Antioxidants attenuate hyperglycaemia-mediated brain endothelial cell dysfunction and blood–brain barrier hyperpermeability

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Aims: Hyperglycaemia (HG), in stroke patients, is associated with worse neurological outcome by compromising endothelial cell function and the blood–brain barrier (BBB) integrity. We have studied the contribution of HG-mediated generation of oxidative stress to these pathologies and examined whether antioxidants as well as normalization of glucose levels following hyperglycaemic insult reverse these phenomena.

Methods: Human brain microvascular endothelial cell (HBMEC) and human astrocyte co-cultures were used to simulate the human BBB. The integrity of the BBB was measured by transepithelial electrical resistance using STX electrodes and an EVOM resistance meter, while enzyme activities were measured by specific spectrophotometric assays.

Results: After 5 days of hyperglycaemic insult, there was a significant increase in BBB permeability that was reversed by glucose normalization. Co-treatment of cells with HG and a number of antioxidants including vitamin C, free radical scavengers and antioxidant enzymes including catalase and superoxide dismutase mimetics attenuated the detrimental effects of HG. Inhibition of p38 mitogen-activated protein kinase (p38MAPK) and protein kinase C but not phosphoinositide 3 kinase (PI3 kinase) also reversed HG-induced BBB hyperpermeability. In HBMEC, HG enhanced pro-oxidant (NAD(P)H oxidase) enzyme activity and expression that were normalized by reverting to normoglycaemia.

Conclusions: HG impairs brain microvascular endothelial function through involvements of oxidative stress and several signal transduction pathways.

Keywords: endothelium, oxidative stress, vascular disease, hyperglycaemia, blood-brain barrier

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Introduction

Vascular endothelium covers the entire inner surface of all the blood vessels and is implicated in the regulation of vasomotor tone, cellular trafficking and the local balance between pro- and anti-inflammatory mediators [1,2]. Endothelial cells in some organs differentiate and develop into specialized barriers to regulate vascular permeability. One such example is the blood–brain barrier (BBB) that stops the passage of substances from blood into the central nervous system. The BBB consists of brain microvascular endothelial cells (BMEC), capillary basement membranes and astrocyte end feet. Astrocytes surround 99% of the BBB endothelia and release soluble factors to maintain the integrity of the BBB [3]. As the brain oedema, associated with the BBB disruption, is more prevalent in stroke patients with diabetes mellitus (DM) than those without, it is safe to suggest
that hyperglycaemia (HG) plays a prominent role in this defect [4–6].

DM is a metabolic disorder associated with microvascular complications. Although several mechanisms including non-enzymatic glycation of proteins and lipids with the irreversible formation/deposition of advanced glycation end products, the activation of polyol pathway, stimulation of protein kinase C (PKC) or oxidative stress accompanied by overproduction of reactive oxygen species (ROS) have been implicated in peripheral vascular pathologies [1], the mechanisms by which HG exerts damage on brain microvasculature remain unknown.

The HG-mediated activation of PKC represents a hallmark in peripheral vasculopathies and is associated with increases in endothelial permeability, angiogenesis and ROS overproduction [7,8]. ROS, in particular superoxide anion (O$_2^-$), increase vascular permeability in various disease models including focal ischaemia and impairs neuronal homeostasis and cerebrovascular tone [9,10]. NAD(P)H oxidase, a pro-oxidant enzyme with a membrane-bound active core consisting of p22-phox and gp91-phox subunits, has been shown as the main enzymatic source of ROS in coronary endothelial cells exposed to HG [11]. Under physiological conditions, once generated O$_2^-$ is neutralized by superoxide dismutases (SODs) to H$_2$O$_2$, it in turn is metabolized to H$_2$O by catalase and glutathione peroxidase (GPx).

NO, another ROS generated by eNOS, is also implicated in diabetic microvasculopathy and BBB hyperpermeability in cerebral pathologies such as middle cerebral artery occlusion where the NOS inhibitors (NG-nitro-L-arginine (L-NNA) and NG-Nitro-L-Arginine Methyl Ester (L-NAME)) have been shown to reduce both brain oedema and BBB damage [12]. However, physiological levels of NO are known to regulate cerebral blood flow, vascular tone and protect against ischaemic stroke by increasing collateral flow to the ischaemic area [13]. It is of note that endothelial cells represent the main cellular source of NO and O$_2^-$ and the prevalence of diabetes is significantly greater in ischaemic stroke patients with small vessel compared with large-artery diseases [14].

It is well documented that HG promotes a pro-coagulant state to compromise blood supply to the penumbral areas in acute ischaemic stroke (AIS) through, in part, increasing the expressions of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [15].

In the light of the currently available data, this study hypothesizes that HG may perturb endothelial function and the BBB integrity by promoting oxidative stress and employing a variety of intermediary molecules. To this end, the present study was designed to investigate whether (i) changes in the expression and/or activities of oxidative stress related parameters, that is, adhesion molecules and pro-oxidant and antioxidant enzymes are involved in HG-mediated cerebral microvascular endothelial cell dysfunction; (ii) exposure of human brain microvascular endothelial cell (HBMEC) and human astrocytes (HA) co-cultures to normoglycaemia (NG) or HG distinctly modulate BBB integrity; (iii) antioxidants and signal transduction pathway inhibitors can help in the maintenance of the BBB integrity during hyperglycaemic insult and (iv) normalization of glucose levels has favourable effects on the aforementioned parameters following hyperglycaemic insult.

**Materials and Methods**

**Cell Culture**

HBMEC and HA were purchased from TCS Cellworks (Buckingham, UK) and cultured in specialized media. HA were characterized by glial fibrillary acidic protein staining, while HBMEC were characterized by staining for Von Willebrand factor (vWF)/factor VIII and CD31 (P-CAM) as well as by uptake of DiI-Ac-LDL. Cells were grown to subconfluence and incubated under NG (D-glucose; 5.5 mM) and hyperglycaemic (HG; D-glucose; 25 mM) conditions for a period of 5 days. Cells were also cultured in equimolar concentrations of D-mannitol (25 mM) to assess if the changes observed in enzyme activities or expressions were because of hyperosmolality rather than HG per se. In a subset of experiments, HBMEC were initially cultured in hyperglycaemic conditions for 5 days followed by a further 5 days culture in NG conditions to assess the specific effects of glucose normalization on aforementioned oxidative stress-related biomarkers.

**Evaluation of Cell Viability**

To detect cytotoxicity of outlined treatment regimens, a small aliquot of cells were incubated in 0.1% trypan blue for 4 min and viewed under a light microscope. By counting 100 cells, the percentage of viable cells was calculated.

**In Vitro BBB Co-culture Model**

HA were seeded on the outside of collagen-coated polycarbonate membrane (0.4 µm) Transwell inserts (12-well type; Corning Coster, High Wycombe, UK) directed upside down in the culture chamber. HA were allowed to adhere to the membrane overnight and then inverted the correct way. HBMEC were seeded on the inside of the
insert and cells were grown to confluence to create the contact co-culture model. The non-contact model is a replica of the contact model but the astrocytes were seeded onto the bottom of the well as opposed to the underside of the Transwell insert. Co-cultures were incubated for a period of 72 h under NG, HG and HG in the presence of an antioxidant agent including vitamin C (0.5 μM), catalase (150 U/ml), a free radical scavenger (MnTBAP; 50 μM), a PKC inhibitor, that is, bisindoylamide 1 (5 μM) or a MAPK inhibitor (SB203580; 10 μM). To investigate the extent of HA involvement in preservation of the BBB integrity under HG conditions, a monolayer of HBMEC were exposed to the aforementioned agents.

**Measurement of BBB Integrity**

This was assessed by measuring transendothelial electrical resistance (TEER) across the membrane using STX electrodes and an EVOM resistance meter (World Precision Instruments, Hertfordshire, UK). Three independent readings were taken every 24 h and blank filter measurements were subtracted from inserts seeded with cells. Values are shown as Ωcm² based on culture insert size.

**Measurement of NAD(P)H Oxidase Activity**

NAD(P)H oxidase activity was measured by cytochrome c reduction assay. Briefly, an aliquot of HBMEC homogenates (obtained from 20 × 10⁶ cells by homogenization of the cell pellet in cold 20 mM HEPES buffer, pH 7.2, containing 1 mM ethylene glycol tetraacetic acid (EGTA), 210 mM mannitol and 70 mM sucrose) was incubated with 50 μM cytochrome c for 60 min at 37°C. O₂⁻ generation was measured as the SOD (10 μg/ml)-inhibitable reduction of cytochrome c and monitored as the change in absorbance at 550 nm using a GENios plate reader (TECAN, Reading, UK). To abrogate all cytochrome c reduction to NAD(P)H oxidase, the specific inhibitors of other ROS-generating enzymes, that is, L-NAME (100 μM, NOS inhibitor), rotenone (50 μM, mitochondrial complex I inhibitor), allopurinol (100 μM, xanthine oxidase inhibitor) or indomethacin (50 μM, cyclooxygenase inhibitor) were added simultaneously to aliquots at the beginning of 60-min incubation period before determining O₂⁻ generation. Absorbancess were recorded for 8 min with 60-s intervals and activity was calculated as pmoles O₂⁻ per mg protein following subtraction of background levels at 550 nm.

**NOS Assay**

NOS activity was determined, in cell homogenates, using the NOSdetect assay kit (Alexis Biochemicals, Nottingham, UK). Briefly, cells were lysed in buffer containing 25 mM Tris, pH 7.4, 1 mM EDTA and 1 mM EGTA and centrifuged for 30 min at 4000 g. About 10 μl supernatant and 40 μl reaction buffer (50 mM Tris, pH 7.4, 6 μM tetrahydrobiopterin, 2 μM flavin adenine mononucleotide, 2 μM flavin adenine dinucleotide, 10 mM NADPH, 6 mM CaCl₂ containing 100,000 dpm [³H]L-arginine were combined. Following 30-min incubation at room temperature, the enzymatic reaction was stopped by the addition of stop buffer (50 mM HEPES, 5 mM EDTA, pH 5.5). Newly formed [³H]L-citrulline, neutral at pH 5.5, was then separated from the incubation mixture by cation exchange resin (Dowex AG 50 W-X8, Bio-Rad Laboratories, Hemel Hespstead, UK) and quantified using a liquid scintillation counter. The contribution of iNOS to overall NOS activity was assessed in similar experiments by replacing the calcium in reaction buffer with EGTA (5 mM). Data were expressed as pmol L-citrulline/mg protein/min.

**Nitrite Detection**

Nitrite levels were measured by Griess reaction as an index of NO generation following conversion of nitrate to nitrite by nitrate dehydrogenase. Cellular homogenate was mixed with an equal volume of Griess reagent (sulphanilamide 1% w/v, naphthyl ethylenediamine dihydrochloride 0.1% w/v and orthophosphoric acid 2.5% v/v) and incubated at room temperature for 10 min before measurement of absorbances at 540 nm. Nitrite formed was compared with those of known concentrations of sodium nitrite and normalized according to the protein concentration.

**SOD Assay**

SOD activity was measured by detecting the level of variation in O₂⁻ levels using a tetrazolium salt. Untreated and treated HBMEC homogenates were diluted 1:20 with radical detector and assayed in triplicate in a 96-well ELISA plate (Calbiochem, Nottingham, UK). Reactions were initiated by the addition of 20 μl diluted xanthine oxidase to generate O₂⁻ and the plate was incubated on a shaker for 20 min at room temperature. Absorbances were read at 450 nm and total SOD activity was determined using the equation obtained from the linear regression of the SOD standard curve. One unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the O₂⁻ (kit range 0.025–0.25 units/ml). MnSOD activity was detected following inhibition of Cu/Zn-SOD activity through incubation with 3 mM potassium cyanide (BDH Chemicals Ltd, Hemel Hespstead, UK).
Dorset, UK), at room temperature for 45 min. CuZnSOD activity was subsequently calculated by the subtraction of MnSOD activity from total SOD activities.

**Catalase Assay**

Catalase activity was measured using an immunoassay kit (Merck Biosciences, Nottingham, UK). The assay was based on the reaction of catalase with optimal concentration of H$_2$O$_2$ in the presence of methanol to produce formaldehyde and detect it spectrophotometrically using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald, Sigma-Aldrich, Dorset, UK) as the chromogen. Enzyme activities were then determined using a standard curve generated with formaldehyde. One unit was defined as the amount of enzyme that generates the formation of 1 nmol formaldehyde per minute, at 25 °C.

**GPx Assay**

GPx activity was measured using a specific immunoassay kit (Merck Biosciences) based on the simultaneous reduction of glutathione and oxidation of NAD(P)H to NADP$^+$, accompanied by a decrease in absorbance at 340 nm. GPx activities were then calculated using the extinction coefficient for NAD(P)H at 340 nm (0.00373/μM). One unit of GPx was defined as the activity that converts 1 mM of reduced glutathione per litre per minute, at 25 °C.

**Cellular Adhesion Molecule Assays**

ICAM-1 and VCAM-1 concentrations were determined using quantitative sandwich enzyme immunoassay kits (R&D Systems, Abingdon, Oxfordshire, UK) and microplates precoated with monoclonal antibodies specific for the markers in question, as per manufacturer’s instructions. Sample absorbances were read at 450 nm and subtracted from readings taken at 540 or 620 nm for VCAM-1 and ICAM-1, respectively. Concentrations were then calculated from a standard curve of known values.

**Western Blotting**

Total cellular proteins were isolated by lysis and equal amounts of protein (50 μg) were run on 10% sodium dodecyl sulphate–polyacrylamide gels. Proteins were transferred to nitrocellulose membranes before overnight incubation with monoclonal primary antibodies. Primary antibodies to catalase, MnSOD, CuZnSOD and GPx were obtained from Merck Biosciences. eNOS was from Transduction Laboratories (Oxford, UK) while those for gp91-phox and p22-phox were from Autogen Bioclear (Wiltshire, UK). Horseradish peroxidase–linked secondary antibodies were then used and the bands were detected by enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK). Blots were assessed by densitometry of bands with subtraction of the background counts measured outside loaded lanes.

**Statistical Analysis**

Results of molecular assays and TEER experiments are presented as mean ± s.e.m. Statistical analyses were performed by two-way analysis of variance followed by Bonferroni-Dunn’s post hoc analysis and p < 0.05 was considered significant.

**Results**

**Effects of HG on Protein Expression**

HG produced significant increases in p22-phox, a subunit of NAD(P)H oxidase, eNOS, CuZnSOD, catalase and GPx protein expression in HBMEC without affecting that of MnSOD. Glucose normalization following HG significantly decreased p22-phox, eNOS, catalase and GPx protein levels (figure 1).

**Effects of HG on Pro-oxidant and Antioxidant Enzyme Activities**

Exposure of HBMEC to HG for 5 days resulted in a twofold increase in pro-oxidant NAD(P)H oxidase enzyme activity compared with cells grown under NG conditions. Normalization of glucose levels for a further 5-day period attenuated the effects of the hyperglycaemic insult (table 1).

Similar to NAD(P)H oxidase, the eNOS activity and nitrite levels, a breakdown product of NO production, also rose in HBMEC cultured under hyperglycaemic conditions that were subsequently reduced by glucose normalization (table 1).

HG significantly enhanced CuZnSOD activity in HBMEC that was diminished by glucose normalization where MnSOD activity appeared to be unaffected by HG (table 1).

Catalase activity was found to be significantly increased in hyperglycaemic HBMEC that was diminished with glucose normalization. Although HG produced a slight increase in GPx activities, glucose normalization caused a profound decrease in its activity (table 1).

**Effects of HG on Adhesion Molecules**

HG produced a significant increase in VCAM-1 but not ICAM-1 concentration. Glucose normalization had no effect on ICAM-1 levels while significantly decreasing that of VCAM-1 compared with the HG group (figure 2).
Effects of Different Treatment Regimens on Cellular Viability

No significant variations were observed in overall viability rates of HBMEC and HA cultured with HG, NG or hyperosmolar media (table 2).

Effects of HG on TEER

As HA–HBMEC co-cultures produced better electrical resistance compared with non-contact and HBMEC monolayer models, this model has been used throughout the study to obtain statistically significant differences (figure 3). HG induced a marked decrease in TEER compared with D-mannitol and NG treatment groups, thereby inferring an increase in BBB permeability through disruption of the BBB where reversal of glucose to normal levels for 72 h restored BBB integrity (figure 4). Treatment of cells with a wide range of antioxidants, namely an NAD(P)H oxidase inhibitor (apocynin; 500 μM), vitamin C (0.5 mM), a free radical scavenger [mercaptopropionylglycine (MPG); 0.5 mM], an antioxidant enzyme (catalase; 25–150 U/ml) and two different SOD mimetics (MnTBAP; 50 μM or Mn(III)TMPyP; 10 μM) restored HG-mediated decreases in TEER (figure 5).

Other studies employing specific inhibitors of PKC (bisindolyamide I, 5 μM) and p38MAPK (SB203580, 10 μM) and PI3 kinase (LY29402, 50 μM) have shown that selective inhibitions of PKC and p38MAPK but not PI3 kinase reverses HG-induced decreases in TEER readings (figure 6).

Discussion

The major conclusions to be drawn from this study are that HG compromises the BBB integrity through

Table 1 The effects of hyperglycaemia and glucose normalization on pro-oxidant NAD(P)H oxidase and antioxidant enzyme activities

| Treatment group | NAD(P)H oxidase (pmol O$_2^-$/mg protein) | eNOS (pmol L-citrulline/mg protein/min) | CuZnSOD (mU/mg protein) | MnSOD (mU/mg protein) | GPx (nmol/min/ml) | Catalase (nmol/min/ml) | Nitrite (μM) |
|-----------------|-----------------------------------------|--------------------------------------|-------------------------|------------------------|-------------------|------------------------|--------------|
| D-mannitol 25 mM| 0.042 ± 0.007                           | 13415.9 ± 886.5                      | 278 ± 46                | 12 ± 1                 | 64.09 ± 6.61      | 22.13 ± 1.97          | 10.44 ± 0.67  |
| D-glucose 5.5 mM| 0.045 ± 0.007†                          | 12952.8 ± 924.4                      | 290 ± 37†               | 24 ± 6                 | 53.40 ± 4.88      | 21.94 ± 0.59          | 6.73 ± 0.86   |
| D-glucose 25 mM | 0.100 ± 0.006*                          | 17361.4 ± 1414.9*                    | 428 ± 39*               | 22 ± 3                 | 58.92 ± 6.40      | 27.00 ± 1.56*         | 13.44 ± 0.24*  |
| D-glucose 25/5.5 mM | 0.067 ± 0.005†                       | 15720.6 ± 277.9†                     | 209 ± 28†               | 7 ± 4†                 | 21.83 ± 5.40†      | 10.36 ± 1.22†         | 10.77 ± 0.37† |

GPx, glutathione peroxidase. The levels of enzyme activities were measured in human brain microvascular endothelial cells cultured with a hyperosmolality control (D-mannitol 25 mM), normal glucose (D-glucose 5.5 mM), high glucose (D-glucose 25 mM) and high glucose followed by normal glucose for a period of 5 days. Data are expressed as mean ± s.e.m. from five different experiments.

*p < 0.05 compared with NG (D-glucose 5.5 mM).
†p < 0.05 compared with HG (D-glucose 25 mM).
mechanisms mediated, in part, by oxidative stress and different signal transduction pathways. Indeed, specific inhibition of PKC or p38MAPK pathways, alleviation of oxidative stress by a series of functionally distinct antioxidants and normalization of high glucose levels restore the BBB integrity.

DM is a major risk factor for ischaemic stroke, while HG without pre-existing diabetes is linked to a worse neurological outcome and stroke mortality [9,16]. Brain oedema constitutes one of the main causes of death following stroke and is associated with the BBB disruption. Considering that the brain oedema is more prevalent in stroke patients with DM than those without, it is reasonable to suggest that HG plays a prominent role in this defect [4–6]. Ischaemic stroke develops through an interference with blood supply to the central nervous system and may be associated with oxidative stress, that is, an imbalance between ROS production and metabolism [17]. Although low-level generation of ROS is critical for neuronal homeostasis, their excess generation, particularly by NAD(P)H oxidase, is implicated in vascular injuries and intracranial haemorrhage [18,19]. Increases in certain NAD(P)H oxidase subunit expressions have been shown in streptozotocin (STZ)-induced diabetic rats with transient middle cerebral artery occlusion, an animal model of human ischaemic stroke and correlated with infarct sizes, more severe neurological deficits and brain oedema volume [20]. Significant increases in NAD(P)H oxidase enzyme activity and p22-phox protein expression, an integral NAD(P)H subunit, have also been found in this study in HBMEC cultured under hyperglycaemic conditions. Given that these increases were suppressed by normalization of glucose levels, collectively these data shed some light on the mechanistic causes of HG-mediated increases of oxidative stress and subsequent endothelial dysfunction.

As deficiencies in MnSOD and/or CuZnSOD activities may also contribute to enhanced oxidative status, the expression and activities of these enzymes were explored in HBMEC exposed to HG that revealed no changes or significant increases in cases of MnSOD and CuZnSOD, respectively. As HG significantly increases the BBB permeability, these data imply that the increase in CuZnSOD activity (approximately twofold), which makes up approximately 80% of total SOD activity [21], is not sufficient to quench the excessive ROS generated under hyperglycaemic conditions. Given that these increases were suppressed by normalization of glucose levels, collectively these data shed some light on the mechanistic causes of HG-mediated increases of oxidative stress and subsequent endothelial dysfunction.

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Table 2 Effects of hyperosmolality, normoglycaemia, HG and HG followed by normoglycaemia on HBMEC and HA viability

| Treatment Group | HBMEC | HA |
|-----------------|-------|----|
| d-mannitol 25 mM | 86 ± 4 | 91 ± 4 |
| d-glucose 5.5 mM | 93 ± 5 | 90 ± 7 |
| d-glucose 25 mM | 89 ± 2 | 88 ± 5 |
| d-glucose 25/5.5 mM | 90 ± 7 | 87 ± 6 |

HBMEC, human brain microvascular endothelial cell; HA, human astrocytes; HG, hyperglycaemia. Percentage viable cells were determined by trypan blue exclusion experiments and counting 100 cells. Data are expressed as mean ± s.e.m. from three different experiments.
A compensatory response to increase subdued enzyme activity by \textit{de novo} protein synthesis to neutralize still considerably high quantities of \( \text{O}_2^\cdot / \text{C}_0\text{O}_2\) during NG phase following a hyperglycaemic insult may, in part, account for this phenomenon.

Once dismutated, \( \text{O}_2^\cdot \) forms \( \text{H}_2\text{O}_2\), which requires further breakdown to \( \text{H}_2\text{O} \) by antioxidant enzymes catalase and GPx. This study has shown significant increases in catalase activity and protein expression in HBMEC exposed to HG that were reversed by glucose normalization and consequent removal of HG-mediated oxidative insult. Similar to catalase, a slight but significant increase was also observed in GPx protein expression in cells cultured under hyperglycaemic conditions. However, the increase in protein levels was not translated into activity perhaps as a result of previously reported instability of the GPx complex over time in diabetic rat tissues including brain [23].

NO, another ROS, has also been implicated in the BBB hyperpermeability in cerebral pathologies such as middle cerebral artery occlusion [12]. It is mainly generated by eNOS whose activity and expression under diabetic conditions remain controversial in that both enhanced and diminished eNOS expressions have been reported in diabetic patients, animal models and also in cells cultured with high concentrations of glucose [24,25]. However, similar to a previous study showing substantial elevations in STZ-induced diabetic rat hippocampus, cortex, cerebellum, brain stem and spinal cord [26], the current study has also revealed considerable increases in both eNOS enzyme activity and nitrite levels in HBMEC subjected to HG that were attenuated by glucose normalization. Although these findings may suggest a possibility that, in contrast to peripheral organs eNOS may be regulated in a similar manner by HG in...
different parts of the central nervous system, this hypothesis requires further detailed investigations. Endothelial dysfunction is, in part, regulated by the expression of adhesion molecules such as ICAM-1 and VCAM-1 on the surface of endothelial cells. Results from this study demonstrate that VCAM-1 but not ICAM-1 concentrations are increased under HG conditions that are dramatically decreased by glucose normalization. Similar to our study, Haubner et al. [27] have also reported a glucose-specific increase in the basal expression of membrane-bound VCAM-1 but not ICAM-1 and E-selectin. Conversely, a recent in vivo study has shown that the numbers of ICAM-1 positively stained microvessels in the cortex were markedly increased at 3 days of reperfusion in diabetic but not in non-diabetic rats, suggesting that inflammatory responses

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**Fig. 5** Effects of antioxidants, apocynin (500 μM), vitamin C (0.5 μM), mercaptopropionylglycine (0.5 μM), and superoxide dismutase mimetic MnTBAP (50 μM) and Mn(III)TMPyP (10 μM) and catalase (25 and 150 U/ml) on transendothelial electrical resistance (TEER) readings. Data are expressed as mean ± s.e.m. from five different experiments. *p < 0.05 compared with NG (5 mM D-glucose); #p < 0.05 compared with HG (25 mM D-glucose).

**Fig. 6** Effects of protein kinase C (bisindolyamide I, 5 μM), p38MAPK (SB203580, 10 μM) and PI3 kinase (LY29402, 50 μM) inhibitors on transendothelial electrical resistance (TEER). Data are expressed as mean ± s.e.m. from six different experiments. *p < 0.05 compared with NG (5.5 mM D-glucose); #p < 0.05 compared with HG (25 mM D-glucose).
mediate HG-aggravated brain damage, induced by ischaemia and reperfusion [28]. Although the reasons behind the differences between in vitro and in vivo studies require exploration, development of intracerebral diabetic ketoacidosis may, in part, account for this dichotomy. Indeed, an in vitro study with increasing concentrations of glucose along with acetoacetate and β-hydroxybutyrate led to increased expression of ICAM-1 and perturbed membrane function [29].

It is well documented that the integrity of the BMEC layer is critical for BBB function as a whole and pathological conditions including HG may compromise this tight continuum through facilitating the formation of openings in transcellular or paracellular pathways. Hence, the last phase of the current study examined the alterations in BBB permeability under hyperglycaemic vs. NG conditions through TEER measurements. As static monolayer and non-contact co-culture models did not produce significant variations in TEER, contact co-cultures have been used throughout the study. The current study has shown that HG exerted a significant decrease in TEER, a reliable indicator of the BBB integrity, which was blocked by a series of antioxidants with unique mechanisms of action thereby proving oxidative stress as a key player in the pathogenesis of this defect. The antioxidant compounds used in the study were vitamin C (an antioxidant vitamin), apocynin (a specific inhibitor of NAD(P)H oxidase), MPG (O2− scavenger), MnTBAP and Mn(III)TMPyP (SOD mimetics), catalase (an antioxidant enzyme) and Bis-I and SB203580 (PKC and p38MAPK inhibitors, respectively). These findings were in agreement with previous studies showing that SOD and catalase administrations reduce the tracer permeation to the cerebral ischaemic brain in rats [30–32].

The findings with the PKC inhibitor were, to a large degree, expected considering undisputed involvement of PKC activation in HG-mediated peripheral vasculopathies, ROS overproduction and cerebral endothelial dysfunction [7,8,33]. PKC may use two mechanisms to impair NO-mediated vasodilatation. Firstly, it may directly modify eNOS post-translationally through its dephosphorylation at Ser1177 and phosphorylation at Thr495 that together result in decreased NO production [34]. Secondly, PKC may impair eNOS function indirectly through effects on NAD(P)H oxidase, the most potent generator of O2− that rapidly scavenges NO. Similar to PKC, inhibition of p38MAPK has also been shown to be neuroprotective in cerebral ischaemia [35,36]. The p38 pathway may mediate neuronal damage by counteracting ERK signalling in models of apoptosis and free radical damage in vitro [37], by controlling the release of granules in neutrophils in the process of inflammation [38] or by induction of cytokines [39]. However, contradictory reports with p38MAPK inhibitor also exist in that rats given SB203580 before transient middle cerebral artery occlusion have been shown to develop widespread cerebral lesions and vascular leakage [40].

Research encompassing the PI3K inhibitor, LY29402 is very limited. However, Gottfried et al. [41] reported loss of polygonal morphology with varying degrees of stellation in rat hippocampal astrocytes treated with LY29402. This may have significance as intact brain astrocytic processes encounter and bind to collagen IV and laminin in the basement membrane of endothelial cells that surround cerebral capillaries and form the BBB [42].

Concurrence between previously published data and our findings suggest that the potential therapies for HG in acute stroke patients should include treatment regimens that establish and maintain normoglycaemia. In support of this notion, a recent retrospective study of AIS patients showed that patients with an admission glucose level of >130 mg/dl had increased mortality compared with those whose glucose levels were normalized within 48 h [43]. In this context, another study has revealed that intensive intravenous insulin therapy to maintain NG reduces the risk of organ failure and death in surgical intensive care patients [44].

In conclusion, the current study has shown that HBMEC incubated with HG possesses enhanced NAD(P)H oxidase enzyme activity and expression compared with cells grown under NG and that the reversal of HG to NG significantly suppress these increases. Secondly, the increased production of free radicals by NAD(P)H oxidase through activation of different signal transduction pathways appear to compromise the integrity of the BBB under diabetic conditions. Effective reversal of high glucose levels exerts beneficial effects on the preservation of the BBB unity and may therefore be therapeutically beneficial in diabetic stroke patients.

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References

1 Bayraktutan U. Free radicals, diabetes and endothelial dysfunction. Diabetes Obes Metab 2002; 4: 224–238.
2 Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001; 414: 813–820.
Abbott N. Astrocyte-endothelial interactions and blood-brain barrier permeability. J Anat 2002; 206: 527.

Baird TA, Parsons MW, Barber PA et al. The influence of diabetes mellitus and hyperglycaemia on stroke incidence and outcome. J Clin Neurosci 2002; 9: 618–626.

Hacke W, Schwab S, Horn M et al. ‘Malignant’ middle cerebral artery territory infarction: clinical course and prognostic signs. Arch Neurol 1996; 53: 309–315.

Kiers L, Davis SM, Larkins R et al. Stroke topography and outcome in relation to hyperglycaemia and diabetes. J Neurol Neurosurg Psychiatry 1992; 55: 263–270.

Bayraktutan U, Yang ZK, Shah AM. Selective dysregulation of nitric oxide synthase type 3 in cardiac myocytes but not coronary microvascular endothelial cells of spontaneously hypertensive rat. Cardiovasc Res 1998; 38: 719–726.

Cai S, Khojo J, Channon KM. Augmented BH4 by gene transfer restores nitric oxide synthase function in hyperglycemic human endothelial cells. Cardiovasc Res 2005; 65: 823–831.

Capes SE, Hunt D, Malmberg K, Pathak P, Gerstein HC. Stress hyperglycemia and prognosis of stroke in non-diabetic and diabetic patients: a systematic overview. Stroke 2001; 32: 2426–2432.

Ago T, Kitazono T, Kuroda J et al. NAD(P)H oxidases in rat basilar arterial endothelial cells. Stroke 2005; 36: 1040–1046.

Ceriello A, dello Russo P, Amstad P, Cerutti P. High glucose induces antioxidant enzymes in human endothelial cells in culture. Evidence linking hyperglycaemia and oxidative stress. Diabetes 1996; 45: 471–477.

Traystman RJ, Moore LE, Helfaer MA et al. Nitro-L-arginine analogues. Dose- and time-related nitric oxide synthase inhibition in brain. Stroke 1995; 26: 864–869.

Morikawa E, Moskowitz MA, Huang Z et al. L-arginine infusion promotes nitric oxide-dependent vasodilation, increases regional cerebral blood flow, and reduces infarct volume in the rat. Stroke 1994; 25: 429–435.

Grau AJ, Weimar C, Buggle F et al. Risk factors, outcome, and treatment in subtypes of ischemic stroke: the German stroke data bank. Stroke 2001; 32: 2559–2566.

Duckrow RB, Beard DC, Brennan RW. Regional cerebral blood flow decreases during hyperglycemia. Ann Neurol 1985; 17: 267–272.

Bell DS. Stroke in the diabetic patient. Diabetes Care 1994; 17: 213–219.

Hou ST, MacManus JP. Molecular mechanisms of cerebral ischemia-induced neuronal death. Int Rev Cytol 2002; 221: 93–148.

Crack PJ, Taylor JM. Reactive oxygen species and the modulation of stroke. Free Radic Biol Med 2005; 38: 1433–1444.

Guzik TJ, West NE, Black E et al. Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. Circ Res 2000; 86: E85–E90.

Kusaka I, Kusaka G, Zhou C et al. Role of AT1 receptors and NAD(P)H oxidase in diabetes-aggravated ischemic brain injury. Am J Physiol Heart Circ Physiol 2004; 286: H2442–H2451.

Didion SP, Ryan MJ, Didion LA et al. Increased superoxide and vascular dysfunction in CuZnSOD-deficient mice. Circ Res 2002; 91: 938–944.

Camada H, Yu F, Nito C, Chan PH. Influence of hyperglycemia on oxidative stress and matrix metalloproteinase-9 activation after focal cerebral ischemia/reperfusion in rats: relation to blood-brain barrier dysfunction. Stroke 2007; 38: 1044–1049.

Ulusu NN, Sahilli M, Avci A et al. Pentose phosphate pathway, glutathione-dependent enzymes and antioxidant defense during oxidative stress in diabetic rodent brain and peripheral organs: effects of stoßdine and vitamin E. Neurochem Res 2003; 28: 815–823.

Cosentino F, Hishikawa K, Katusic ZS, Luscher TF. High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. Circulation 1997; 96: 25–28.

Chakravarthy U, Hayes RG, Stitt AW, McAuley E, Archer DB. Constitutive nitric oxide synthase expression in retinal vascular endothelial cells is suppressed by high glucose and advanced glycation end products. Diabetes 1998; 47: 945–952.

Ates O, Yuceil N, Cayll SR et al. Neuroprotective effect of etomodine in the central nervous system of streptozotocin-induced diabetic rats. Neurochem Res 2006; 31: 777–783.

Haubner F, Lehle K, Munzel D et al. Hyperglycemia increases the levels of vascular cellular adhesion molecule-1 and monocoyte-chemoattractant-protein-1 in the diabetic endothelial cell. Biochem Biophys Res Commun 2007; 360: 560–565.

Ding Y, Li J, Rafols JA, Phillis JW, Diaz FG. Pre-reperfusion saline infusion into ischemic territory reduces inflammatory injury after transient middle cerebral artery occlusion in rats. Stroke 2002; 33: 2492–2498.

Hoffman WH, Cheng C, Passmore GG, Carroll JE, Hess D. Acetoacetate increases expression of intercellular adhesion molecule-1 (ICAM-1) in human brain microvascular endothelial cells. Neurosci Lett 2002; 334: 71–74.

Armstead WM, Mirro R, Thelin OP et al. Polyethylene glycol superoxide dismutase and catalase attenuate increased blood-brain barrier permeability after ischemia in piglets. Stroke 1992; 23: 755–762.

Liu ZF, Fang YZ. Observations on the activity and immunologic properties of CuZnSOD of whole blood in cancer patients. Zhonghua Yi Xue Za Zhi 1989; 69: 212–213.

Nelson CW, Wei EP, Povlishock JT, Kontos HA, Moskowitz MA. Oxygen radicals in cerebral ischemia. Am J Physiol 1992; 263: H1356–H1362.
33 Mayhan WG, Didion SP. Activation of protein kinase C does not participate in disruption of the blood-brain barrier to albumin during acute hypertension. Brain Res 1995; 696: 106–112.

34 Fleming N, Mellow L. Distribution and translocation of isoforms of protein kinase C in rat submandibular acinar cells. Life Sci 1995; 57: 2003–2010.

35 Barone FC, Irving EA, Ray AM et al. Inhibition of p38 mitogen-activated protein kinase provides neuroprotection in cerebral focal ischemia. Med Res Rev 2001; 21: 129–145.

36 Sugino T, Nozaki K, Hashimoto N. Activation of mitogen-activated protein kinases in gerbil hippocampus with ischemic tolerance induced by 3-nitropropionic acid. Neurosci Lett 2000; 278: 101–104.

37 Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995; 270: 1326–1331.

38 Mocsai A, Jakus Z, Vantus T et al. Kinase pathways in chemoattractant-induced degranulation of neutrophils: the role of p38 mitogen-activated protein kinase activated by Src family kinases. J Immunol 2000; 164: 4321–4331.

39 Lee JC, Laydon JT, McDonnell PC et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 1994; 372: 739–746.

40 Lennmyr F, Ericsson A, Gerwings P, Ahlstrom H, Terent A. Increased brain injury and vascular leakage after pretreatment with p38-inhibitor SB203580 in transient ischemia. Acta Neurol Scand 2003; 108: 339–345.

41 Gottfried C, Cechin SR, Gonzalez MA, Vaccaro TS, Rodnight R. The influence of the extracellular matrix on the morphology and intracellular pH of cultured astrocytes exposed to media lacking bicarbonate. Neuroscience 2003; 121: 553–562.

42 Tilling T, Engelbertz C, Decker S et al. Expression and adhesive properties of basement membrane proteins in cerebral capillary endothelial cell cultures. Cell Tissue Res 2002; 310: 19–29.

43 Gentile NT, Seftchick MW, Huynh T, Kruus LK, Gaughan J. Decreased mortality by normalizing blood glucose after acute ischemic stroke. Acad Emerg Med 2006; 13: 174–180.

44 van den Berghe G, Wouters P, Weekers F et al. Intensive insulin therapy in the critically ill patients. N Engl J Med 2001; 345: 1359–1367.