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Oxygen-Glucose Deprivation (OGD) and Interleukin-1 (IL-1) Differentially Modulate Cathepsin B/L Mediated Generation of Neuroprotective Perlecan LG3 by Neurons

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Abstract

Brain extracellular matrix (ECM) is highly degraded after cerebral ischemia. The perlecan c-terminal fragment LG3 is generated at increased levels by proteolytic processing as long as 3 days after ischemia. It has previously been shown that oxygen-glucose deprivation (OGD), reperfusion and interleukin-1 α (IL-1 α) stimulate brain cells to yield increased levels of LG3. This LG3, in turn, is neuroprotective against OGD, and may therefore represent one of the brain's defenses against ischemic injury. Here, we investigate whether, in neurons, this increased LG3 is the result of increased perlecan generation and cellular release, increased protease release (to generate LG3 from previous extracellularly deposited perlecan) or both. We found that pre-synthesized perlecan may be exocytosed by neurons during OGD and *de novo* synthesis of perlecan is increased during reperfusion, even 24 h after OGD. Furthermore, while cathepsin L activity was seen to be marginally important to generate LG3 during normoxic conditions, cathepsin B activity was found to be important to generate increased levels of LG3 following OGD and reperfusion. On the other hand, IL-1 α treatment raised levels of cathepsin L in neuronal media, and both cathepsin L and cathepsin B were demonstrated to be important for increasing LG3 levels after IL-1 α treatment.

Keywords

Perlecan LG3; Oxygen-glucose deprivation (OGD); interleukin-1 (IL-1); neurons; cathepsin; perlecan synthesis

1 Introduction

Extracellular matrix (ECM) is very important for brain function and homeostasis. It is composed of proteins including collagen, fibronectin, laminin and heparan sulfate proteoglycans (HSPGs) (Dityatev et al., 2010). Perlecan is one such HSPG which forms the basal lamina-like structures in the brain (Dityatev et al., 2010; Iozzo, 1994; Iozzo, 1998). It

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has previously been shown that components of ECM, especially perlecan, are rapidly degraded following cerebral ischemia (Fukuda et al., 2004). In agreement with this, we have recently demonstrated that levels of the c-terminal most domain of perlecan, domain V (DV), are persistently elevated in the brain after focal cerebral ischemia for at least as long as 7 days after ischemia (Lee et al., 2011). Also, the levels of the c-terminal most laminin globule (LG) repeat of DV, LG3, were shown to be elevated even 3 days after cerebral ischemia (Saini et al., 2011). This increase in perlecan DV and LG3 fragments appears to have significance in the post-stroke brain, in as much as these fragments were shown to have beneficial effects including modulation of astrogliosis and neuroprotection (Al-Ahmad et al., 2011; Lee et al., 2011; Saini et al., 2011). DV, originally characterized to be anti-angiogenic outside of the brain due to interaction with endothelial cell $\alpha 2\beta 1$ integrin (Bix et al., 2004), is also pro-angiogenic in the brain due to both the absence of $\alpha 2\beta 1$ integrin in brain microvasculature, and its binding to brain endothelial cell $\alpha 5\beta 1$ integrin and subsequent release of the pro-angiogenic growth factor VEGF (Lee et al., 2011). LG3, on the other hand, has a thousand fold lower affinity for $\alpha 5\beta 1$ integrin as compared to DV, and is unable to replicate the pro-angiogenic effects of DV in brain endothelial cells (Clarke et al., 2011; Saini et al., 2011), despite it still possessing anti-angiogenic activity outside of the brain (Bix et al., 2004). Importantly, however, LG3 appears to have other distinct activities from DV including being anti-apoptotic for neurons, fibroblasts and mesenchymal stem cells (Laplanche et al., 2006; Saini et al., 2011; Soulez et al., 2010).

One of the classes of proteases which have been implicated in the generation of LG3 from perlecan is that of lysosomal cathepsins, specifically cathepsin L (Cailhier et al., 2008; Fukuda et al., 2004). Cathepsins are known to have similar sequence and structure, and thus may have redundant function (Turk et al., 2001). In support of redundant function between cathepsin L and B, it has been shown that while cathepsin B null mice have normal phenotype (Deussing et al., 1998), cathepsin L null mice show non-fatal phenotypes such as reduction in major histocompatibility complex (MHC) invariant chain degradation (Nakagawa et al., 1998) and periodic hair loss (Roth et al., 2000). But, cathepsin B and cathepsin L double knockout mice die in infancy and show massive neuronal apoptosis (Felbor et al., 2002). Both these cathepsins, L and B, have been shown to be elevated within hours of cerebral ischemia (Fukuda et al., 2004; Kohda et al., 1996), suggesting their importance in the post-ischemic brain.

It has been previously established that oxygen glucose deprivation (OGD)/reperfusion and IL-1 α treatment may cause an increase in the LG3 generated by brain cells (Saini et al., 2011). Specifically for neurons, the same study also showed that it was not only OGD and reperfusion (with reperfusion referring to an in vitro model for in vivo reperfusion involving re-oxygenation and re-addition of glucose), but also low concentrations of both IL-1 α and IL-1 β that cause an increase in LG3 levels. However, the molecular mechanisms causing this increased LG3 in neuronal media were not investigated. Therefore, in this work, we investigate how neurons respond to OGD and inflammatory stimuli in terms of perlecan and protease release. We found that while pre-synthesized perlecan is exocytosed during OGD, reperfusion causes an increase in *de novo* perlecan synthesis. Increased release of perlecan may be partly responsible for increased levels of LG3 after OGD/reperfusion, both *in vitro* and *in vivo*. Importantly, the cathepsin L specific inhibitor, Z-FY-CHO, could not abrogate the increased LG3 levels resulting from OGD and reperfusion of neurons. However, with cathepsin B inhibitor, we saw a significant decrease in LG3 levels after OGD and reperfusion when compared to normoxic conditions, collectively suggesting the involvement of cathepsin B in neuronal perlecan degradation in response to OGD/reperfusion. We also observed that IL-1 α treatment causes a slight, but significant increase in cathepsin L levels secreted by neurons and that IL-1 α mediates increased levels of LG3 through cathepsin L and cathepsin B activity. These results suggest that the release of proteases, and subsequent

generation of LG3 from perlecan (previously generated and exocytosed, or generated *de novo*), are differentially regulated depending on the type of stimulus.

2 Results

2.1 Increased *De Novo* Synthesis of Perlecan Occurs Following Reperfusion but NOT During OGD

It was recently demonstrated that LG3 levels are increased in the conditioned media of fetal cortical neurons (FCN) during exposure to OGD and following reperfusion (Saini et al., 2011). To further decipher the molecular source of such an increase in LG3 levels, we performed Q-PCR analysis to quantify the levels of FCN perlecan mRNA following OGD and reperfusion. FCN were exposed to 1h of OGD, after which they were either lysed to extract RNA, or they were reperfused with normoxic-high glucose media for 24 h followed by cell lysis for RNA extraction. We observed that while expression of perlecan mRNA remained unchanged compared to normoxic control after 1 h of OGD, it increased significantly during reperfusion (Fig. 1A-B).

It was also reported that treatment with low concentrations of IL-1 α and IL-1 β increased LG3 levels in the media of FCN (Saini et al., 2011). Therefore, we wanted to investigate if the levels of perlecan mRNA also increased following treatment with a low concentration (0.1 ng/ml) of IL-1 α and IL-1 β . Q-PCR analysis showed that levels of perlecan mRNA remain unchanged following 0.1 ng/ml IL-1 α / β treatments (Fig. 1C).

2.2 Neurons Show an Increase in the Number of Perlecan Positive Vessicle-like Structures during OGD

To further evaluate the increased LG3 levels seen after exposure to OGD, we performed perlecan immunocytochemistry. FCN were exposed to 1 h of OGD and were fixed immediately following OGD to stain for perlecan. It was observed that FCN exposed to OGD showed an increase in perlecan positive intracellular vesicle-like structures located towards the periphery of the cell (Fig. 2A-B). This was in contrast to the more homogenous perlecan staining seen in control cultures. In both cases, we also noted high staining for perlecan around the nuclei. Also, FCN treated with IL-1 α and IL-1 β did not show any changes in the pattern of perlecan staining when compared to control cultures (data not shown).

2.3 Levels of Secreted Cathepsin-L Increase Following Treatment with IL-1 α

Cathepsin-L released by apoptotic human umbilical vein endothelial cells (serum starved for 4 h) has previously been shown to cleave LG3 from perlecan (Cailhier et al., 2008). To further elucidate the molecular mechanisms causing an increase in LG3 levels following various neuronal stressors, we decided to evaluate the release of cathepsin L in neuronal media.

FCN were exposed to 1 h of OGD and their conditioned media was collected subsequently. Following this, cultures were changed to normoxic-high glucose media and this was collected after 24 h (termed reperfusion media). Although we did not observe any significant changes in the levels of pro-cathepsin L (42kDa), 36kDa mature cathepsin L and 29kDa mature cathepsin L, the levels of different forms of cathepsin L decreased almost unanimously to some level in OGD and reperfusion media of FCN when compared to corresponding normoxic media (Fig. 3A-B).

Next, we treated cells with low concentrations of IL-1 α and IL-1 β and collected the culture supernatants 12 h later. We observed a small but significant increase in the levels of pro-

cathepsin L following IL-1 α treatment, but, non-significant increases in the levels of 36kDa and 29kDa mature cathepsin L were observed following IL-1 α treatment (Fig. 3C). Similarly, IL-1 β treatment resulted in small, but non-significant increases in all three forms of cathepsin L (Fig. 3D).

2.4 Cathepsin L Specific Inhibitor Does not Prevent the Increase in LG3 levels Following Neuronal Exposure to OGD/Reperfusion

Next, we wanted to see whether the cathepsin L specific inhibitor Z-FY-CHO could prevent the increase in LG3 levels seen after FCN exposure to OGD and reperfusion (Saini et al., 2011). FCN were treated with Z-FY-CHO and were then exposed to 1 h of OGD. Their media was collected for analysis and then changed to normoxic-high glucose media containing Z-FY-CHO. This reperfusion media was collected 24 h later.

We observed that while Z-FY-CHO could not prevent the increase in LG3 levels caused during 1 h of OGD, it caused a small, non-significant decrease when normoxic cultures were treated with it for 1 h (Fig. 4A). Also, we observed that LG3 levels decreased significantly in normoxic cultures which were treated with Z-FY-CHO for 24 h, when compared to untreated normoxic cultures (Fig. 4B). But, Z-FY-CHO treated, OGD exposed cells showed a significant increase in LG3 levels in their reperfusion media when compared to Z-FY-CHO treated-normoxic cells (Fig. 4B).

2.5 Cathepsin B Specific Inhibitor Reverses the Increase in LG3 levels Following OGD/Reperfusion

Since cathepsin L specific inhibitor was unable to obliterate the increase seen in levels of LG3 during FCN exposure to OGD or OGD plus reperfusion, we repeated these experiments with the cathepsin B specific inhibitor, CA074. This was also done since the inhibitors used to previously establish the role of cathepsin L in LG3 generation (Cailhier et al., 2008), have been used to inhibit cathepsin B elsewhere (Kennedy et al., 2009; Schotte et al., 2001).

Although an increase in LG3 levels after treatment with 1 h of OGD has been reported previously (Saini et al., 2011), it was observed that CA074 suppressed such an increase in LG3 levels (Fig. 4C). Unexpectedly, 1 h of CA074 treatment caused a significant increase in LG3 levels under normoxic conditions (Fig. 4C). In the reperfusion media of OGD treated FCN, CA074 treatment resulted in a significant decrease in LG3 levels when compared to both untreated normoxic cultures and normoxic cultures treated with CA074 for 24 h (Fig. 4D).

2.6 Both Cathepsin L and Cathepsin B Specific Inhibitors Cause Decreased LG3 levels Following IL-1 α but NOT after IL-1 β Treatment

Following the investigation into the effects of cathepsin inhibitors on LG3 levels following OGD/reperfusion, we were interested in investigating whether these inhibitors could lower the increased LG3 reported following treatment with low concentrations of IL-1 α and IL-1 β (Saini et al., 2011). FCN were treated with IL-1 α or IL-1 β along with Z-FY-CHO or CA074. Control cultures were treated with just IL-1 α or IL-1 β . It was observed that while both Z-FY-CHO and CA074 caused a decrease in LG3 following IL-1 α treatment, no such decrease was seen following IL-1 β treatment (Fig. 4E-F).

3 Discussion

3.1 *De Novo* Synthesis of Perlecan May be Partially Responsible for Upregulated Levels of Perlecan Fragments Post-Stroke

Upregulated levels of perlecan c-terminal fragments DV and LG3 have been observed to persist up to 7 days and 3 days post focal cerebral ischemia, respectively (Lee et al., 2011; Saini et al., 2011). This suggests that at least some level of elevation in *de novo* perlecan synthesis must occur after cerebral ischemia. Here, we report that the stimulus of reperfusion post OGD, but not OGD itself, is enough to cause an increase in perlecan expression in FCN *in vitro*. Our findings are consistent with a previous study linking hypoxia to an increase in perlecan expression in macrophages (Asplund et al., 2010), although in that study, hypoxia alone, and not additional reperfusion with normoxic media, was sufficient to induce perlecan expression. This suggests that the regulation of perlecan production in neurons and macrophages may be similar, but is clearly not identical. Although we didn't observe any changes in the perlecan mRNA levels in neurons exposed to 1 h of OGD, we observed an increased number of vesicle-like perlecan immunoreactive intracellular structures, unlike the more diffused intracellular perlecan staining observed in normoxic cells. This change in the morphology of cells, which may explain the increase in LG3 levels seen previously, is similar to the change observed during lysosomal exocytosis described previously (Huynh et al., 2004). Indeed, such lysosomal secretion was reported to happen as a direct result of cell damage (Huynh et al., 2004). On the other hand, there is a possibility that the perlecan immunopositive vesicle-like structures observed here are secretory vesicles. However, unlike the individually resolved vesicle-like structures we observe here, secretory vesicles may not be individually resolved by simple light microscopy because of their small size (Anlauf and Derouiche, 2005). Thus, our results suggest that neurons exposed to OGD may increase perlecan positive lysosomal exocytosis. This would also mean that OGD may result in the formation of perlecan positive lysosomes. If true, this would suggest that perlecan can be degraded intracellularly, a possibility that has been established for other ECM components such as collagen (Premzl et al., 2006). Overall, our results, along with a previous study showing increased expression of fibronectin in the brain of mice exposed to hypoxia (Milner et al., 2008), may represent a general increase in expression of significant components of ECM in the brain after ischemia, and thus may suggest functional outcomes of increased ECM expression for the post-stroke brain.

Interestingly, our results which demonstrate that treatment of FCN with IL-1 α or IL-1 β has no effect on perlecan mRNA levels apparently stand in contrast with a previous study which showed that IL-1 α stimulates perlecan expression in hippocampal derived-astrocytes (Garcia de Yebenes et al., 1999). Interestingly, this same study, in seeming agreement with our results, demonstrated that IL-1 α had no effect on perlecan mRNA levels in striatal-derived astrocytes. This suggests that IL-1 effects on cellular perlecan production are both brain region and cell type dependent. Importantly, however, astrocytes have been previously demonstrated to release a variety of factors, both beneficial and harmful to neurons, upon sensitization with IL-1 and may respond very strongly to IL-1 by displaying astrogliosis (Pinteaux et al., 2009). The fact that astrocytes, but not neurons, have been shown to increase perlecan mRNA after IL-1 stimulus may be representative of the strong secretory response astrocytes may generate in response to IL-1 stimulation. On the other hand, compared to neurons, astrocytes are known to be resilient to OGD and have been shown to decrease secretion of proteins during reperfusion, after non-lethal exposures to OGD (Goldberg and Choi, 1993; Yan et al., 2010). On the contrary, however, neurons have been shown to significantly increase the overall synthesis of proteins 24 h after non-lethal exposures to OGD (Ying et al., 1997). This evidence supports not only the present study but also our previous study where astrocytes, when compared to neurons, displayed a weak response to OGD in terms of increase in LG3 levels. Moreover, this also points towards an

important difference between the behavior of astrocytes and neurons to non-lethal doses of OGD, where neurons show a strong secretory response while astrocytes don't. Indeed, the role of both these cell types under inflammatory and ischemic stressors seems to be different. Clearly, the differences between how astrocytes and neurons respond to OGD and inflammation, and the interplay between the two cell types ultimately plays a profound role in the brain's response to hypoxic injury.

3.2 IL-1 α may Increase LG3 levels through Cathepsin-L Release while OGD/Reperfusion Decrease Cathepsin-L Release

Transient ischemia has previously been shown to elevate the levels of Cathepsin B and L in hippocampal neurons *in vivo* (Kohda et al., 1996). Also, OGD has previously been shown to affect the distribution of cathepsins inside cells (Windelborn and Lipton, 2008). Thus, we examined whether OGD/reperfusion of FCN could affect the levels of cathepsin secreted into the cell media. We observed that OGD and OGD/reperfusion tended to decrease all 3 forms of cathepsin L in FCN conditioned media. These results suggest that cathepsin L may not play a significant role in the generation of LG3 when neurons are stimulated by OGD and/or reperfusion.

Since we did not see an increase in perlecan expression, we investigated if an increase in cathepsin L levels in the neuronal conditioned media could cause the increase in LG3 levels seen with the addition of low concentrations of IL-1 α/β , as was observed previously. We saw a small significant increase in secreted pro-cathepsin L levels after treatment with IL-1 α . Since IL-1 α is upregulated within hours of cerebral ischemia (Hill et al., 1999), it is possible that it contributes to the increase in the level of Cathepsin L seen after cerebral ischemia. Also, we saw that both IL-1 α and IL-1 β cause small increases in the levels of both forms of mature cathepsin L. These results would support a previous study which showed an increase in cathepsin activity in the cultured media of chondrocytes upon treatment with IL-1 (Mehraban et al., 1997). Thus, this evidence may suggest that it is an increase in protease release which elevates levels of LG3 following treatment with low concentrations of IL-1.

3.3 Cathepsin L Degrades Perlecan under Normoxic Conditions but a 'Shift' in Relative Importance of Proteases Happens During OGD/Reperfusion

The cathepsin L selective inhibitor, Z-FY-CHO has previously been shown to be membrane permeable (Klose et al., 2006; Pacheco et al., 2005) and may, therefore, act both outside and inside the cell. Hence, the results presented here do not distinguish extracellular cathepsin L activity from intracellular activity. We saw that under normoxic conditions, short-term (1 h) exposure to Z-FY-CHO did not cause any changes to LG3 levels, while longer exposure (24 h) significantly decreased LG3 levels. This difference may merely be due to the cumulative effect of a longer exposure with the degree of inhibition remaining the same. During OGD, Z-FY-CHO was unable to prevent increase in LG3 levels, which has been reported before (Saini et al., 2011). Furthermore, OGD exposed and Z-FY-CHO treated neurons showed an increase in LG3 levels during reperfusion when compared to normoxic and Z-FY-CHO treated neurons. This would seemingly indicate that the increase in LG3 levels seen after OGD and reperfusion is not cathepsin L mediated. The decrease in relative importance of cathepsin L would also be supported by the decrease in cathepsin L levels which we saw in neurons treated with OGD/reperfusion. This also indicates that another protease becomes relatively more involved in perlecan degradation during OGD and reperfusion. The action of this other protease causes an increase in LG3 levels despite cathepsin L being inhibited. Therefore, these results indicate a shift in the complement of proteases released by neurons as a result of OGD and reperfusion stress

3.4 Cathepsin B Becomes an Important Protease for Perlecan Degradation after OGD/Reperfusion

The effects of the cathepsin B specific inhibitor CA074 were examined due to the redundancy of function between cathepsin L and cathepsin B. Also, we were concerned that the previous work which implicated cathepsin L in the generation of LG3 from apoptotic human umbilical vein endothelial cells used non-specific protease inhibitors that could block the function of both cathepsin L and cathepsin B (Caillhier et al., 2008). CA074 is charged and is unable to penetrate the cell membrane (Bogyo et al., 2000), while its cell permeable methyl ester form has previously been shown to act on other cysteine proteases as well (Montaser et al., 2002). But, CA074 has previously been shown to be endocytosed such that its concentration inside the cell increases by 12 h of incubation (Szpaderska and Frankfater, 2001). Thus, it may be assumed that treatment with CA074 during OGD (1 h) mainly inhibited extracellular cathepsin B, while treatment with CA074 during reperfusion (24 h) inhibited both intracellular and extracellular cathepsin B.

Interestingly, treatment of normoxic neurons with CA074 for 1 h caused an increase in the LG3 levels in the cell media when compared to normoxic-untreated neurons. Such an increase in matrix degradation upon inhibition of cathepsins has been reported before and was attributed to the release of other compensatory proteases by the cell (Sondergaard et al., 2006). Here we show that these compensatory mechanisms may apply even if cathepsin B is inhibited only outside the cell. OGD and CA074 exposed neurons showed a significant decrease in LG3 levels when compared to LG3 levels of normoxic, CA074 treated neurons. This indicates that CA074 can prevent the increase in LG3 levels seen during OGD previously (Saini et al., 2011), which further indicates that cathepsin B becomes more involved in LG3 cleavage from perlecan during neuronal exposure to OGD as compared to cathepsin L.

Unexpectedly, the increase in LG3 levels seen upon treatment of normoxic FCN with CA074 for 1 h was not observed after longer durations (24 h) of treatment, during which intracellular cathepsin B might have also been inhibited. But, during reperfusion of OGD exposed neurons, CA074 treatment caused a significant decrease in the levels of LG3, further indicating that cathepsin B becomes more involved in the processing of perlecan following OGD/reperfusion. This may also indicate unknown/indirect mechanisms via which cathepsin B assumes importance in the perlecan degradation process since cathepsin B has previously been shown to activate other proteases (Kobayashi et al., 1991). Thus, cathepsin B may also be a part of the cascade leading to ECM degradation.

3.5 IL-1 α , but NOT IL-1 β , Stimulates LG3 Generation via a Cathepsin L/B Dependent Mechanism

Since it has already been reported that treatment of FCN with 0.1 ng/ml IL-1 α and IL-1 β increases LG3 levels in neuronal media (Saini et al., 2011), we were also interested to see if treatment with cathepsin L and B inhibitors has any effect on LG3 levels in IL-1 α or IL-1 β treated neurons. We saw that both cathepsin L and cathepsin B inhibitors decreased LG3 levels increased by IL-1 α . These results not only support the hypothesis that IL-1 α causes increased secretion of cathepsin L which generates LG3, but also indicate that IL-1 α may stimulate perlecan degradation through both cathepsin L and cathepsin B. On the other hand, neither Z-FY-CHO nor CA074 could decrease the levels of LG3 elevated by IL-1 β , indicating that IL-1 β may increase LG3 levels via a non-cathepsin L/B mediated proteolytic mechanism. This also shows that there is a difference in the effects of IL-1 α and IL-1 β on neurons, which has been reported before for brain cells (Saini et al., 2011).

In summary, we found that while release of pre-synthesized perlecan causes an increase in LG3 levels during OGD in FCN, it is the increased *de novo* synthesis of perlecan in FCN which may be responsible for increased LG3 levels during reperfusion. Furthermore, we found evidence that cathepsin B, but not cathepsin L, is involved in increasing LG3 levels during OGD and reperfusion in FCN. In contrast, however, both cathepsin L and cathepsin B caused the increase in LG3 levels caused by IL-1 α exposure in FCN. Our results shed new light on how different neuronal stressors (OGD/reperfusion and IL-1 α and β) differentially regulate the generation of the neuroprotective LG3 protein.

4 Experimental Procedure

4.1 Cell Cultures

Primary fetal cortical neurons (FCN) were extracted from C57BL6 embryonic day 17-18 mice as previously described (Harris et al., 2007) and in accordance with Texas A&M College of Medicine IACUC. Briefly, cervical dislocation was used to euthanize pregnant females and embryos were dissected in order to get cerebral cortices. FCN were extracted using mechanical dislocation of the cortices and treatment with trypsin. The cells were seeded on Poly-D-Lysine (Sigma, St Louis, Mo, USA) coated plates in Dulbecco's modified Eagle Medium (DMEM) high glucose containing 2% (v/v) B27 supplement (Invitrogen, Carlsbad, CA, USA) and 1% (v/v) penicillin-streptomycin. The FCN were cultured in conditioned incubators (37°C/5% CO₂) and were used after 3-4 days in vitro (DIV).

4.2 *In vitro* Oxygen Glucose Deprivation (OGD)

Oxygen Glucose Deprivation (OGD) was performed using a previously described setup (Tauskela et al., 2003). OGD media was composed of DMEM with no glucose (Invitrogen) containing 1% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin and 2% (v/v) B27 supplement. FCN were washed twice with OGD media and were then incubated in OGD media inside a hypoxic chamber (Billups-Rothenberg Inc, Del Mar, CA, USA) which was flushed with anoxic gas (90% N₂, 5% CO₂ and 5% H₂) for 5 min, based on the manufacturer's instructions. The incubation under OGD conditions was carried out for 1 h. After OGD, some cultures were lysed using the reagents in the RNeasy mini kit (Qiagen, Valencia, CA, USA), to extract total RNA, according to manufacturer's protocol. For other FCN cultures, after OGD, the media was collected for western blot analysis and cultures were incubated with oxygenated DMEM high glucose with 1% (v/v) FBS, 1% (v/v) penicillin-streptomycin and 2% B27 supplement (Invitrogen). After 24 h, reperfusion media was collected for western blot analysis and cultures were lysed using the RNeasy mini kit (Qiagen).

4.3 IL-1 α and IL-1 β treatment

Stocks of IL-1 α and IL-1 β (R&D Systems, Abingdon, UK) were a kind gift from Dr. Emmanuel Pinteaux (Faculty of Life Sciences, University of Manchester). The stocks were diluted in 0.1 % (w/v) BSA / 0.9 % (w/v) NaCl. IL-1 α and IL-1 β , up to a concentration of 0.1 ng/ml, were added directly to the culture media, which was composed of normal FCN media (as described above) containing 5% (v/v) FBS. Cells were washed once with their normal media before being incubated in the media containing IL-1 α/β . For Q-PCR analysis, FCN were treated with IL-1 α/β for 12 h. After this, lysates were prepared using RNeasy mini kit (Qiagen). For inhibitor studies (see below) and for studying cathepsin L levels, FCN were treated with IL-1 α/β for 24 h, before their supernatants were collected for western blot analysis.

4.4 Treatment with Cathepsin Inhibitors

Cathepsin L specific inhibitor, Z-FY-CHO (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Cathepsin B specific inhibitor, CA074 (Calbiochem, EMD Chemicals, Gibbstown, NJ, USA) were diluted in DMSO to a concentration of 10 mM. Cultures were treated with 10 μ M of Z-FY-CHO or CA074, a concentration range which has previously been shown to be active (Klose et al., 2006; Matarrese et al., 2010). Control cultures were treated with an equal volume of vehicle, DMSO. Cultures were treated with cathepsin inhibitors during OGD, during reperfusion and during IL-1 α / β treatment, as described above.

4.5 Quantitative Polymerase Chain Reaction (Q-PCR)

Total RNA was extracted from FCN cultures treated with OGD or IL-1 α / β , as described above. The integrity of RNA was checked using agarose gel electrophoresis and UV spectrophotometer (Beckman, Brea, CA, USA) was used to measure its quantity. Total RNA was used for preparing cDNA using random primers (Invitrogen), dNTP (Invitrogen) and Cloned AMV Reverse Transcriptase (Invitrogen), according to manufacturer's protocol. Q-PCR was performed using Fast SybrGreen Master Mix (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) was used as the housekeeping control and primers used for perlecan were:

Forward (5'-3'): ATGGGCAGAAGCGGAGTC

Reverse (5'-3'): GCCTGCATCAAATCGAAAC

The reaction was carried out using the Applied Biosystems 7500 Fast Real-Time PCR System, which also calculated the Δ Ct values. $\Delta\Delta$ Ct values were later calculated and used to calculate the fold change in mRNA expression.

4.6 Western blot analysis

Western blot analysis was performed on cell culture supernatants, prepared as described above. Protein concentrations were determined using Bradford assays (Bio-Rad) and equal concentrations of protein were loaded on SDS-PAGE. After separation, proteins were transferred to nitrocellulose membrane which was later blocked in 5% (w/v) non-fat dry milk in Tris buffer saline containing 1:1000 Tween 20. After overnight incubation with anti-endorepellin (1:1000 dilution; R&D Systems, Minneapolis, MN, USA) or anti-cathepsin L (1:1000 dilution; Abcam, Cambridge, MA, USA) at 4°C, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000 dilution; Genetex, Irvine, CA, USA) for 2 h at room temperature (24°C). Membranes were developed using chemiluminescent ECL kit (Thermo Scientific, Rockford, IL, USA). Quantification was performed using Image J software (NIH) as described by Luke Miller (<http://lukemiller.org/journal/2007/08/quantifying-western-blot-without.html>). 0.1% (w/v) Ponceau S. solution (Sigma) was used to carry out ponceau staining, which was used to normalize the blots, as done previously (Saini, Pinteaux et al. 2011). Finally, in several cases, black bars have been displayed on blots to indicate regions of the blot on either side of the black bar that are not continuous despite being on the same blot.

4.7 Immunocytochemistry

FCN were extracted as described above and were seeded on Poly-D-Lysine coated glass chambers (Lab-Tek; Nunc, Naperville, IL, USA) in normal FCN media (described above). At 3-4 DIV, they were exposed to OGD or IL-1 α / β treatment, as described above. Immediately following 1 h OGD or 4 h of IL-1 α / β treatment, the cells were fixed in 4% (w/v) paraformaldehyde and permeabilized with 0.1% (v/v) Triton-X 100 solution. After blocking with 5% (w/v) BSA in PBS for 2 h, cells were incubated overnight at 4°C with

anti-perlecan antibody (1:125 dilution; Santa Cruz). This was followed by washes with 1% (w/v) BSA in PBS and incubation with goat anti-rabbit alexa secondary antibody conjugated with Alexa 576 (Invitrogen) for 45 min at room temperature (24°C). After washes with 1% BSA (w/v) in PBS, cells were mounted in Vectashield containing DAPI (Vector Labs, Burlingame, CA, USA). Axiovision A1m microscope (Carl Zeiss, Thornwood, NY, USA) was used to observe the slides and images were taken using a Retiga-SRV camera (Qimaging, Surrey, BC, Canada) and iVision software (Biovision technologies, Exton, PA, USA). At 60x, approximately 200 cells (recognized by DAPI fluorescence) were counted in random directions, in order to quantify the percentage of cells showing the perlecan positive vesicle-like structures.

4.8 Statistical Analysis

All experiments were performed at least 3 independent times, each time in duplicate or more unless otherwise stated. The study was randomized by seeding cells on different wells of the plates for each repetition with no particular set order for the treatments. For all immunocytochemistry subjective analyses, the examiner was blinded to the various treatment conditions. The data is presented as mean $\pm$ standard error of the mean (SEM). Groups were compared using two-tailed Student's t-test or one-way ANOVA with Tukey as a post test, as appropriate, using GraphPad Prism 4.0. Significance was defined as $P < 0.05$.

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Highlights

- Perlecan c-terminal fragment, LG3, elevated after OGD/reperfusion and IL-1 treatment in neurons
- Increased release of pre-synthesized perlecan during OGD
- Increase in perlecan synthesis during reperfusion
- Cathepsin B involved in cleaving perlecan to generate LG3 during OGD/reperfusion
- Cathepsin L and B elevate LG3 levels following IL-1 α treatment

Figure 1. Q-PCR analysis of perlecan mRNA levels after OGD/reperfusion and IL-1 α / β treatment. Fold change in perlecan mRNA levels in FCN exposed to A) 1 h of OGD and B) 24 h of reperfusion after OGD, when compared to respective normoxic controls (Norm). C) Fold change in perlecan mRNA levels after 12 h treatment with 0.1 ng/ml IL-1 α and IL-1 β when compared to vehicle treated control. Data were analyzed by Student's t-test and were significant at * $p < 0.05$ when compared to respective controls.

Figure 2.

OGD results in formation of perlecan positive vesicle-like structures in FCN. A) Perlecan immunocytochemistry on FCN treated with 1 h of OGD. FCN were fixed and stained with perlecan antibody (red) and DAPI (blue) immediately after OGD exposure. The 'vesicle-like' structures are indicated with arrows. Scale bar=10 μ m. B) Representative quantification of perlecan staining on FCN showing absolute percentage of cells showing perlecan positive vesicle-like structures in normoxic FCN (Norm) and FCN exposed to 1 h of OGD. Data were analyzed by Student's t-test. * $p < 0.05$ when compared to control.

Figure 3.

Effect of various stressors on cathepsin L levels in FCN conditioned media. Representative anti-cathepsin L western blots were normalized to respective Ponceau S. stains and depict fold change in 42kDa pro-cathepsin L, 36kDa mature cathepsin L, 29kDa mature cathepsin L levels in conditioned media of FCN treated with A) 1 h of OGD, B) 24 h of reperfusion after OGD, C) 0.1 ng/ml IL-1 α and D) 0.1 ng/ml IL-1 β , when compared to cathepsin L released by respective control cultures, normoxic (Norm) for a) & b) and vehicle treated (veh) for c) & d). Data were analyzed by Student's t-test. * $p < 0.05$ when compared to control cultures.

Figure 4.

Effect of cathepsin L and cathepsin B inhibitors on LG3 levels in conditioned media of FCN exposed to various stressors. Representative anti-LG3 western blots were normalized to respective Ponceau S. stains and represent fold change in LG3 levels in conditioned media of A) normoxic or OGD exposed FCN treated with 10 μ M Z-FY-CHO for 1 h; B) normoxic FCN or FCN reperused after OGD and treated with 10 μ M Z-FY-CHO for 24 h; C) normoxic or OGD exposed FCN treated with 10 μ M CA074 for 1 h; D) normoxic FCN or FCN reperused after OGD and treated with 10 μ M CA074 for 24 h, when compared with their respective normoxic-untreated controls (Norm). Representative western blots showing fold change in LG3 levels in conditioned media of E) FCN treated with 10 μ M Z-FY-CHO or 10 μ M CA074 and 0.1ng/ml IL-1 α ; F) FCN treated with 10 μ M Z-FY-CHO or 10 μ M CA074 and 0.1ng/ml IL-1 β , when compared to respective IL-1 α and IL-1 β treated controls. Data were analyzed by one-way ANOVA and was found significant at * p <0.05 and ** p <0.01.