MICROARRAY ANALYSIS OF GENE EXPRESSION DURING ANOXIA IN *C. ELEGANS*

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Abstract:

In humans, periods of hypoxia (low oxygen) or anoxia (no oxygen) are associated with many diseases, including cancer, stroke and cardiac failure. For example, during cerebral ischemia, lack of oxygen due to a decrease in blood flow to the brain can case severe brain damage if it lasts more than five minutes. A better understanding of the cellular changes occurring during oxygen deprivation will be important in increasing the survival rate for patients suffering from a stroke. The nematode *C. elegans* is an excellent model organism for these studies, because their neurons go through cell death pathways similar to humans and their entire genome has been sequenced. Importantly, *C. elegans* can also adapt to environments of anoxia by entering a state of suspended animation. The aim of the current study is to develop a better understanding of the changes in gene expression that occur during anoxic conditions in *C. elegans*. We first conducted viability assays at 24, 36, 48, 72, and 96 hours of exposure to anoxia to confirm the ability for *C. elegans* to survive extended periods in anoxic conditions. Anoxia treatments at 4 and 24 hours were performed and DNA microarrays were hybridized and prepared for analysis in order to identify changes in gene expression due to anoxic conditions.

Introduction:

In humans, the state of anoxia for over four minutes can result in severe brain damage (Glickman-Simon 2005). Periods of oxygen deprivation such as hypoxia (low oxygen between 0.5% to 1% oxygen) or anoxia (0% oxygen) as defined by Shen and Powell-Coffman (2003) are associated with many diseases, including solid tumor progression, pulmonary disease and stroke (White *et al.* 1984; Hajeria *et al.* 2005). On a cellular level, the absence of oxygen can reduce the amount of energy produced by mitochondrial respiration and increase the amount of damaging free radicals available in the cells. This can impact a variety of cellular processes and possibly lead to apoptosis or programmed cell death (Nystul and Roth 2004).

Stroke, a cerebrovascular disease, is the third leading cause of death in the United Sates according to the National Institute of Neurological Disorders and Stroke (2006). A stroke, or cerebral ischemia, is caused when the glucose and blood oxygen supply to part of the brain is interrupted. In this anoxic or hypoxic state, mitochondrial energy production is low, ion gradients are not maintained and neuronal death can occur. The low production of energy for the cell, in

the form of ATP, causes a cascade effect. For example, the active transport of the sodium-potassium pump (Na^+K^+ ATPase) requires ATP to move three Na^+ out of the cell and two K^+ into the cell. This normally causes an electrical gradient that is essential for the action potential of neurons. However, without ATP, sodium ions leak into the cell and depolarize the membrane triggering the release of neurotransmitters (Adibhatla and Hatcher 2006). One of these neurotransmitters is glutamate. Excess glutamate can activate phospholipase A_2 which cleaves an arachidonic acid containing phospholipids to release arachidonic acid, a precursor for molecules involved in apoptosis (Adibhatla and Hatcher 2006). Therefore, when glutamate levels increase above normal during a stroke, it can become neurotoxic (Choi 1990). This is only one activated pathway in the cell due to depletion or lack of oxygen, however that are many more (Figure 1).

An attractive model organism for studying human related disease is *Caenorhabditis elegans*. This organism has a quick generation time and about 35% of its genes are homologous to that of humans (Genome Sequencing Center 2004). *C. elegans* was also the first multicellular organism to have its entire genome sequenced in 1998 (Consortium TCeGS). Most importantly, *C. elegans* have similar cell death pathways as humans and can survive 24 hours of anoxia with a high survival rate (Kourtis and Tavernarakis 2007).

Through previous studies, the anoxia response in *C. elegans* has been recognized to cause suspended animation where cell-cycle progression and development is arrested, but within a few hours of reoxygenation (normoxia) development of worms continues as normal (Hajeri *et al.* 2005). It is thought that this reversible arrest is caused by highly condensed DNA that is untranscribable (Hajeri *et al.* 2005). Although *C. elegans* can survive 24 hours of anoxia, after 72 hours of anoxia exposure, arrest is not reversible and the rate of mortality in *C. elegans* significantly increases (Van Voorhies and Ward 2000).

The pathways that respond to anoxia are still unclear; identifying genes that change expression levels in *C. elegans* during an anoxic condition may help the understanding of organismal survival under stressful conditions. It may also narrow the focus of areas and pathways that contribute to anoxic response. This response to anoxia can be related to the absence of oxygen observed during stroke. Genes that are significantly related to anoxia survival may be useful in determining future targets in stroke prevention or treatment.

The transcriptional regulation of genes in an entire genome can be identified through microarrays (DeRisi 1997; Yeung *et al.* 2004). This is an unbiased approach to determine which

genes are upregulated or downregulated in response to anoxia. Since it has been noted in literature that *C. elegans* can survive at least 24 hours without oxygen selecting time points between 0 and 24 hours will be relevant to identify early changes in genes expression that allow the worm to cope with anoxia. We first verified our anoxia treatment by conducting *C. elegans* viability assays at 0, 24, 36, 48 and 96 hours of anoxia which were similar to other literature. Anoxia treatments at 4 and 24 hours were performed and RNA was extracted and used for microarray experiments.

Methods:

Growth Conditions for Worms:

Wilde-type (N2) worms were cultured at 24°C for anoxia viability assays (St. Lawrence University) and at 25°C for anoxia treatments (Virginia Commonwealth University) on standard agar plates seeded with *Escherichia coli* OP_{50}. Adult worms used for both assays and treatments were synchronized by treating gravid adults with hypochlorite solution to collect embryos (Emmons *et al.* 1979). Embryos were starved overnight on unseeded plates to allow all worms to develop into the L1 larval stage (Figure 2). Worms at L1 stage were transferred to plates with food and used for assays and anoxia treatments once worms reached young adulthood.

Anoxia Viability Assays:

Adult worms were exposed to anoxic conditions for 24, 36, 48, 72, and 96 hours using a 2.5L Oxoid AnaeroJar System (Thermo Fisher Scientific, Waltham, MA). After exposure, worms were returned to normoxic conditions for 24 hours before viability was assessed. A worm was counted dead if it showed no movement after a gentle poke with an eyelash pick (n = 2-6 at 24, 36, 48, and 72 hours; n = 2 at 96 hours).

Anoxic Treatments for Microarrays:

The 2.5L Oxoid AnaeroJar System (Thermo Fisher Scientific, Waltham, MA) was used to expose worms to anoxia for 4 and 24 hours. Three replicates from each time point were performed. After each treatment, worms were washed off plates with M9 and stored for RNA isolation in microfuge tubes with 800 μL TRIzol® at -80°C.

RNA Isolation:

RNA was isolated using a modified TRIzol® method from Burdine and Stern (1996). After separation of layers with chloroform, the clear aqueous solution for each sample was transferred to a new RNase free tube. The RNA was further extracted and purified with a spin-column using a PureLink™ Micro-to-Midi™ Total RNA Purification Kit (Invitrogen, Carlsbad, CA). RNA quality and quantity was assessed through absorbance readings at 260 nm and 280 nm. Quality of RNA was also assessed by running 1% agarose gel with four randomly selected RNA samples to observe 28S and 18S ribosomal bands.

Microarray Protocol:

C. elegans cDNA microarrays were obtained from the Genome Sequencing Center (St. Louis, MO). The 40ug RNA was initially used for reverse transcription (RT) to make DNA. The DNA was labeled by RT amino-allyl dye coupling and hybridization was conducted as described by DeRisi based on the protocol developed for yeast microarrays (DeRisi 2001). Amino-allyl dye coupling is an alternative method for labeling DNA. Usually, DNA is labeled during reverse transcription (RT) from RNA by using a fluorescently tagged nucleotide; however, this alternative method uses a modified uracil base, 5-5-aminoallyl-2'-deoxyuridine 5'-triphosphate (aa-dUTP), during RT which can later be coupled to a fluorescent tag (Cy3 or Cy5). The advantage to using the amino-allyl dye coupling protocol is that the cDNA can be purified before and after labeling thereby increasing fluorescent signal after cDNA hybridized to the microarray.

All microarrays were scanned within one hour after hybridization using GenePix 4200A Scanner (Axon Instruments) and GenePix Pro5.1 Software (Axon Instruments).

Results:

Anoxia Viability Assays:

Greater than 98% of the adult worms survived exposure to 24 hours of anoxic conditions (Figure 3). At all time points after 24 hours, viability significantly decreased. By 72 and 96 hours all adult worms were unable to survive anaerobic conditions.

RNA Isolation:

All RNA samples had 260nm/280nm ratios between 1.9 and 2.1. The overall quality of RNA was assessed by observing 28S and 18S ribosomal bands of one control and anoxia treated sample from each time point of 4 and 24 hours (Figure 4).

Microarray Results:

After scanning the microarrays it was observed that not all of the arrays were optimal. The arrays from the 24 hour time point displayed a "Christmas tree" effect meaning that most spots were either red (up-regulated genes) or green (down-regulated genes). In addition, parts of the 4 hour microarrays had sections where there was no fluorescence at all.

Discussion:

After 24 hours without any oxygen, the viability of the nematode is not significantly affected (Figure 3). This result was similar to those obtained by Van Voorhies *et al.* (2000). However, at time points greater than 24 hours, viability began to decrease and by 72 and 96 hours of anoxia viability was 0% (Figure 3). Van Voorhies *et al.* (2000) observed a decline in viability to about half at 96 hours and 0% at 144 hours, which differ from the viability assays reported here. Van Voorhies and colleges (2000) maintained their worms at 20°C and measured anoxia viability in liquid culture instead of exposing worms to anoxia on agar plates. This may have somehow favored their increased survival in anoxic conditions.

Despite the differences between literature viability after 72 hours of anoxia, the consistent data suggesting that viability is not greatly affected after 24 hours of anoxia is of primary focus. Between 0 and 24 hours I predict that there must be a variety of transcriptional changes occurring to allow the worm to prevent death and handle oxygen deprivation. Therefore analyzing the transcriptional regulation of genes at 4 and 24 hours of anoxia may help identify genes that play a crucial role in anoxia survival.

The quality of RNA used for reverse transcription was determined to be pure and uncontaminated by examining the 28S and 18S rRNA bands on the 1% agarose gel (Figure 4). This was also verified by measuring absorbance ratios of 260nm/280nm between 1.9-2.0. Therefore, the cDNA hybridized to the microarrays were pure samples.

After scanning the microarrays, a “Christmas tree” effect was observed in the 24 hour time point microarray set (n=3). This suggests that the results obtained from the analysis of these arrays may be faulty. Usually most spots on a microarray appear yellow with equal amount of red and green signifying no change in expression and only few spots are either upregulated genes identified by a red spot or downregulated genes identified by a green spot. Therefore, overall only few genes are activated or repressed during a response to in this case anoxia. It is not realistic for almost every gene in the genome to be changing expression due to a change in environment such as lack of oxygen. The 4 hour time point microarray set (n=3) did not have a “Christmas tree” effect, but there were portions of the array without signal. This was caused by a bubble between the slide and the coverslip during hybridization. Therefore, not all of the spots were hybridized because of these air pockets and not all genes are consistently accounted for within this set of arrays.

For further research, I will analyze these two sets of microarrays (4 and 24 hours) using Ramhorn Array Database (www.ramhorn.cbsc.vcu.edu; Sherlock *et al.* 2001; Killion *et al.* 2003; Stanford Microarray Database (SMD) and Longhorn Array Database (LAD) and identify genes that have been significantly upregulated or downregulated in response to anoxic conditions at 4 and 24 hours. Once genes have been identified and verified through qRT-PCR, transgenic worms will be constructed to determine the location and function of selected genes.

Works Cited:

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Figures:

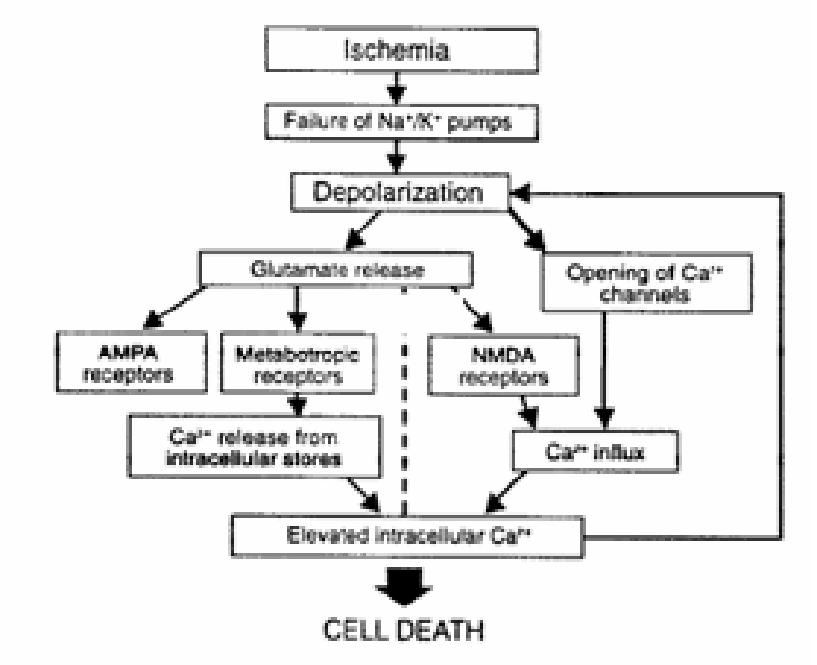

Figure 1. Ischemic excitoxic cascade in stroke (Lyden and Wahlgren 2000).

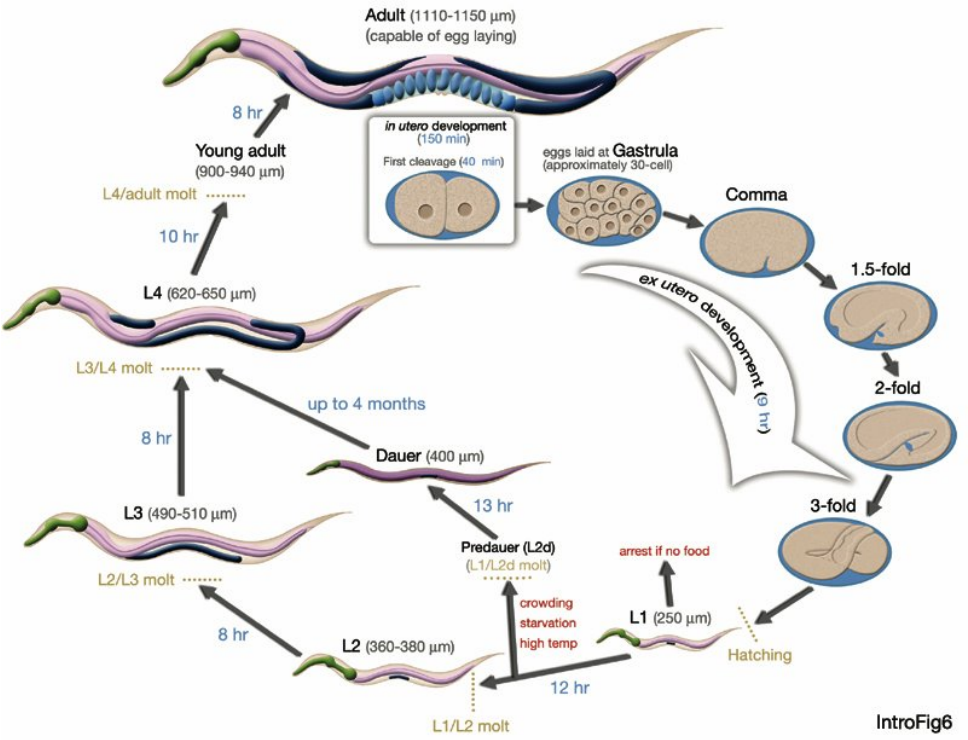

Figure 2. Life cycle of *C. elegans* at 22°C. (www.wormatlas.org)

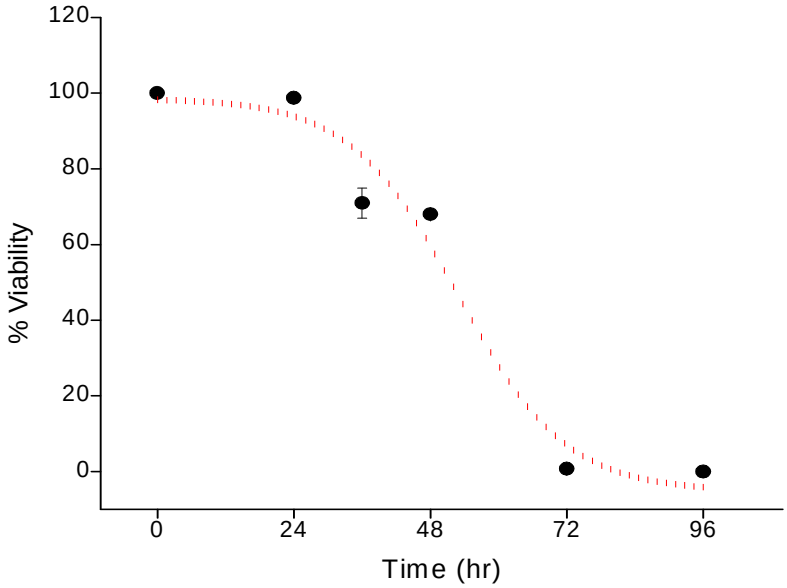

Figure 3. Percent viability of *C. elegans* after 0, 24, 36, 48, 72, and 96 hours of anoxic exposure (n = 2 for 96 hr; n = 4 for 36 and 72 hours; n = 6 for 24 and 48 hours). The curve was fit using a Boltzmann model.

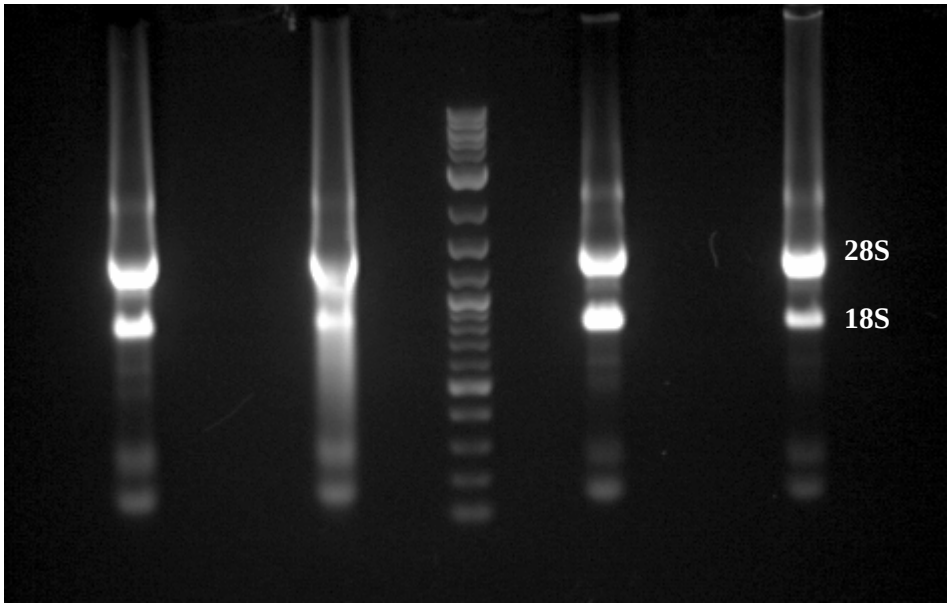

Figure 4. RNA of one control and one anoxia treated sample at 4 and 24 hours. 1) 4 hour anoxia sample; 2) 24 hour anoxia; 3) DNA Ladder; 4) 4 hour control sample; 5) 24 hour control sample