed in MMP-2 activity (one-way ANOVA followed by Tukey post hoc analysis: n ≥ 3, F$^{2,5}_{4.5} = 15.94$, P < 0.05 (Fig. 5A); n ≥ 3, F$^{2,7}_{5.9} = 6.96$, P < 0.05 (Fig. 5B)). Inhibition of
uPA and NAD(P)H oxidase activities reversed the increases observed during reperfusion, and confirmed the notion that both enzymatic systems operate upstream of MMP-2 [one-way ANOVA followed by Tukey post hoc analysis: $n \geq 3$, $F_{4,58} = 17.58$, $P < 0.05$ (Fig. 5C); $n \geq 3$, $F_{5,83} = 7.27$, $P < 0.05$ (Fig. 5D)]. Interestingly, apocynin reduced MMP-2 activity in cells treated with OGD alone to below the levels seen in control cells [one-way ANOVA followed by Tukey post hoc analysis: $n \geq 3$, $F_{4,59} = 32.71$, $P < 0.05$ (Fig. 5E); $n \geq 3$, $F_{5,83} = 12.93$, $P < 0.05$ (Fig. 5F)]. As serum causes an increase in secreted MMP-2 activity and was present during the reperfusion phase of the experiments, two different controls were used to normalize secreted MMP-2 activities. N4 h lacked serum and served as controls for OGD experiments; N20 h containing equal amounts of serum served as controls for OGD + RAB

Fig. 4. The effect of p22-phox cDNA transfection on the NAD(P)H oxidase system, tPA protein levels, and the BBB. HBMECs were transfected with p22-phox or control cDNA cultured alone or with human astrocytes. Cells were then exposed to amiloride, a uPA inhibitor, or concurrently transfected with an anti-uPAR or IgG antibody. As compared with controls, transfection markedly increased NAD(P)H oxidase activity and total $O_2^-$ levels, which were normalized by treatment with amiloride or anti-uPAR antibody. Transfection with vehicle, control cDNA or IgG antibody had no effect on NAD(P)H oxidase activity or $O_2^-$ levels (A and B). p22-phox cDNA transfection led to significant increases in both p22-phox and tPA protein expressions (C and D). Treatment with amiloride and transfection with anti-uPAR antibody abolished the p22-phox cDNA transfection-mediated changes in BBB integrity (E–G) and function (H–J). Data are expressed as means ± standard errors of the mean from $n \geq 3$ experiments, and were analysed with a one-way ANOVA followed by Tukey post hoc analysis for A, B, E, G, H, and J, and with Student’s t-test for C, D, F, and I. *$P < 0.05$ vs. vehicle and control cDNA, and †$P < 0.05$ vs. p22-phox cDNA-transfected cells. Ab, antibody; RAU, relative absorbance unit; RLU, relative luminescence unit.

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experiments. In order to illustrate the actual fold differences in enzymatic activities, both N4 h and N20 h values were normalized to 1.

Effects of OGD ± R on plasminogen-plasmin system components

As well as increasing p22-phox protein expression, OGD ± R increased uPA, tPA and PAI-1 protein expression without affecting that of uPAR in HBMECs [one-way ANOVA followed by Tukey post hoc analysis: \( n \geq 3, F_{2,23} = 10.94, P < 0.05 \) (Fig. 6A); \( n \geq 3, F_{2,33} = 0.70, P \text{ NS} \) (Fig. 6B); \( n \geq 3, F_{2,24} = 10.46, P < 0.05 \) (Fig. 6C); \( n \geq 3, F_{2,12} = 4.82, P < 0.05 \) (Fig. 6E)]. The increases in uPA and tPA activities were somewhat reflective of those observed in the expression of the relevant proteins. To determine the putative regulatory effects of NAD(P)H oxidase and MMP-2 on PA activities, uPA and tPA activities were measured in HBMECs subjected to OGD ± R in the absence or presence of apocynin or MMP-2 inhibitor III. Whereas the inhibition of MMP-2 had no effect on the activity of either PA, inactivation of NAD(P)H oxidase selectively suppressed tPA activity. As expected, amiloride diminished the OGD ± R-mediated increases seen in uPA activity, but not those seen in tPA activity, to the levels recorded in control cells [one-way

Fig. 5. The effects of OGD ± R on intracellular and secreted MMP-2 activities. HBMECs were exposed to 4 h of OGD alone, and OGD followed by 20 h of reperfusion (OGD + R), with and without inhibitors for uPA (amiloride), NAD(P)H oxidase (apocynin), and MMP-2 (MMP-2 inhibitor III). In the normoxia groups, cells were exposed to cell culture medium for either 4 h or 20 h to correspond to 4 h of OGD or 20 h of reperfusion. OGD + R, but not OGD alone, caused significant increases in intracellular and secreted MMP-2 activities in HBMECs (A–F). Whereas treatment with apocynin significantly reduced intracellular and secreted MMP-2 activities in all conditions (E and F), the inhibitory effects of amiloride were confined to the OGD + R groups (C and D). Data are expressed as means ± standard errors of the mean from \( n \geq 3 \) experiments, and were analysed with a one-way ANOVA followed by Tukey post hoc analysis. *\( P < 0.05 \), †\( P < 0.05 \) and ‡\( P < 0.05 \) vs. normoxia groups (N4 h or N20 h), 4 h of OGD, and corresponding untreated cells, respectively.
ANOVA followed by Tukey post hoc analysis: \( n \geq 3, F_{8,18} = 14.55, P < 0.05 \) (Fig. 6D); \( n \geq 3, F_{8,19} = 8.56, P < 0.05 \) (Fig. 6F).

**PAs contribute to BBB damage during 20 h of OGD**

To consolidate the specific contributions of PAs to barrier damage, co-cultures were established with HBMECs transfected with anti-uPAR or anti-PAI-1 antibody to quench uPA activity and enhance overall PA activities, respectively. Electroporation of anti-PAI-1 antibody impaired barrier integrity and function under normoxic conditions and after 20 h of OGD as compared with the corresponding anti-uPAR antibody and control groups. In contrast, electroporation of anti-uPAR antibody did not affect BBB integrity under normoxic and ischaemic conditions as compared with controls [one-way ANOVA followed by Tukey post hoc analysis: \( n \geq 3, F_{7,26} = 40.18, P < 0.05 \) (Fig. 7A); \( n \geq 3, F_{7,24} = 35.49, P < 0.05 \) (Fig. 7B)]. To confirm that tPA and uPA were being affected, PA activities were also measured after the above transfections. As expected, electroporation with anti-PAI-1 antibody increased both tPA and uPA activities in both the control and 20-h OGD groups. Transfection with anti-uPAR antibody had no effect on tPA activity, but did decrease uPA activity in the control and 20-h OGD groups.
The use of an IgG control had no effect on PA activities [one-way ANOVA followed by Tukey post hoc analysis: \( n \geq 3, F_{7,25} = 28.34, P < 0.05 \) (Fig. 7C); \( n \geq 3, F_{7,26} = 16.46, P < 0.05 \) (Fig. 7D)].

**Discussion**

The BBB serves as a highly selective, but permeable, barrier between the systemic circulation and the central nervous system. Several pathologies, including ischaemic injury, compromise its integrity, thereby allowing leakage of circulating molecules into the brain and triggering the formation of vasogenic oedema as a consequence; this is the main cause of death within the first week after a stroke (Hayashi et al., 2004). Microvascular endothelial cells constitute the main cellular component of the BBB, owing to their ability to express many tight junction proteins, notably occludin and claudin-5, to prevent vascular permeability. As endothelial cells express and retain these proteins only when co-cultured with astrocytes (Hayashi et al., 1997; Persidsky et al., 2006), a contact co-culture model of human BBB comprising HBMECs and human astrocytes was used in the current study. This model showed normoxic TEER values that are consistent with those in similar endothelial cell models reported previously, and mimicked the characteristics of the BBB, showing significant physical and functional impairments when subjected to OGD \( \pm R \), as demonstrated by marked decreases in TEER and simultaneous increases in paracellular flux, respectively (Allen & Bayraktutan, 2009a; Allen et al., 2010).

As well as being the primary cells forming the cerebral barrier, endothelial cells can act as key generators of \( \text{O}_2^- \) in acute ischaemic settings, to initiate or worsen cerebrovascular injury (Chrysobolis & Faraci, 2008; Raat et al., 2009). Indeed, exposure of HBMECs to OGD \( \pm R \) in the present study led to substantial increases in \( \text{O}_2^- \) production, which were attributed to the enhanced NAD(P)H oxidase activity during a clinically relevant period of experimental ischaemia, either 4 h of OGD alone or OGD followed by reperfusion (Hacke et al., 2004). Although high concentrations of peroxidizable lipids, low antioxidant capacity and reactions involving glutamate oxidation may further explain the susceptibility of the cerebral barrier to prolonged oxidative injury, putative contributions of mechanisms that are functionally intertwined with NAD(P)H oxidase cannot be fully dismissed in this context (Allen & Bayraktutan, 2009b).

Recent studies with matrix-degrading proteolytic enzymes have implicated PAs in cerebral barrier disruption, because of their ability to digest the basal lamina around the capillaries or modulate MMP-2 activity via their end-product, plasmin (Baramova et al., 1997). Consistent with previous studies reporting higher proteolytic activity in mice after the induction of focal cerebral ischaemia, significant increases have also been observed in the expression and/or activities of tPA, uPA and PAI-1 in HBMECs following exposure to OGD \( \pm R \) (Wang et al., 1998; Ahn et al., 1999; Hosomi et al., 2001). Similar levels of uPA expression in HBMECs before and after ischaemic insult imply that there is a sufficient amount of this receptor on cerebral endothelial cells to enable full uPA proteolytic activity (Wang & Kurpakus-Wheater, 2003), and the continuing high level of uPA activity despite high PAI-1 levels under the same conditions may be attributable to its resistance to inhibition once complexed with uPAR (Higazi et al., 1996).

In terms of MMP activities, whereas dramatic increases were recorded in the intracellular and secreted activities of MMP-2 only after induction of reperfusion, the activity of MMP-9 stayed below the detection threshold of gelatin zymography throughout (Cavdar et al., 2010; Tahanian et al., 2011). Similarly to the findings of
recent studies (Suofu et al., 2012), attenuation of MMP-2 activity with MMP-2 inhibitor III resulted in improvements in both barrier integrity and function during the reperfusion stage. As MMP-2 activity was not found to be increased during OGD alone, it is not surprising that inhibition of MMP-2 during OGD did not have a beneficial impact. A dose-dependence study was conducted with MMP-2 inhibitor III, and a concentration of 100 μM was selected, as further increasing the dose had no effect in reducing MMP-2 activity further during OGD ± R (data not shown). To determine the actual relevance of NAD(P)H oxidase activity to the OGD ± R-mediated activation of MMP-2 and PAs, in some experiments the oxidase activity was quenched with apocynin, which has previously been shown to reduce NAD(P)H oxidase activity in endothelial cells (Bayraktutan, 2004). Apocynin significantly suppressed MMP-2 and tPA activities without affecting that of uPA. Taken together, these data suggest that NAD(P)H oxidase operates downstream of uPA and upstream of tPA and MMP-2. The severity of BBB damage during reperfusion, when MMP-2 activity and O$_2^-$ levels are at their highest, further confirms the existence of a close link between MMP-2 and NAD(P)H oxidase. Furthermore, the attenuation of constitutive MMP-2 activity in cells exposed to OGD alone may indicate that the O$_2^-$-independent beneficial effects emerged from targeting of NAD(P)H oxidase (Chen et al., 2009, 2011).

Having deduced that uPA may be the determinant of the oxidative stress-related BBB damage that emerges during ischemic injury, we tested the effects of uPA inhibition with amiloride on barrier integrity, MMP-2 and NAD(P)H oxidase activities and O$_2^-$ generation during OGD ± R. A dose-dependence study was conducted, and a concentration of 2.5 μM was observed to be effective in normalizing uPA activity during OGD ± R (data not shown). At lower concentrations, amiloride acts as a specific inhibitor of uPA, and shows no effects on tPA expression and activity, despite being able to inhibit angiogenesis, capillary morphogenesis and the Na$^+/H^+$ exchange pathway at considerably higher concentrations, i.e. ≥130 μM (Haworth et al., 1993). Similarly to apocynin, amiloride selectively suppressed O$_2^-$ release and NAD(P)H oxidase activity in cells exposed to OGD ± R, confirming the supposed regulatory effect of uPA on NAD(P)H oxidase activity. Again, like apocynin, it attenuated reperfusion-mediated increases in intracellular and secreted MMP-2 activities. Amiloride also improved the physical and functional aspects of the BBB in the same settings. To discover the specific contributions of PAs to OGD-evoked BBB damage, the intracellular levels of both PAs and uPA alone were manipulated by electroporation of anti-PAI-1 or anti-uPAR antibodies into HBMECs before the establishment of co-cultures and exposure of them to a condition that causes considerable barrier damage, i.e. 20 h of OGD alone (Allen et al., 2010). This revealed that selective increases in total PA activity, but not in tPA activity alone, led to severe impairments in BBB integrity and function under both normoxic and ischemic conditions. Part of the modulation of uPAR function involves the internalization of the uPA–PAI-1–uPAR complex. Once internalized, the uPA–PAI-1 complex dissociates from uPAR and is transferred to the lysosomes, and the free uPAR returns to the plasma membrane. To suppress this recycling, which regulates uPAR signalling, in these experiments the uPAR antibody was introduced into the cells. The use of a relatively high, but non-toxic, concentration of anti-uPAR IgG (3 μg) ensured concurrent neutralization of uPAR on the plasma membrane.

Despite the increased release of O$_2^-$, treatment of acute ischemic stroke patients with free-radical scavengers has produced somewhat contradictory results – beneficial vs. neutral – in terms of lesion volume regression and clinical outcomes (Shuaib et al., 2007; Nakase et al., 2011). To explore the specific involvement of NAD(P)H oxidase and O$_2^-$ in barrier integrity and function, and also to assess the therapeutic role of interventions in uPA activity, the levels of enzyme activity and O$_2^-$ availability were elevated in HBMECs through electroporation of p22-phox cDNA and treatments with pyrogallol, respectively (Ear et al., 2001). Both applications resulted in pathologically relevant quantities of O$_2^-$, similar to the levels observed after 4 h of OGD + R, and caused substantial impairments in BBB integrity and function. Similarly to ischemic insult, elevations in NAD(P)H oxidase activity and the ensuing O$_2^-$ release also increased the protein expression of tPA. Attenuation of uPA proteolytic activity with amiloride or cessation of its binding to uPAR, a prerequisite for enzyme activity (Fuhrman et al., 2008), completely abolished these changes.

In conclusion, the data presented in the current study suggest that suppression of uPA activity may protect the cerebral barrier against ischemia–reperfusion injury by reducing MMP-2 activity in a direct fashion or through attenuation of NAD(P)H oxidase-dependent O$_2^-$ generation.

**Abbreviations**

- BBB, blood–brain barrier
- EBA, Evan’s blue-labelled albumin
- FBS, fetal bovine serum
- HBMEC, human brain microvascular endothelial cell
- HBSS, Hank’s Balanced Salt Solution
- MMP-2 inhibitor III: 2-[isoproxy]-(1,1’-biphenyl-4-ylsulfonyl)-aminol)-H-hydroxyacetamide
- MMP, matrix metalloproteinase
- NAD(P), nicotinamide-adenine dinucleotide (phosphate)
- PA, plasminogen activator
- PAI-1, plasminogen activator inhibitor-1
- tPA, tissue-type plasminogen activator
- TEER, transendothelial electrical resistance
- uPAR, urokinase-type plasminogen activator receptor
- uPA, urokinase-type plasminogen activator

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