Studies on Aquaporin 4 protein expression in Oxygen- Glucose deprived Astrocytes.

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ABSTRACT

Aquaporins (AQPs) are small hydrophobic, integral membrane proteins expressed in many cell types, especially, in epithelial and endothelial cells which are involved in fluid transport; which is important in preserving the electrochemical potential across the membrane. Aquaporins are made up of 13 members namely AQP 0-12. They can be classified into 3 groups: classical AQPs (AQP 0, 1, 2, 4, 5, 6, & 8), aquaglyceroporins (AQP 3, 7, 9& 10) and subcellular AQPs (AQP 11 & 12). In this experiment, the focus will be on AQP 4 and its protein expression in oxygen and glucose deprived (OGD) astrocyte cells that simulate ischemic conditions. The objective of this experiment was to compare the protein expression of AQP4 in astrocytes; that are subjected to OGD at four different time points of 2hrs, 4hrs, 6hrs & 8hrs against normal astrocytes that were grown under normal conditions. The findings of this experiment have shown that Aquaporin 4 protein expression was up-regulated at 2hrs OGD and down-regulated at the other 3 time points (4hrs, 6hrs and 8hrs OGD).

INTRODUCTION

Aquaporins (AQPs) are small hydrophobic, integral membrane proteins expressed in many cell types, especially, in epithelial and endothelial cells which are involved in fluid transport (Park et al., 2008). They are transmembrane water channels which play critical roles in controlling the water contents flowing in and out of the cells; also facilitates the efficient permeation of water, which is important in preserving the electrochemical potential across the membrane (Papadopoulos and Verkman, 2007). Aquaporins are made up of 13 members AQP 0-12 (Park et al., 2008). They can be classified into 3 groups: classical AQPs (AQP 0, 1, 2, 4, 5, 6, & 8), aquaglyceroporins (AQP 3, 7, 9& 10) and subcellular AQPs (AQP 11 & 12) (Hibuse et al., 2006).

Astrocytes are the most abundant cell type in the Central Nervous System which protect and support normal neuronal activities (Suzuki et al., 2006). Oxygen and glucose deprived (OGD) astrocyte cells simulate the condition of ischemic stroke in the brain (Xie et al., 2008). Ischemic stroke occurs due to blockage of arteries in the brain. The brain depends on these arteries to supply fresh oxygenated blood and glucose from the heart and lungs (Gao et al., 2008). Brain Edema has been found to be the major cause of death and disability in various neurological conditions such as stroke (Tait et al., 2008). The main aquaporins that maintain water homeostasis in the brain are Aquaporin 1, 4 & 9 (Papadopoulos and Verkman, 2007).

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The purpose of this study was to quantitate the protein expression of Aquaporin 4 (AQP4) in astrocytes that were subjected to OGD for different time points of 0 (control), 2, 4, 6 & 8hrs by western blotting using antibodies against AQP 4 proteins.

MATERIALS AND METHODS

Culture work & Induction of glucose and oxygen deprivation (OGD):
Astrocytes were grown with RPMI complete media at 37°C in a humidified atmosphere (5% CO2 and 95% air) till confluence followed by addition of Trypsin. After which RPMI complete media was added and the cultures were centrifuged. The cell pellet was resuspended in RPMI complete media. The cells were seeded on 24- wells plate with 1x 10^5 cells per well (Phelan, 2006). Ischemia- like conditions were obtained by omitting glucose (RPMI media without glucose) and by displacing dissolved oxygen with nitrogen. The time course study of OGD was conducted for 2, 4, 6, 8 hours. The OGD cultures and control cultures were washed with basal RPMI and left in a humidified atmosphere for 24 hours (Xie et al., 2008).

Protein Extraction:
TRizol Method: Protein Extraction was carried out following the instructions given in Invitrogen TRIzol Reagent protocol. Direct Lysis Method: The cell cultures were subjected to protein extraction using lysis buffer suggested in Alexis Biochemical Caspase Assay Instruction Manual.

Protein Quantification:
Quantification was carried out following the instructions given in the Bio- Rad Protein Assay Manual.

Western Blot:
Protein samples were mixed with loading buffer and boiled before loading onto a gel made up of 10% resolving gel and 4% stacking gel. The SDS- PAGE electrophoresis was carried out following the Mini-Protean Tetra cell (Bio-Rad) instructional manual. The gel was then electroblotted onto a nitrocellulose membrane following the instructions provided by the Mini Trans-Blot Electrophoretic Transfer cell Instruction Manual. Following this, the nitrocellulose membrane was blocked in a blocking solution incubated in primary antibody followed by the secondary antibody. The membrane was visualized by photography (Gallagher, 2007).

Quantitation of the bands of interest:
The blot photos were scanned and specific bands of interest were quantified using the imaging software by Amersham Biosciences Image Master TM 2D Platinum Version 5.0. The intensity of the Aquaporin and the Beta- Actin bands were quantified. After which the data was normalized by dividing AQP 4 band’s intensity with the corresponding Beta- Actin band’s intensity. The expression of the aquaporin protein was deduced by comparing the ratios of the OGD against the normal at each time point.
RESULTS AND DISCUSSION

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**Figure 1:** Represents the Beta Actin band (43kDa) being a loading control and AQP 4 band (62kDa- dimeric form) corresponding to the 9 samples which were extracted through the TRIzol method.

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**Figure 2:** Represents the Beta Actin band (43kDa) being a loading control and AQP 4 band (62kDa- dimeric form) corresponding to the 9 samples which were extracted through the direct cell lysing method.

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<td>Ratio of Intensity of AQP 4/ Intensity of Beta Actin</td>
<td>2HR OGD</td>
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**Figure 3:** Represents the regulation pattern of AQP 4 protein expression at the 4 different OGD timings when using the TRIzol method of protein extraction.

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**Figure 4:** Represents the regulation patterns of AQP 4 protein expressions at the 4 different OGD timings using the direct cell lysing method of protein extraction.

Two methods of protein extraction were carried out to evaluate the regulation of AQP 4 on OGD induced astrocytes. In the first case, TRIzol method of protein extraction was carried out. But difficulties were faced in dissolving the protein pellet with the recommended lysis buffer (1% SDS) by the Invitrogen TRIzol protocol. Thus a different lysis buffer (Urea, Thiourea, CHAPS, IPG Buffer, DTT, Protease inhibitor and Nuclease Inhibitor) was used in dissolving the pellet. This lysis buffer also posed a problem in the protein quantification step, resulting in inaccurate protein concentrations. There were inconsistent Beta Actin bands (Fig.1).

Thus, direct cell lysing and extraction of protein were carried out with lysis buffer (Tris, NaCl, EDTA, and Triton X-100) (Fig. 2). Experiments with the direct cell lysing method were repeated to confirm the expression patterns of AQP4. Beta Actin acting as a loading control was detected at 43kDa and AQP4 bands in their dimeric form were detected at 62kDa (Trinh-Trang-
Tan et al., 2005). AQP4 protein expression at 2hr OGD was up-regulated and at the other three time-points they were all down-regulated (Fig 3 & 4).

It was discovered that AQP4’s expression increased at the early stages of OGD to promote water influx into astrocytes which results in cytotoxic edema. And in the later stages of OGD, there was a down-regulation of AQP4’s expression which may be a part of a self-protective response to ischemia (Fu et al., 2007). Similarly, there was an up-regulation of AQP4 protein expression at 2hr OGD (water influx into the astrocytes) and a down-regulation of AQP4 protein expression at 4, 6 and 8 hr OGD (self-protective response).

The future areas of study could be to experiments on AQP1 and AQP9 to evaluate their regulation of protein expression during OGD at the 4 different time points.

ACKNOWLEDGEMENTS

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REFERENCES


Gao, Q., Y. Li, et al. (2008), "Bone marrow stromal cells reduce ischemia-induced astrocytic activation in vitro." Neuroscience.


