Cathepsin D Alteration in Oxygen Glucose Deprivation (OGD)

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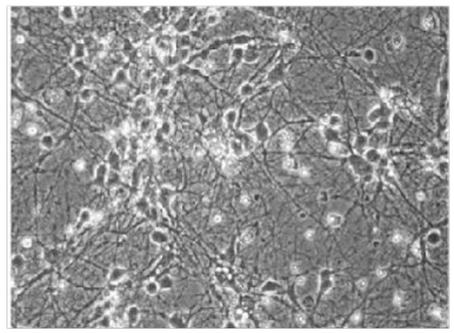
Abstract

Stroke is a leading cause of death and disability around the world and results in neuronal death in affected areas of the brain due to deprivation of oxygen and glucose. The pathology of stroke is very complex and not fully understood. Cathepsin D (Cath D) is a protein that in humans is encoded by the CTSD gene and is implicated in many neurodegenerative diseases such as congenital neuronal ceroid lipofuscinosis (NCL), Parkinson's disease and Alzheimer's disease. Regulation of Cath D in neurodegenerative diseases has been studied elaborately. Recent studies show that the defects in protein clearance processes may contribute to the pathology of stroke. A study using global proteomics approach revealed that expression of Cath D decreases following stroke in rats. Whether Cath D is neuroprotective and maintains proteins homeostasis in stroke is not known. Here we used oxygen–glucose deprivation (OGD) to neurons to mimic ischemic stroke in *in-vitro.* We validated the model by performing cell death assay and found that the model replicated ischemic condition in neurons. Interestingly, Cath D level in these neurons were decreased significantly suggesting that Cath D may play a vital role in stroke pathology.

Objective

Our objective for conducting research on the protein of question, cathepsin D, and its alterations in a state of OGD is to further advance our understanding of strokes. Cathepsin D along with other proteins, are highly associated with strokes, seeing as they are implicated in oxidative stress, inflammatory response and apoptosis. Not only this, but it also alters the permeability of the blood-brain barrier. This in turn increases the disruption of neuronal-microglial interaction and vulnerability in neuronal cells. Preventing a human from having a stroke is a case on its own. If we were to implicate a screening of specific proteins such as Cath-D, it could potentially save more lives than we could have ever thought possible.



Primary cortical neuron (DIV 10)

Methods

Primary cortical neuronal culture
Embryos obtained from mice at Day 15–16 of gestation were used to prepare primary cortical neuron culture. Briefly, the cortical regions of the embryonic brains were aseptically dissected, freed of meninges and dissociated in dissecting medium (DMEM + 20% FBS) and subjected to trypsin digestion at 37°C for 5 min. Tryptic digestion was stopped by the addition of dissecting medium and the cell suspension were centrifuged at 1000 g for 5 min. Next, the pelleted cells were subjected to mechanical trituration in complete Neurobasal medium (10 mM glucose, 1 mM GlutaMAX-I, 1 mM Na-Pyruvate and 2% B-27) and passed through a 40 µm filter. The cells were plated to a density of 5 ×10^5 cells/ml. On day in vitro (DIV) 1, the cultures were treated with 5-fluoro 2-deoxyuridine (40 µM) to inhibit glial growth and proliferation. Experiments were performed at DIV 11. Under these conditions, mature neurons represent 90% of the cells in the culture.

Oxygen-glucose deprivation (OGD) in primary cortical neurons
OGD (an in-vitro model ischemic stroke) treatment was initiated by incubating the cells for 90 min with OGD buffer (NaCl 116 mM, KCl 5.4 mM, MgSO4 0.8 mM, $NaHCO_3$ 26.2 mM, NaH_2PO4 1 mM, $CaCl_2$ 1.8 mM, glycine 0.01 mM, pH 7.4) saturated with 95% N_2 and 5% CO_2 in a hypoxia chamber (temperature 37°C, atmosphere 5% CO_2 and 95% N_2) connected to an O_2 sensor/monitor (Biospherix Ltd. USA) and maintained at 37°C. OGD is terminated by resupplying complete Neurobasal medium to the culture and transferring back the culture to the normal incubator.

Cell death Assay
Cell viability after OGD was determined by using Alamar blue reagent, a water-soluble resazurin dye (blue colored) which is reduced to red fluorescent resorufin dye by metabolically active cells.

Alamar blue reagent [10% (v/v)] was added to each well containing and incubated for 3 hours at 37°C. Following 3 hours of incubation, 100 µL of the medium was collected from each well and transferred to a 96-well microplate. The fluorescence was measured at the excitation and emission wavelength of 540 and 595 nm, respectively using a microplate reader. The fluorescence values were normalized by the control (PBS) and expressed as percent viability. Neuronal death was examined using propidium iodide (PI) staining. In brief, following exposure to OGD under indicated conditions, neurons in culture medium were incubated for 5 min containing 5 µg/ml PI and 1 µM Hoechst 33342 (ThermoFisher). Images were captured using an Zeiss Fluorescence microscope (Zeiss Technologies). ImageJ software was used for analysis of neurons stained with PI and Hoechst.

Western blotting
The neuronal cells were lysed using RIPA buffer (1% Triton-X 100) and the cell lysates were centrifugation at 12,000 × g for 10 min at 4°C. Total protein concentration was determined by BCA protein assay kit. A 15 µg of proteins were loaded in each well of SDS-PAGE gel and separated using running buffer (Bio-Rad) for approximately 90 min at 100 V, and then transferred onto a nitrocellulose membrane at constant voltage (100 V) for 75 min using Western transfer buffer (Bio-rad). The membranes were then blocked with 5% (w/v) non-fat dry milk in Tris buffered saline with 0.1% Tween 20 (TBST). After blocking, the membranes were washed three times (10 min each) with TBST and then probed with primary antibodies overnight at 4°C and afterwards with horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature.

Results

Figure 1

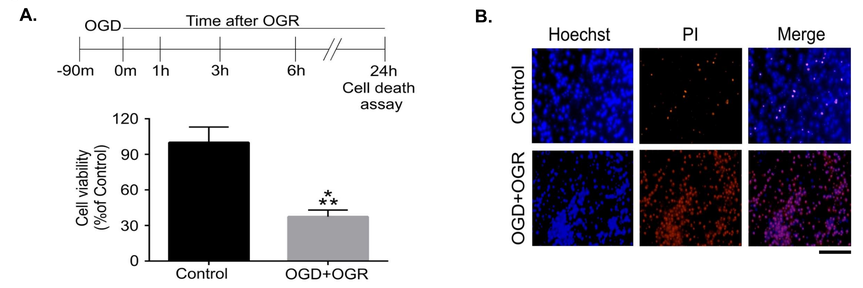


Figure 1: Cell viability in neurons exposed to OGD. (A) Schematic diagram of the experimental design. Neurons were exposed to OGD for 90 minutes and cell viability was assessed 24 hrs after OGR using Alamar Blue assay. Results are presented as percent of control. Data are mean ± SEM of n = 5. ***$P < 0.001$ vs indicated groups, Student's t-test **(B)** Representative images showing propidium iodide (PI) and Hoechst 33258 labeled cells in control neurons and neurons exposed to OGD. Scale bar: 50 µm. Neurons were stained 24 hrs after OGR. PI positive cells represent dead cells.

Figure 2

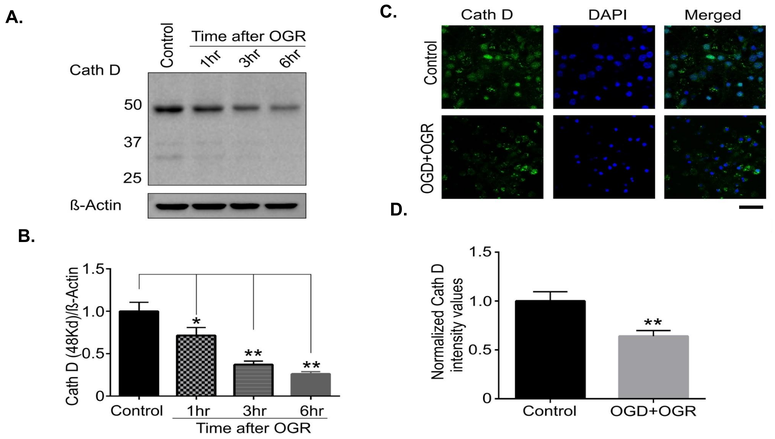


Figure 2: Cathepsin D level in neurons exposed to OGD treatment. (A) Western blot showing levels of Cath D in neurons after OGD. Neurons were exposed to OGD for 90 min and cell lysates were collected at indicated time points after OGR to assess Cath D expression. **(B)** Quantification of Cath D levels normalized with the values of the corresponding β-Actin levels. Data represent mean ± SEM, n = 4. **(C)** Immunostaining of control and OGD neurons using Cath D antibody. Scale bar: 20 µm. **(D)** Quantification of Cath D fluorescence intensity in control and OGD neurons. Data are mean ± SEM, control n = 10, OGD n = 10. *$P<0.05$, **$P<0.01$, ***$P<.0.001$ vs indicated groups, One-way ANOVA with Tukey's post hoc test.

Conclusion

Here we demonstrate that Cath D protein levels decrease in neurons exposed to OGD and loss of Cath D may play a key role in lysosomal dysfunction, protein aggregation and cell death in stroke. This study suggests that pharmacological intervention targeting Cath D and thus lysosomal function may be effective in limiting brain damage following stroke and related neural diseases.

Acknowledgements

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