Investigation of the Insulin/IGF-1 Signaling Pathway and the Epigenetic Memory of Thermotolerance in *Caenorhabditis elegans*

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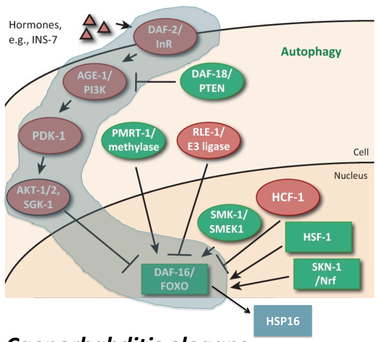
Abstract

The goal of this study was to see if the Insulin/IGF-1 Signaling pathway (IIS pathway) is involved in the epigenetic memory of thermotolerance in *Caenorhabditis elegans* (*C. elegans*). The IIS pathway mediates longevity, metabolism and stress-resistance, which includes thermotolerance. This pathway activates a family of small heat shock proteins (such as hsp16), which help maintain protein homeostasis by preventing other proteins from unfolding during exposure to extreme heat. It has been shown that higher temperatures can induce a heat resistance that can be inherited in *C. elegans*. The two experiments presented in this report explore the connection between the IIS pathway and this inherited thermotolerance. The first experiment investigated the duration of the inherited thermotolerance by analyzing the death rates of *C. elegans* at extreme temperatures (37ºC) for progeny of nematodes grown in warmer temperatures (25ºC) and in optimal temperatures (20ºC) over 5 generations. The control group was the progeny of generation 1 nematodes grown at 20ºC and the experimental group was the progeny of generation 1 nematodes grown at 25ºC to induce thermotolerance. Wild type worms and two strains of *C. elegans* with mutations in the IIS pathway (*daf-16* and *age-1*) were compared to see if the number of generations that inherited thermotolerance in the experimental group would change. The *daf-16* was a null mutation (less thermotolerant) and the *age*-1 was a down-regulated mutation (more thermotolerant). Wild type and *daf*-16 mutants inherited the thermotolerance for 4 generations whereas *age*-1 mutants inherited the thermotolerance for 5 generations. However, an ANOVA test among the three strains for each generation showed that there was no statistical difference in the rate at which the thermotolerance was lost among the three strains ($p > 0.05$). This suggests that this pathway is not responsible for how long the epigenetic memory of thermotolerance lasts. The second experiment compared the relative expression levels of heat shock protein 16 (*hsp-16.1* and *hsp-16.2*) between the control (Gen. 1 at 20ºC) and experimental (Gen. 1 at 25ºC) groups after heat shock using quantitative real-time PCR (RT-PCR). There was no observed trend in reference to the association between heat shock protein and inherited thermotolerance due to a large degree of variation in the levels of *hsp-16.1* and *hsp-16.2*. Though initial results are inconclusive, this experiment established a reliable RNA extraction and RT-PCR protocol for future use in our lab to repeat the experiment.

Introduction

Insulin/IGF-1 Signaling Pathway (IIS pathway) is a conserved pathway defined as being present in multiple species and unchanged through evolution. Accordingly, the IIS pathway has a similar pathway in humans. The first component of the IIS pathway is daf-2/IGFR, which is the receptor. When insulin-like peptides bind to the receptor, a phosphoinositide 3-kinase, known as age-1, is activated. This activates a cascade of kinases that negatively regulate the nuclear localization of daf-16/FOXO, the primary target of the IIS pathway. It mediates longevity, stress resistance and dauer-constitutive phenotypes.



Caenorhabditis elegans

Wildtype: Control nematodes
daf-16(mu86): Less thermotolerant (compared to wild type) due to a null mutation of the *daf-16* gene, eliminating a main target of the IIS pathway
age-1(hx546): More thermotolerant (compared to wild type) due to a down-regulation of the *age-1* gene and a reduced IIS pathway activation.

Heat Shock Proteins are proteins that cells release when exposed to stressors. When *C. elegans* are exposed to extreme heat, small heat shock proteins are amplified and help maintain protein homeostasis by preventing other essential proteins from denaturing and unfolding due to the heat. The family of small heat shock proteins, *hsp16*, is one of the target genes of *daf-16.*

Epigenetics is the study of heritable changes of gene expression that don't involve a change in the DNA base sequence. An organism's experiences can cause genetic alterations (methylation of nitrogen bases) not associated with sequence changes in DNA.

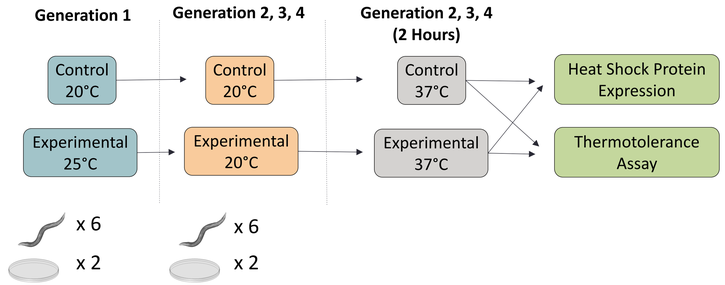
Previous Research I showed that thermotolerance to extreme temperatures can be inherited for at least four generations in wild type *C. elegans*

Research Questions

Is the **IIS pathway** responsible for the thermotolerance in successive generations of *C. elegans?*

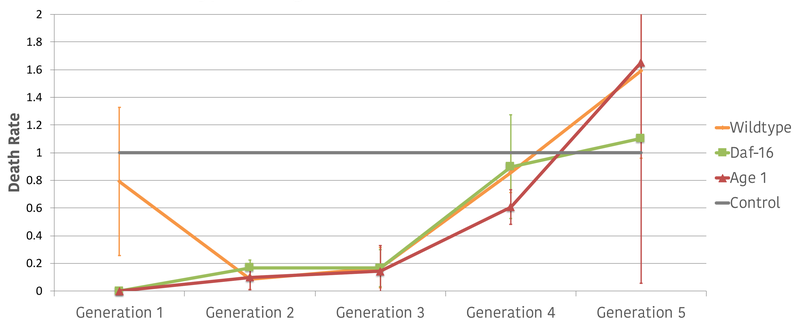
- Do mutations in the IIS pathway cause changes in the **duration** of the thermotolerance epigenetic memory?
- Do the progeny of nematodes grown at higher temperatures demonstrate higher **gene expression levels** of hsp16?

Methods



Thermotolerance Assay –
Three days after the plates started culturing, 4 plates were inoculated with 6 L4/adult nematodes each from the experimental plates (25ºC) and 4 plates were inoculated with 6 L4/adult nematodes each from the control plates (20ºC). All 8 plates were treated with a heat shock treatment of 2 hours at 37ºC. After 2 hours, all 8 plates were removed from the incubators and observed under a microscope. The nematodes were classified as either dead or alive.
This was repeated for all generations and for all strains of *C. elegans*.

Thermotolerance Results & Discussion



ANOVA Test Results for Comparison of Data between Three Strains of *C. elegans*	
Generation	**p-value**
Generation 1	0.00713
Generation 2	0.40665
Generation 3	0.94839
Generation 4	0.11056
Generation 5	0.65157

We conclude that the IIS pathway is involved in the duration of inherited thermotolerance in *C. elegans*, since as seen in the graph above, there is no statistically difference between the death rate of the three strains of *C. elegans* for each generation.

Methods (continued)

Heat Shock Protein Expression
Two experimental and two control group plates were placed at 37ºC for 2 hours (heat shock treatment). RNA extraction and RT-PCR protocol is run following the heat shock.

RNeasy kit (Qiagen) →RNA extraction
iScript cDNA synthesis kit (Bio-Rad) and myCycler (Bio-Rad) thermal cycler → cDNA synthesis
iQ SYBR green supermix (Bio-Rad) and Bio-Rad iCycler → RT-PCR

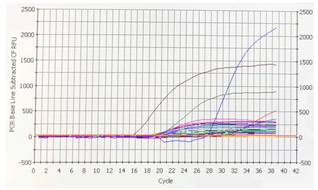
Primer sequences used:
pmp-3 forward: TGGTGTCGCGATTACTGTAG
pmp-3 reverse: GATTTGTTGTCGCAGAGTGG

hsp-16.1 forward: TTTTGTTCAACGGGCGCTTGC
hsp-16.1 reverse: GAGGCTCTCCATCTGAATCTTCTGAG

hsp-16.2 forward: CGTCGAAGAGAAATCTGCTGAA
hsp-16.2 reverse: TGCAGCGAACAATACTGTAATTTATG

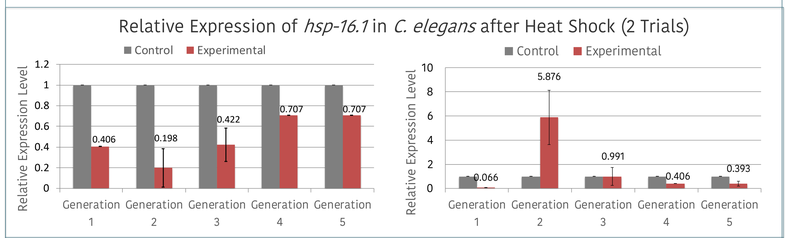
actin forward: GTGTTCCCATCCATTGTCGGAAGA
actin reverse: GCACTTGCGGTGAACGATGGATGGG

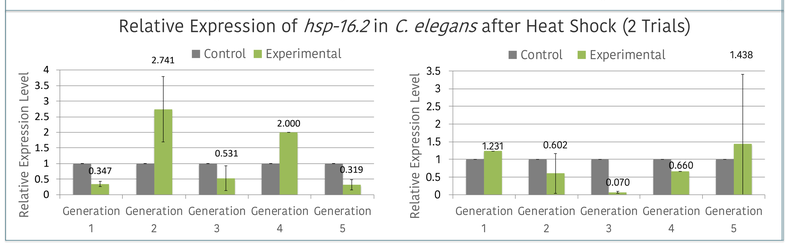
Heat Shock Protein Expression Results & Discussion



The results from the expression levels of hsp-16.1 and hsp-16.2 are inconclusive, as there is no clear trend in the graphs below.

The figure to the left are direct results from the RT-PCR cycler





Acknowledgements

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