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ORIGINAL ARTICLE

Oxygen–glucose deprivation and interleukin-1α trigger the release of perlecan LG3 by cells of neurovascular unit

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Abstract

Two of the main stresses faced by cells at the neurovascular unit (NVU) as an immediate result of cerebral ischemia are oxygen–glucose deprivation (OGD)/reperfusion and inflammatory stress caused by up regulation of IL-1. As a result of these stresses, perlecan, an important component of the NVU extracellular matrix, is highly proteolyzed. In this study, we describe that focal cerebral ischemia in rats results in increased generation of laminin globular domain 3 (LG3), the c-terminal bioactive fragment of perlecan. Further, *in vitro* study of the cells of the NVU was performed to locate the source of this increased perlecan-LG3. Neurons, astrocytes, brain endothelial cells and pericytes were exposed to OGD/reperfusion and IL-1α/β. It was observed that neurons and pericytes showed increased levels of LG3 during OGD in their culture media. During *in vitro* reperfusion, neurons, astrocytes and pericytes showed elevated levels of LG3, but only after exposure to brief durations of OGD. IL-1α and IL-1β treatment tended to have opposite effects on NVU cells. While IL-1α increased or had minimal to no effect on LG3 generation, high concentrations of IL-1β decreased it in most cells studied. Finally, LG3 was determined to be neuroprotective and anti-proliferative in brain endothelial cells, suggesting a possible role for the generation of LG3 in the ischemic brain.

Keywords: cerebral ischemia, interleukin-1 (IL-1), neurovascular unit, oxygen–glucose deprivation, perlecan LG3.

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Extracellular matrix (ECM) comprises about 20% of the brain (Nicholson and Sykova 1998) and plays a key role in the maintenance of CNS homeostasis and function. It is composed of proteins including fibronectin, collagen, laminin and heparan sulfate proteoglycans (Webersinke *et al.* 1992; Dityatev *et al.* 2010). One of the most important ECM structures in the brain is the basal lamina-like structure (Dityatev *et al.* 2010), which forms a thick sheath over pericytes and endothelial cells of the neurovascular unit (Farkas and Luiten 2001; Hawkins and Davis 2005). An important component of these structures are heparan sulfate proteoglycans, the most prominent being perlecan, which forms an integral part of the basement membrane of the endothelial cells in mature tissues (Iozzo 1994, 1998). Perlecan is also expressed by neurons and astrocytes in addition to endothelial cells (Shee *et al.* 1998). Thus, perlecan forms an important part of the neurovascular unit (NVU) which consists of neurons, astrocytes, brain endothelial cells, pericytes and ECM (Hawkins and Davis 2005).

Acute brain injury, such as transient cerebral ischemia, damages the NVU by oxygen–glucose deprivation (OGD), and by the reactive oxygen species produced during reperfusion (Chan 2001). This triggers proteolysis of the ECM proteins, especially perlecan, within hours of ischemic insult

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Abbreviations used: DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle Medium; DV, Domain V; ECM, extracellular matrix; FBS, fetal bovine serum; hCMEM, human brain vascular endothelial cell; LG, laminin globular domains; MBEC, mouse brain endothelial cell; MCAo, middle cerebral artery occlusion; NVU, neurovascular unit; OGD, oxygen–glucose deprivation; PBS, phosphate-buffered saline.

due to up regulation of various proteases (Fukuda *et al.* 2004; Vikman *et al.* 2007). ECM degradation is accompanied by a local inflammatory response due to up regulation of cytokines (Vikman *et al.* 2007). One of the main pro-inflammatory cytokines is Interleukin-1 (IL-1) (Allan *et al.* 2005). Two of the main agonists of the IL-1 family, IL-1α and IL-1β are up-regulated within hours of cerebral ischemia (Hill *et al.* 1999; Allan *et al.* 2005) and both of them contribute to further ECM degradation, exacerbation of inflammation, and neuronal injury (Thornton *et al.* 2008).

Perlecan is an extracellular matrix protein which is made up of 5 domains (Bix and Iozzo 2008). Its c-terminal most domain, Domain V (DV), is made up of three laminin globular domains (LG) each separated by two epidermal growth factor (EGF)-like repeats (Bix and Iozzo 2008). DV and the fragment resulting from the cleavage of its last laminin globular domain, LG3, have been found to be biologically active (Bix *et al.* 2004; Wright *et al.* 2010). Indeed, LG3 has been found to be lowered in the plasma of breast cancer patients, elevated in the urine of patients with end-stage renal failure, and has also been found to be anti-apoptotic for mesenchymal stem cells, thus, suggesting an important physiological role for LG3 (Oda *et al.* 1996; Chang *et al.* 2008; Soulez *et al.* 2010).

Our group recently found that perlecan DV is generated and remains elevated up to 7 days in the brains of mice and rats subjected to transient middle cerebral artery occlusion (MCAo), and that this DV not only has important neuroprotective and proangiogenic effects on the post-stroke brain, but also modulates astrogliosis (Al-Ahmad *et al.* 2011; Lee *et al.* 2011). Immunohistochemistry studies using antibodies directed to perlecan Domain V (specifically recognizing the LG3 region) and perlecan Domain IV, which were used to differentiate free Domain V from Domain V attached to full length perlecan, showed that free Domain V is generated mainly at the NVU following cerebral ischemia (Lee *et al.* 2011). Furthermore, one of the cysteine proteases elevated during cerebral ischemia, cathepsin L, was found to cleave DV and generate LG3 (Fukuda *et al.* 2004; Cailhier *et al.* 2008). Thus, we were interested to know if LG3 was also elevated after cerebral ischemia. In the present study, we found that LG3 levels were indeed elevated after transient focal cerebral ischemia in rats. We further investigated the cellular source and mechanisms of LG3 production after cerebral ischemia, and here demonstrate that LG3 production is triggered by OGD/reperfusion insult and IL-1α treatment of the cells of NVU.

Materials and methods

Human LG3 production/purification

Human perlecan LG3 was produced and purified as described previously (Wright *et al.* 2010). Briefly, LG3 was cloned into the pCEP-pu plasmid which was then transfected into 293-Epstein-Barr nuclear antigen (EBNA) cells (vector and cells kindly provided by Maurizio Mongiat, Aviano, Italy). Cell-secreted LG3 was purified from the culture supernatant using an added c-terminal 6xHis-tag and Ni-ATA agarose beads (Qiagen, Valencia, CA, USA). Eluted fractions containing LG3 were combined and dialyzed against phosphate-buffered saline (PBS), and the purity of LG3 was confirmed using sodium dodecyl sulphate–gel electrophoresis under denatured conditions. LG3 was visualized by staining with Coomassie Brilliant Blue (Sigma Aldrich, St Louis, MO, USA), silver stain, and western blot with an LG3 antibody (R&D systems, Minneapolis, MN, USA; data not shown).

Experimental *in vivo* stroke model

Middle cerebral artery occlusion was performed on adult male 3-month-old Sprague–Dawley rats, as approved by Texas A&M College of Medicine IACUC, and as described previously (Lee *et al.* 2011). Briefly, ischemic injury was performed by tandem common carotid artery and middle cerebral artery occlusion. Animals with diminished perfusion reading (12–15% of the initial value), which was confirmed using laser Doppler flow meter (Perimed, Dickinson, TX, USA), were included in further experiments. Occlusions were removed after 60 min and animals were sacrificed 1 day and 3 days post-MCAo. Brains were extracted, contralateral and ipsilateral (ischemic) hemispheres were separated, and total homogenates were prepared. Lysates were prepared in RIPA lysis buffer complemented with protease inhibitor cocktail (Calbiochem, EMD Chemicals, San Diego, CA, USA).

Cell cultures

All primary cells were extracted from rats or mice as approved by Texas A&M College of Medicine IACUC. All cells were routinely cultured in conditioned incubators (37°C/5% CO_2).

Primary fetal cortical neurons were extracted from C57BL6 embryonic day 17–18 mice as previously described (Harris *et al.* 2007). Briefly, pregnant females were euthanized using cervical dislocation and embryos were dissected in order to obtain cerebral cortices. After mechanical dissociation and treatment with trypsin, neurons were seeded on poly-D-lysine (Sigma Aldrich) coated plates in Dulbecco's modified Eagle Medium (DMEM) high glucose (Invitrogen, Carlsbad, CA, USA) containing 2% (v/v) B27 supplement and 1% (v/v) penicillin-streptomycin. Experiments were performed after 3–4 days *in vitro* (DIV).

Primary astrocytes were prepared from 1 day old Sprague–Dawley rat pups as described previously (Chow *et al.* 2001; Cole and de Vellis 2001). Briefly, pups were anesthetized using hypothermia and brains were extracted and dissociated by mechanical trituration and trypsin/Dnase I treatment. Cells were seeded on Poly-D-lysine (Sigma Aldrich) coated flasks in DMEM/F12 (Invitrogen) containing 10% (v/v) fetal bovine serum (FBS; Denville Scientific Inc., South Plainfield, NJ, USA) and 1% (v/v) penicillin–streptomycin. After 7–9 DIV, the cultures were shaken at 37ºC and 180 rpm for 24 h to remove contaminating microglia and precursor oligodendrocyte cells. Cultures were then trypsinised and cells were seeded into fish gelatin (Sigma Alderich) coated flasks. Cultures contained 95% astrocytes as assessed by glial fibrillary acid protein immunocytochemistry.

Primary mouse brain endothelial cell (MBEC) cultures were established as described previously (Song and Pachter 2003). Briefly, 4- to 12-week-old C57BL6 mice were killed using CO_2

inhalation. Brains were extracted and homogenized. Microvessels were separated after density centrifugation in 18% (w/v) Dextran. Microvessels were digested using collagenase/dispase (Roche Applied Science, Burgess Hill, West Sussex, UK) solution containing Dnase (Invitrogen) and then suspended in DMEM-F-12 containing 10% (v/v) plasma-derived serum (FirstLink, Birmingham, UK), 10% (v/v) fetal calf serum (FCS), 100 μg/mL endothelial cell growth supplement (BD Biosciences, Oxford, UK), 100 μg/mL heparin, 2 mM glutamine, 1% (v/v) penicillin–streptomycin. Cells were then seeded on murine collagen-IV coated (50 μg/mL; BD Biosciences) plates, and cultures were used after 14 DIV.

Primary brain pericytes cultures were established as described previously (Dore-Duffy 2003; Al Ahmad *et al.* 2009). Briefly, adult Sprague–Dawley rats were euthanized using CO_2 inhalation. Brains were extracted and homogenized. Microvessels were separated after centrifugation in 20% (w/v) dextran. Microvessels were digested in collagenase/dispase solution (Roche) and seeded on tissue culture plates coated with 250 μg/mL collagen I (BD Biosciences) in DMEM high glucose (Invitrogen) with 20% (v/v) FBS and 1% (v/v) penicillin–streptomycin–amphotericin. After cultures became confluent, cells were trypsinised and seeded on uncoated tissue culture plates, and were cultured in media containing 10% (v/v) FBS and were used at passage 2 (P2). Cultures contained 95% pericytes as assessed by vimentin-positive, Von-Willenbrand factor-negative and glial fibrillary acid protein-negative immunostaining as described previously (Kim *et al.* 2006).

Human brain vascular endothelial cells (hCMEC/D3, kindly provided by Pierre-Olivier Couraud, Institute Cochin Institute, University of Paris, France) were used as a model for human brain endothelial cells (Weksler *et al.* 2005). hCMEC/D3 were cultured on fish gelatin coated plates in endothelial basal medium (EBM)-2 (Lonza, Walkersville, MD, USA) containing 10% (v/v) FBS, 1% (v/v) penicillin–streptomycin, 1.4 μM hydrocortisone, 5 μg/mL ascorbic acid, 1 : 100 chemically defined lipid concentrate, 1 ng/mL basic fibroblast growth factor and 10 mM HEPES. They were used from passages 29–38. The rat brain endothelial cell line, RBE4, was also used as a model for rat brain endothelial cells in some experiments (Roux *et al.* 1994), and were cultured on fish gelatin (Sigma) coated plates from passages 28–35 in minimum essential media-alpha containing nucleosides and ribonucleosides (Invitrogen) and F10 media (Invitrogen) in the ratio of 1 : 1, and containing 1% (v/v) penicillin–streptomycin.

In vitro oxygen–glucose deprivation

Oxygen–glucose deprivation was performed using a previously described setup (Tauskela *et al.* 2003). Non-neuronal cells were serum-starved overnight by changing culture media with DMEM high glucose (Invitrogen) with 1% (v/v) FBS and 1% (v/v) penicillin–streptomycin. For OGD, both neuronal and non-neuronal cultures were washed twice with OGD media, composed of DMEM with no glucose (Invitrogen) containing 1% (v/v) FBS and 1% (v/v) penicillin–streptomycin, with the addition of B27 for neuronal cultures. Cultures were then incubated with OGD media and transferred into a hypoxic chamber (Billups-Rothenberg) which was flushed with anoxic gas (90% N_2, 5% CO_2 and 5% H_2) for 5 min, according to the manufacturer's instructions. After OGD, the media was collected for western blot analysis and cultures were incubated with oxygenated DMEM high glucose with 1% (v/v) FBS and 1% (v/v) penicillin–streptomycin (and B27 for neuronal cultures). After 24 h, reperfusion media were collected for western blot analysis.

IL-1α and IL-1β treatment

Stocks of IL-1α and IL-1β (R&D Systems) were diluted in 0.1 % (w/v) bovine serum albumin (BSA)/0.9 % (w/v) NaCl. For non-neuronal cells, 100 ng/mL IL-1α and IL-1β were prepared in normal culture media of the cells (described above) and lower treatment concentrations were prepared by serial dilution. The media used for neurons contained 5% (v/v) FBS in addition to their normal culture media. Cells were washed once with their normal culture media before being treated with IL-1α or IL-1β. Treatment was carried out for 24 h and supernatants were collected for western blot analysis.

Western blot analysis

Western blot analyses were performed on brain lysates, which were prepared in RIPA lysis buffer complemented with protease inhibitor cocktail (Calbiochem; EMD chemicals), or were performed on cell culture supernatants. Culture media were also added to empty, acellular wells and were analyzed by western blot. Cell culture supernatants were concentrated 5- to 10-fold using vacuum centrifugation (Speedvac, Savant; Thermo Scientific, Rockford, IL, USA). Bradford assays (Bio-Rad Laboratories, Hercules, CA, USA) were used to determine protein concentration, and equal amounts of protein were separated on sodium dodecyl sulphate–gel electrophoresis and transferred to nitrocellulose membranes. Non-specific binding sites were blocked in 5% (w/v) non-fat milk in Tris buffer saline containing 1 : 1000 Tween20. Membranes were then incubated overnight with anti-endorepellin (1 : 1000; R&D Systems), hereby referred to as anti-LG3, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1 : 5000; Genetex, Irvine, CA, USA) for 2 h. Membranes were then developed using ECL kit (Thermo Scientific). Bands were quantified using Image J software (NIH) as described by Luke Miller (http://lukemiller.org/journal/2007/08/quantifying-western-blots-without.html). Ponceau staining (Sigma Aldrich) was carried out using 0.1% (w/v) Ponceau S. solution and was used to normalize the blots obtained from supernatants (Romero-Calvo *et al.* 2010). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize the blots from brain lysates.

Viability assay

Fetal cortical neurons were seeded on poly-D-lysine (Sigma) coated 96-well plates and used at 3–4 DIV. Cultures were washed twice with DMEM no glucose (Invitrogen) containing 1% (v/v) penicillin–streptomycin (OGD media). They were then incubated with OGD media in a hypoxic chamber for 2 h, as described above, to expose them to OGD. Media was then changed to DMEM high glucose (Invitrogen) containing 1% (v/v) L-glutamine and 1% (v/v) penicillin–streptomycin. Cultures were treated with PBS vehicle (control) or with 150 nM LG3, a dose that has previously been demonstrated to be biologically active (Bix *et al.* 2004; Wright *et al.* 2010). After 2 days, Alamar blue (AbD Serotec, Kidlington, Oxford, UK) was added to the wells and fluorescence readings were taken after 24 h with excitation at 560 nm and emission at 590 nm using a florescent plate reader (Victor X3; Perkin Elmer, Shelton, CT, USA), according to manufacturer's instructions.

Immunocytochemistry

Fetal cortical neurons were seeded on poly-D-lysine-coated glass chambers (Lab-Tek; Nunc, Naperville, IL, USA). At 3–4 DIV, they were exposed to OGD and treated with LG3 or PBS vehicle control, as described above. After 72 h, the cells were fixed in 4% (w/v) paraformaldehyde and permeabilized with 0.1% (v/v) Triton-X 100 solution. Cells were then blocked with 5% (w/v) BSA in PBS at 24°C for 2 h and were subsequently incubated with anti-active caspase 3 antibody (1 : 125 dilution; Abcam, San Francisco, CA, USA) overnight at 4°C, followed by washes with 1% (w/v) BSA and incubation with goat anti-rabbit alexa secondary conjugated with Alexa 576 (Invitrogen) for 45 min at 24°C. Cells were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). Samples were observed on an Axiovision A1m microscope (Carl Zeiss). Images were captured using a Retiga-SRV camera (Qimaging, Surrey, BC, Canada) and iVision software (BioVision Technologies, Exton, PA, USA) on an Apple Macintosh computer. Approximately 200 cells (recognized by DAPI nuclear florescence and phase contrast imaging) were counted in randomly selected high power fields, 10 fields per treatment condition, in order quantify the number of the cells showing active caspase 3 staining as a percentage of total cells.

Proliferation assay

hCMEC/D3 cells were seeded on uncoated wells at a density of 5000 cells/well in 96-well plates in hCMEC/D3 cell media (see above) containing 10% (v/v) FBS. After 24 h, the media was changed to hCMEC/D3 media containing 1% (v/v) FBS with PBS vehicle (control) or 150 nM LG3 (treated). After 48 h, MTS (Cell titer[96]; Promega, Madison, WI, USA) was added to the cultures and the absorbance was read after 4 h at 590 nm using a spectrophotometer (Phoenix Sunrise; Tecan, San Jose, CA, USA).

Statistical analysis

All experiments were performed at least three independent times, each time in duplicate or more unless otherwise stated. The data are presented as mean ± standard error of the mean (SEM). Comparison between groups was performed using two-tailed Student's *t*-test, one-way ANOVA with Tukey as a post-test, or two-way ANOVA, as appropriate, using GraphPad Prism 4.0. Significance was defined as $p < 0.05$.

Results

Focal ischemia induces the release of LG3

As our group recently demonstrated the active generation of perlecan DV following stroke injury (Lee *et al.* 2011), we wanted to investigate if perlecan LG3 is similarly increased after MCAo. As expected, we detected increased LG3 levels in the ipsilateral, ischemic hemisphere as compared with the contralateral hemisphere (of the same animal) at post-stroke day 1 (Fig. 1a). No LG3 was detected in sham surgical controls (data not shown). This increase was sustained, although slightly diminished at post-stroke day 3.

Oxygen–glucose deprivation causes neurons and pericytes, but not astrocytes, to release more LG3

Next, we investigated the possible source(s) of increased LG3 levels seen after MCAo *in vivo*. To this end, we chose to perform an *in vitro* analysis, as this approach affords the ability to study cellular components of the NVU individually. As OGD is the primary stimulus injuring cells during cerebral ischemia, we assessed LG3 levels in the media of neurons, astrocytes, RBE4 brain endothelial cells and pericytes exposed to OGD. The cells were changed to OGD media and were incubated in hypoxic conditions for various durations, after which the OGD media was collected and analyzed for LG3 levels by western blot. These were compared with the LG3 levels present in the media collected from control cultures (normoxic-high glucose) after the same durations. LG3 levels strongly and significantly increased in the media of neurons exposed to 1 h and 2 h OGD (Fig. 2a). Exposure of primary astrocyte cultures to OGD for 2, 6 or 24 h had no effect on LG3 levels (Fig. 2b), whereas 2 h OGD, but not 6 or 24 h, induced a slight increase in LG3 levels in the media of brain endothelial cells (Fig. 2c). In contrast, significantly increased levels of LG3 were detected in the media of pericyte cultures exposed to OGD for 2, 6 or 24 h, with maximum release observed during 6 h OGD (Fig. 2d). Finally, culture media that had been added to empty, acellular wells did not contain any detectable levels of LG3 (data not shown), demonstrating that the media itself does not appreciably contribute to detectable LG3.

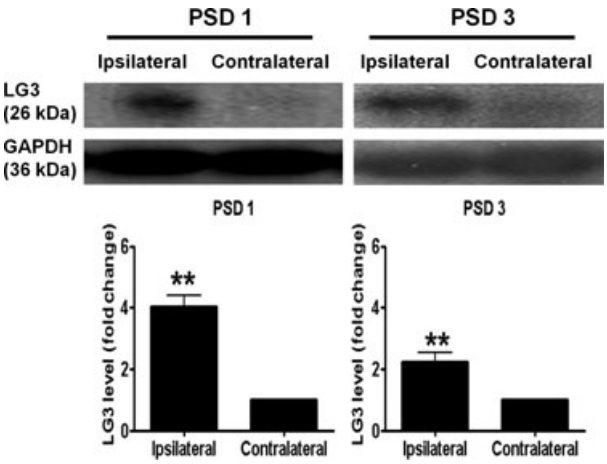

Fig. 1 Perlecan LG3 levels are elevated after stroke. Representative anti-LG3 western blots were normalized to their respective GAPDH blots and show that LG3 levels are elevated in ipsilateral hemisphere (stroked) when compared with contralateral hemisphere (of the same corresponding animal) at post-stroke day (PSD) 1 and PSD 3. Data were analyzed using Student's *t*-test and is significant at $^{**}p < 0.015$ as indicated.

Reperfusion stress causes neurons, astrocytes and pericytes to generate more LG3 only after brief durations of OGD

Following OGD, another stress which the cells of the NVU face is reperfusion, which results in the generation of reactive oxygen species (Chan 2001). Therefore, we were interested to see how reperfusion media of cultures treated with OGD

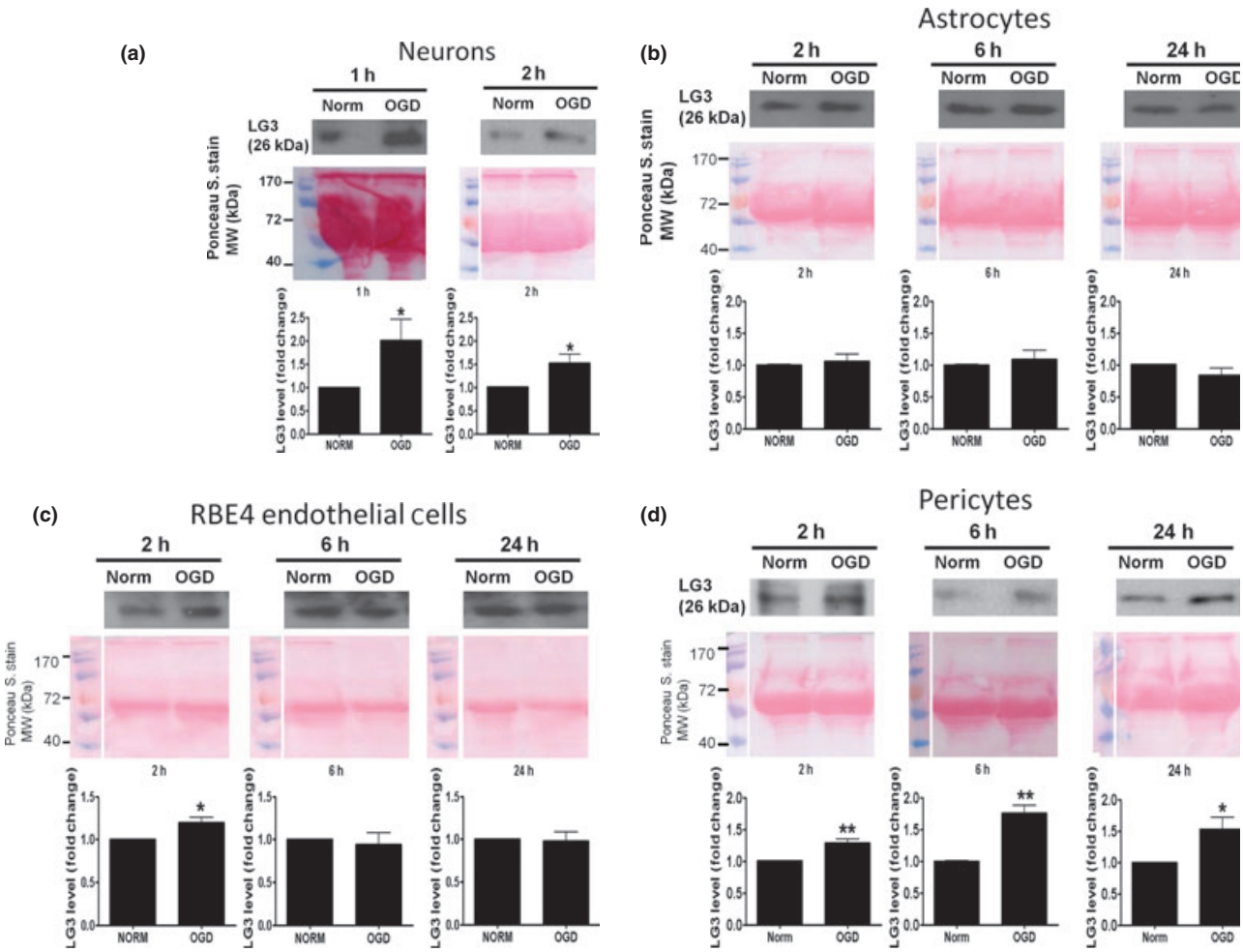

Fig. 2 Analysis of perlecan LG3 levels in OGD media of the cells of the NVU. Representative anti-LG3 western blots were normalized to their respective Ponceau S. stains and represent the fold change in LG3 released by (a) neurons, (b) astrocytes, (c) RBE4 brain endothelial cells and (d) Brain pericytes during various durations of OGD when compared with LG3 released by control cultures (Norm). Data were analyzed by Student's *t*-test and is significant at $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared with respective control culture LG3 levels.

would differ in LG3 levels when compared with that of control cultures. Apart from the reoxygenation-induced stress, we were also interested in examining the amounts of LG3 released by OGD-damaged cells when they return to normal conditions.

For cultures exposed to OGD, media were changed to normoxic-high glucose media, which was collected after 24 h and termed reperfusion media, as this can be considered to be an *in vitro* model for *in vivo* reperfusion. The reperfusion media was analyzed for LG3 levels and compared with LG3 levels in the media from control cultures which were also given a similar change of media. Neurons, astrocytes and pericytes showed an increase in LG3 levels only when OGD was of shorter durations (Fig. 3a, b and d). A decrease in the release of LG3 was observed after longer durations of OGD exposure to neurons, brain endothelial cells and pericytes (Fig. 3a, c and d).

Neurons reperfused after 1 h OGD, showed the strongest significant increase in LG3 levels (Fig. 3a). This increase was lost when neurons were reperfused after a 2 h OGD, with LG3 levels being significantly lower than control values. LG3 levels were significantly increased in reperfusion media of astrocyte cultures exposed to 2 h OGD, but this increase was lost in media of astrocytes exposed to 6 or 24 h OGD (Fig. 3b). Although RBE4 endothelial cells showed no change in LG3 levels in their reperfusion media after a 2 h OGD, LG3 levels progressively and significantly decreased after 6 and 24 h OGD (Fig. 3c). Pericytes, however, accumulated significantly more LG3 in their reperfusion media after a 2 h OGD, when compared with control cultures (Fig. 3d). However, longer durations of OGD induced a progressive decrease in the LG3 levels in the media of pericytes, which became significantly lower after 24 h OGD/reoxygenation.

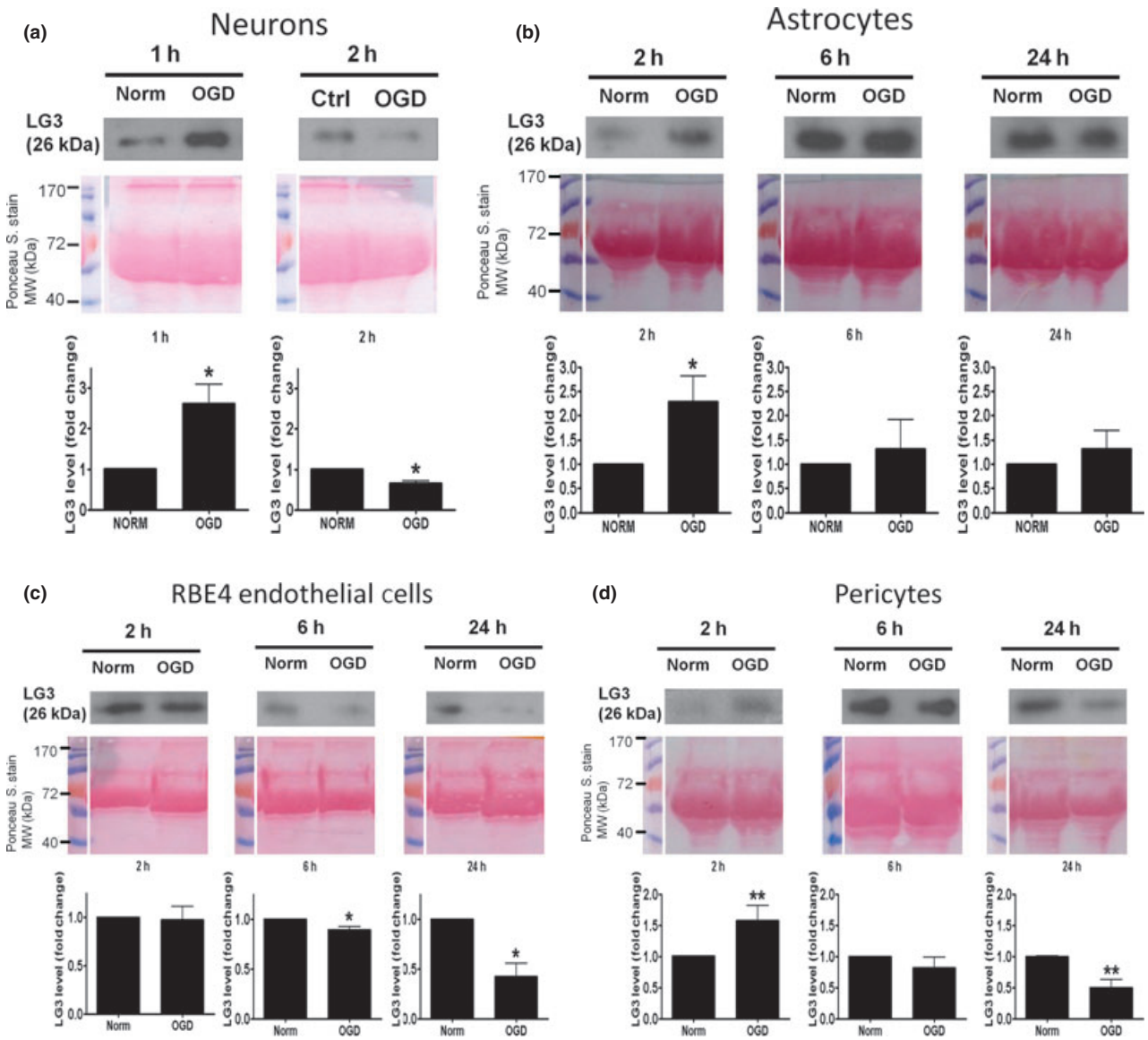

Fig. 3 Analysis of perlecan LG3 levels in the reperfusion media of the cells of the NVU. Representative anti-LG3 western blots were normalized to their respective Ponceau S. stains and represent the fold change in LG3 levels by (a) neurons, (b) astrocytes, (c) RBE4 brain endothelial cells and (d) brain pericytes after various durations of OGD (during 24 h of reperfusion) when compared with LG3 released by control cultures (Norm). Data were analyzed by Student's *t*-test and is significant at $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared with respective control culture LG3 levels.

Interleukin-1α and -1β differentially regulate LG3 generated by neurons, astrocytes, brain endothelial cells and pericytes

The central neuroinflammatory response driven by IL-1 plays a key role in the pathogenesis of cerebral ischemia, and is a key regulator of ECM degradation and remodeling in the brain. As the effect of IL-1 on LG3 production by brain cells is unknown, we investigated whether IL-1α and IL-1β could induce changes in LG3 produced by neuron, astrocyte, brain endothelial cell and pericyte cultures.

In neurons, IL-1α (0.1–100 ng/mL) induced an increase in LG3 levels in the culture media, although this was not found to be statistically significant (Fig. 4a). However, IL-1β, at 0.1 ng/mL, caused an increase in the amount of LG3 released, but, higher concentrations progressively caused a decrease in the amount of LG3 released in culture media, which became significant at 100 ng/mL of IL-1β (Fig 4a). As a result, a statistically significant difference was found between the effects of IL-1α and IL-1β at 100 ng/mL treatment.

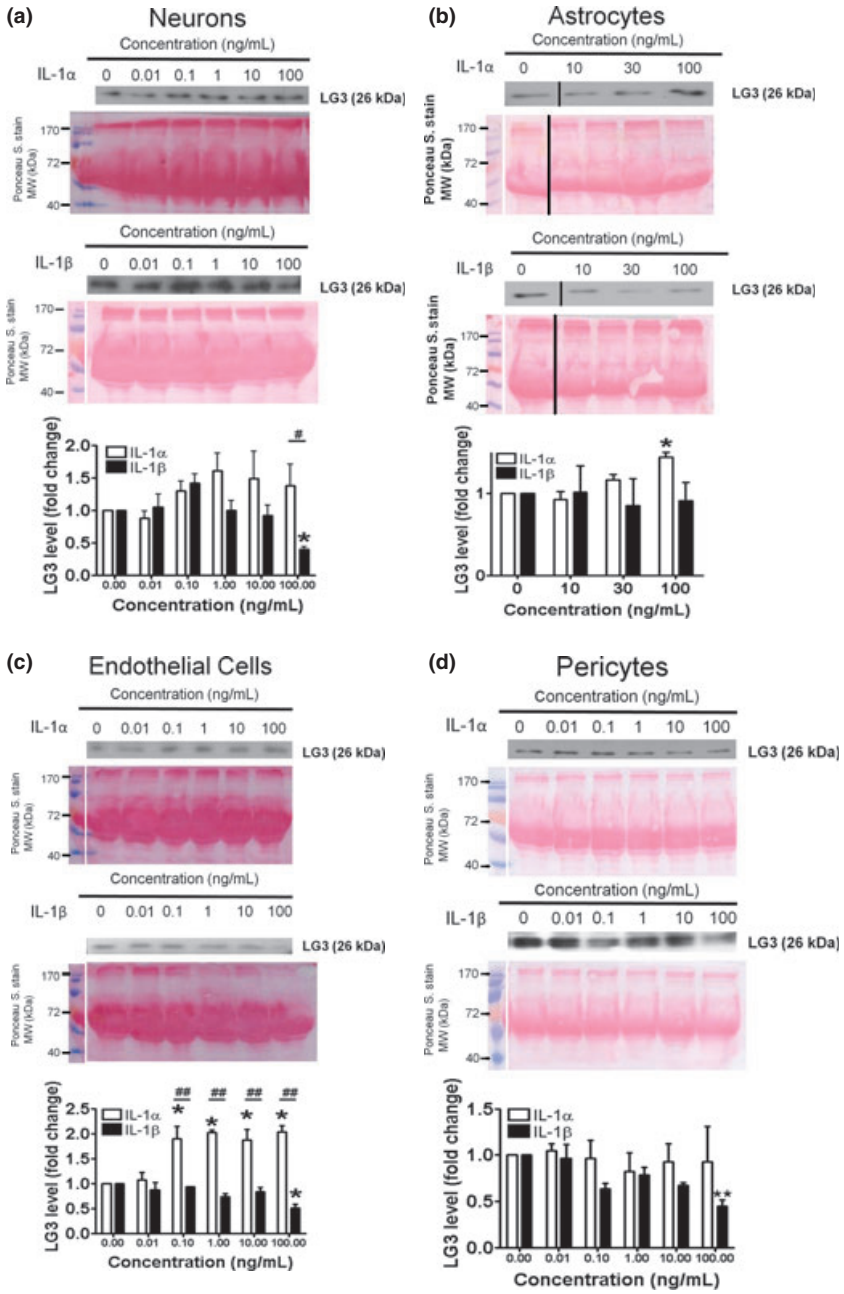

Fig. 4 Effect of IL-1α and IL-1β treatment on the LG3 released by cells of the NVU. Representative anti-LG3 westerns blots were normalized to their respective Ponceau S. Stains and depict LG3 levels in the media of (a) neurons, (b) astrocytes, (c) primary mouse brain endothelial cells and (d) brain pericytes after treatment with various concentrations of IL-1α and IL-1β. The data were analyzed by one-way ANOVA and was found to be significant at $^{*}p < 0.05$ and $^{**}p < 0.01$. For each cell, the dose response to IL-1α treatment was compared with the dose response generated to IL-1β treatment by two-way ANOVA and significance was found at $^{\#}p < 0.05$ and $^{\#\#}p < 0.001$.

In astrocytes, IL-1β failed to induce changes in LG3 levels at all of the concentrations tested (Fig. 4b). By contrast, adding increasing concentrations of IL-1α induced a concentration dependent increase in LG3 release, which was significantly higher after a 100 ng/mL treatment.

In MBECs, significant differences were found between the effects of IL-1α and IL-1β at concentrations of 0.1–100 ng/mL (Fig. 4c). IL-1α (0.1–100 ng/mL) strongly and significantly induced LG3 release from MBECs (Fig. 4c); IL-1β, however, failed to induce changes in LG3 levels at 0.01–10 ng/mL (Fig. 4c), but caused a slight, but significant decrease at high concentration (100 ng/mL). Finally, IL-1α had no effect on LG3 levels released by pericyte cultures, but, IL-1β reduced LG3 release at the highest concentration (100 ng/mL).

LG3 increases neuronal survival after OGD and decreases brain endothelial cell proliferation *in vitro*

After investigating the cellular source of increased LG3 levels as a result of ischemic / inflammatory stimuli, we investigated whether increased LG3 levels could have biological activity in brain cells after ischemia. As perlecan domain V, which is elevated after cerebral ischemia, has been found to be neuroprotective and pro-angiogenic (Lee *et al.* 2011), we tested the hypothesis that LG3 could regulate cell survival and/or proliferation *in vitro*.

The effect of LG3 on neuronal viability was studied because of particular neuronal susceptibility to damage as a result of ischemia/reperfusion (I/R). Fetal cortical neurons were exposed to OGD and then were treated with LG3 or PBS vehicle control. Their viability was measured 72 h later using Alamar blue assays. We found that LG3 treatment resulted in about a 300% increase in neuronal viability compared with PBS-treated cells (Fig. 5a). To further validate and elucidate the neuroprotective effect of LG3, we also performed active caspase 3 immunocytochemistry of similarly treated fetal cortical neurons (Fig. 5b). We observed that LG3 treatment caused a significant decrease in the percentage of cells staining positive for active caspase 3 (Fig. 5c).

We also studied the effect of LG3 on the proliferation of the hCMEC/D3 brain endothelial cell line. hCMEC/D3 cells treated with LG3 proliferated significantly less than untreated cells (Fig. 5d). In addition to a decrease in proliferation, the brain endothelial cells also showed a 'shriveled' morphology in response to LG3 treatment (Fig. 5e).

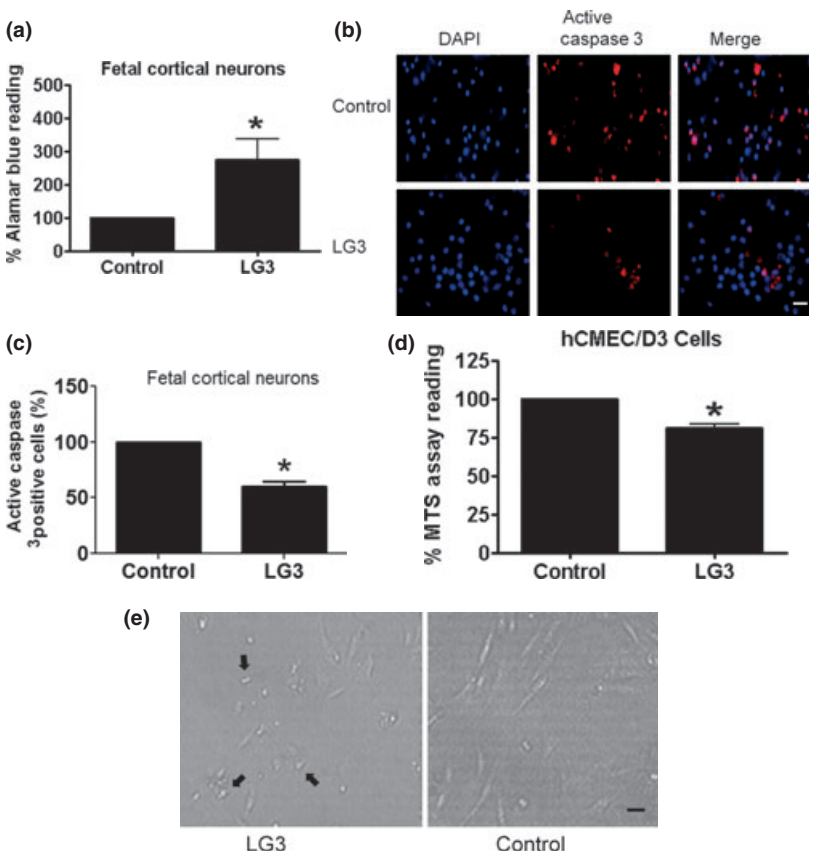

Fig. 5 LG3 is neuroprotective and anti-proliferative for brain endothelial cells. (a) Fetal cortical neurons were treated with 2 h of OGD and then treated with 150 nM LG3 or PBS (control). Alamar blue readings were taken 72 h later and the elevation in Alamar blue readings obtained after LG3 treatment is shown as a percentage of the Alamar blue readings obtained for control cultures. Data were analyzed by Student's *t*-test and significance was found at $*p < 0.05$ when compared with control. (b) Active caspase 3 immunocytochemistry on OGD-treated neurons. Fetal cortical neurons were treated with 2 h of OGD and then treated with 150 nM LG3 or PBS (control). They were fixed and stained with active caspase 3 antibody (red) and DAPI (blue) 72 h later. Scale bar = 20 μm. (c) Representative quantification of active caspase 3 staining on fetal cortical neurons (FCN) showing the active caspase 3-positive cells as a percentage of total cells. The data were analyzed by Student's *t*-test and is significant at $*p < 0.05$. (d) hCMEC/D3 brain endothelial cells were treated with 150 nM LG3 and their proliferation was analyzed 48 h later using MTS assay. The mean MTS reading of LG3-treated cells is shown as a percentage of mean MTS reading of untreated cells. Data were analyzed by Student's *t*-test and is significant at $*p < 0.05$. (e) LG3 causes hCMEC/D3 cells to express a 'shriveled' morphology (arrows). Cells were treated with LG3 and images were taken after 48 h of LG3 treatment using a light microscope. Scale bar = 10 μm.

Discussion

In this study, we observed a significant increase in the generation of the perlecan c-terminal fragment, LG3, in the brain after transient MCAo. The elevation of LG3 levels in the ipsilateral hemisphere occurred within 24 h after ischemia suggesting that events taking place during or immediately after cerebral ischemia cause increased perlecan synthesis/proteolysis. We therefore investigated the effects of ischemia/reperfusion stress and the effect of elevation of cytokines, which takes place within hours of cerebral ischemia (Hill *et al.* 1999), on LG3 generation from neurons, astrocytes, brain endothelial cells and pericytes *in vitro*, in an attempt to identify the potential cellular source(s) of the post-stroke generated LG3. Even though we demonstrated increased levels of LG3 both *in vivo* and *in vitro* post-ischemic conditions, the exact molecular source(s) of the increased LG3 remains to be identified. The increase in LG3 may result from an increase in cellular perlecan synthesis following ischemic conditions, increased cellular release of proteases cleaving previously secreted perlecan in the ECM, or both. However, the relative persistence of LG3 at 3 days post-stroke could suggest that its source at that time is at least partially from *de novo* brain cell-generated perlecan. It also remains to be investigated whether matrix can be degraded intracellularly in the brain, a possibility which has been established in human umbilical vein endothelial cells (Premzl *et al.* 2006).

Even though we show a significant increase in LG3 levels in rats following MCAo, a similar increase was also observed in stroked mice and non-human primates (data not shown), demonstrating the multi-species similarity in terms of perlecan synthesis/proteolysis. Furthermore, human perlecan domain V (DV) and LG3 also show a high degree (nearly 100%) of amino acid sequence similarity to their rat and mouse counterparts, and have thus been used successfully with mouse, rat, and human cells previously (Mongiat *et al.* 2003; Bix *et al.* 2004, 2007; Lee *et al.* 2011). Thus, these evidences indicate a conserved nature of perlecan and its DV and LG3 fragments, in terms of sequence, metabolism, and function.

In this study, brain lysates were generated from ipsilateral-stroked brain hemispheres and contralateral, non-stroked brain hemispheres. Unfortunately, this method does not afford sufficient spatial resolution to determine exactly where LG3 has been generated, that is, at the ischemic core versus the ischemic border area, or how much has been generated in each area. Therefore, assuming that LG3 is produced at both the ischemic core and the ischemic border area, it is conceivable that LG3 generated in the core would also be neuroprotective. This core-generated LG3 might then contribute to increased heterogeneity/viability within the core such that it could potentially reduce the evolution of 'mini-cores' (associated with multiple, more viable 'mini-penumbras') within the core proper into a more homogenous core over time (Del Zoppo *et al.* 2011).

OGD may stimulate the release of cysteine proteases and degradation of perlecan causing release of LG3

A key factor in cerebral ischemia-induced brain injury and inflammation is reduction in oxygen and glucose supply. Hence, we hypothesized that OGD could be a direct inducer of perlecan proteolysis. Neurons and pericytes were highly responsive during OGD in terms of LG3 levels. Brain endothelial cells showed a small increase only during 2 h OGD while astrocytes showed no changes. The increase seen during lower durations of OGD might be due to the release of lysosomal proteases and breakdown of already established matrix instead of *de novo* protein synthesis of perlecan. These results are also in agreement with a previous *in vivo* study which showed a significant decrease in perlecan positive microvessels 2 h after MCAo, which was caused by an increase in the levels of cysteine proteases and hence, proteolysis of perlecan (Fukuda *et al.* 2004). This study shows that neurons (primarily), pericytes and brain endothelial cells may be responsible for such an increase in proteolysis, and that merely OGD may initiate the release of proteases.

Cells release decreased levels of LG3 after longer exposures to OGD

We also assessed the effects of reperfusion/reoxygenation induced oxidative stress on the generation of LG3. Based on the LG3 levels observed after reperfusion, we could also assess the damage caused to the cells by OGD. Reperfusion of neuron and astrocyte cultures after 1 and 2 h of OGD, respectively, caused a substantial increase in LG3 levels. This increase in LG3 levels during reperfusion after short durations of OGD, by cells present in large numbers in the brain, may partially account for the increased LG3 levels detected *in vivo,* 1 day after MCAo. Brain pericytes also showed a significant increase in LG3 levels during reperfusion after 2 h of OGD.

Interestingly, it appears that longer durations of OGD induce a decrease in LG3 levels, as detected in neurons (after 2 h OGD), brain endothelial cells (after 6 and 24 h OGD) and pericytes (after 24 h OGD). Astrocytes, however, show no such decrease even after exposure to 24 h of OGD. These data for RBE4 endothelial cells and astrocytes are corroborated by the changes in viability seen in these cell types after exposure to various durations of OGD, where astrocytes show no significant decrease in viability even after 24 h of hypoxic OGD (Schmid-Brunclik *et al.* 2008; Lee *et al.* 2009). For RBE4 endothelial cells, in our OGD model, the decrease in viability after longer durations of OGD/reperfusion was confirmed by lower metabolism of cell medium, as confirmed by lack of change in color of the phenol red ph indicator in the cell medium (data not shown). For neurons, a

decrease in viability 24 h after 1 h of OGD has previously been reported (Jones *et al.* 2004). However, we did not see any significant decrease in neuronal viability 24 h after a 1 h OGD (data not shown). Given the very short duration of OGD involved, this may represent a difference in experimental setup. Much of cell death caused by OGD is triggered and occurs after the cells are returned to normal conditions (Nakajima *et al.* 2006). As we show that dying cells generate lesser amounts of LG3 than healthy cells, our results would seemingly be in contrast with a previous study which showed that apoptotic human umbilical vein endothelial cells generate greater amounts of LG3 (Laplante *et al.* 2006). This discrepancy may be due to the difference in the mechanism of OGD/reperfusion mediated cell death. Thus, we can collectively conclude that shorter durations of OGD that stress, but do not kill cells of the NVU, lead to an increase in LG3 levels, while longer periods of OGD that result in cell death lead to decreased LG3 levels.

An increase in LG3 levels may partially be maintained by IL-1α even 3 days after stroke

Given that IL-1 is established as a key mediator of inflammation and neuronal injury, we next investigated whether IL-1 could regulate LG3 production by cells associated with the NVU. Both IL-1α and IL-1β induce similar biological actions by binding to the same signaling receptors (Allan *et al.* 2005), and therefore, have redundant functions (Boutin *et al.* 2001). However, IL-1α and IL-1β have also been found to have clear differential effects (Trebec-Reynolds *et al.* 2010). In agreement with this, we found here that IL-1α and IL-1β have generally opposite effects in neurons and endothelial cells, in that, IL-1α induced increased levels of LG3 whereas IL-1β induced a decrease in LG3 levels. Our data strongly correlate with a previous study which reported that IL-1α increases global perlecan mRNA in the hippocampus and in astrocytes (Garcia de Yebenes *et al.* 1999), and we can speculate that IL-1α may cause an increase in LG3 levels via an increase in perlecan synthesis.

Both IL-1α and IL-1β are elevated within hours of cerebral ischemia and IL-1α may stay elevated up to 4 days after stroke (Hill *et al.* 1999). But, unlike IL-1β, IL-1α is constitutively expressed in the brain (Boutin *et al.* 2001). Furthermore, IL-1α (but not IL-1β) has been recently shown to be released by blood platelets and induce brain endothelium activation in the ischemic core within 24 h after stroke (Thornton *et al.* 2010). As IL-1α has also previously been shown to be transported across blood–brain barrier (Banks *et al.* 1994), IL-1α is more likely to be present at a higher concentration than IL-1β in the brain. Thus, even though IL-1β is elevated after cerebral ischemia (Hill *et al.* 1999), high enough concentrations may not be reached for it to produce an effect because it only decreases LG3 levels at high concentrations. IL-1α may, on the other hand, contribute towards maintaining the levels of LG3 generated by brain cells, given the sensitivity of cells, such as brain endothelial cells, to low concentrations of IL-1α, and the possibility of its higher concentrations in the brain, even 3 days after stroke. Additionally, we conclude that brain pericytes express an IL-1 receptor because they respond to IL-1β.

Increased LG3 levels have functional significance

We observed a beneficial effect of LG3 on neuronal survival. Our group previously showed that LG3 blocks neuronal toxicity induced by amyloid beta through α2β1 integrin binding (Wright *et al.* 2010). Here, we demonstrate that LG3 may act as an agonist to promote neuronal survival after OGD induced damage. We further showed that LG3 caused a decrease in active caspase 3 immunostaining following OGD-reperfusion, which suggests that LG3 has anti-apoptotic effects on neurons, which have previously been observed in other cell types (Laplante *et al.* 2006; Soulez *et al.* 2010).

LG3 also causes a small decrease in the proliferation of hCMEC/D3 brain endothelial cells. This is in direct contrast to perlecan DV's enhancement of brain endothelial cell proliferation (Lee *et al.* 2011) and may reflect relative differences in their affinities for the perlecan DV pro-angiogenic receptor α5β1 integrin (Clarke *et al.* 2011). Furthermore, it is possible that the post-stroke pro-angiogenic activity of DV is regulated by its further proteolytic cleavage yielding the anti-angiogenic LG3.

In conclusion, we have demonstrated that perlecan LG3 is persistently generated after focal cortical ischemia, provided evidence that specific cell types of the NVU are responsible for the generation of LG3 after OGD or reperfusion, respectively, and demonstrated that IL-1α and IL-1β differentially affect LG3 production. Finally, we provide evidence that this post-ischemia generated LG3 is neuroprotective, suggesting its potential relevance to stroke pathophysiology, and hinting at its therapeutic potential.

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