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Loss of Methionine Sulfoxide Reductase B Leads to Higher Failure Rates in *Drosophila melanogaster* after Hyperthermic Stress

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Abstract

When an organism is facing excessive metabolic production of heat, there is a risk of entering into a hyperthermic state. Hyperthermia causes an increased production of dangerous reactive oxygen species (ROS) that often lead to cell damage. ROS are free radicals that attack electron rich molecules. Methionine, a common amino acid in proteins, is especially susceptible to oxidation by ROS. The oxidized methionine can be restored to normal functionality by reduction through the activity of methionine sulfoxide reductase (Msr). Msr is a highly conserved enzyme from bacteria to humans. The gene is an antioxidant defense that reverses oxidative damage created by ROS by a reduction process through two enzymes: *MsrA* and *MsrB*. *MsrA* and *MsrB* reduce the two enantiomers of methionine sulfoxide back to functional methionine. RNA interference (RNAi) was used to knock down expression of either *MsrA* or *MsrB* to better understand the role of these genes in response to hyperthermia. Results have shown that knockdown of *MsrB* expression in all tissues leads to a higher failure rate in older aged flies. However, specific knockdown of *MsrB* in just motor neurons did not show this effect. When the flies were aged to 5 days and 24 days, knockdown of *MsrB* did not prove to be detrimental as flies had the same failure rates as wild type. However, when aged to 35 days the flies began to increase in failure rates. Currently, the molecular basis of *MsrB* is unknown. The reduced thermotolerance may be attributed to increase levels of ROS, hence *MsrB's* anti-oxidative function may contribute to hyperthermic tolerance.

Keywords: Methionine Sulfoxide Reductase, MsrB, ROS, Hyperthermia

1.Introduction

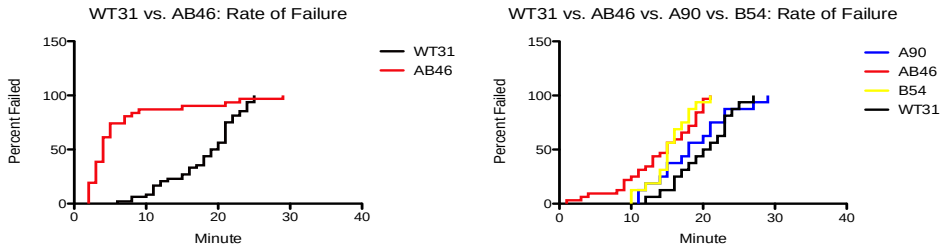
Organisms have adapted to cope with a variety of environmental stresses like elevated temperatures[1]. Exposure to unusually high temperatures is called hyperthermia. Hyperthermia happens when the thermoregulatory mechanisms become overwhelmed by the excessive metabolic production of heat, excessive environmental heat, or impaired heat dissipation[1]. To combat this, organisms have developed ways to reduce the biological consequences of hyperthermia. *Drosophila melanogaster* exhibits a phenomenon called spreading depression. Spreading depression is a condition where an invertebrate such as *Drosophila* enters into a reversible coma like state. The electrical activity in the nervous and muscular system is shut down until conditions are returned to normal or normoxia[5]. It is also known that when faced with hyperthermic conditions *Drosophila* also use heat shock proteins to survive against the stressful condition. Heat shock proteins help in coping with the effects of heat, hypoxia, and exposure to toxic substances[6]. Heat shock proteins are differentiated into groups based on their molecular mass and increase during times of stress[7]. It has been demonstrated that heat shock proteins develop a thermotolerance when exposed to

moderate heat shock. Furthermore, heat shock protein 70 (hsp70) in *Drosophila* has been shown to be the biggest factor inhibiting cell apoptosis[7].

Recent research studies have shown that heat shock proteins play a vital role in response to thermal stress and that reactive oxygen species (ROS) production increases during times of hyperthermia[4]. ROS are highly reactive free radicals that contain oxygen. ROS are produced by oxygen metabolism and are crucial players in cell signaling. When the cells are faced with environmental stress, the ROS levels begin to increase dramatically[2]; this stress eventually causes significant damage to the cell structures. Oxidative modification through one or more forms of ROS can affect a number of different amino acid residues of proteins[3]. Methionine is especially susceptible to oxidative damage by ROS. Oxidation of methionine residues causes the formation of the S and R epimers of methionine sulfoxide (Met-(o)). The enzyme methionine sulfoxide reductase (Msr) functions to repair this oxidative damage and restore functional methionine. *MsrA* specially reduces the S epimer of Met-(o) while *MsrB* reduces only the R epimer of Met-(o)[3]. Msr genes are highly conserved among organisms ranging from bacteria to humans. There is evidence that the *MsrA* gene plays a role in protecting against other forms of stress that produce ROS such as hyperoxia or exposure to hydrogen peroxide[3] However, a possible role of *MsrB* is unknown.

In this study, we investigated the role of the repair and scavenging enzymes: methionine sulfoxide reductases (Msr). In *Drosophila*, this family of enzymes is encoded in two genes designated *MsrA* and *MsrB*. For this study, we are using a fairly new genetic tool called RNA interference (RNAi) to control expression of *MsrA* and *MsrB* in specific tissues *to better understand the role of this genes in counteracting thermal stress induced ROS.*

Previous studies from our lab have shown that *Drosophila* lacking Msr activity had decreased thermotolerance and a higher failure rate after exposure to thermal stress when compared to wild-type (i.e. normal) flies. Previous research from our lab has indicated that the absence of Msr was detrimental to the fly (Figure 1A). The flies had increased failure rates when both *MsrA* and *MsrB* were deleted from the fly. More specifically, the deletion of *MsrB* lead to the highest failure rates despite the presence of *MsrA* (Figure 1B). Therefore, it became apparent that *MsrB* played a bigger role in thermotolerance.



(a) (b)

Figure 1. (a) Rate of failure comparisons between wild type (WT31) with *MsrA* and *MsrB* against flies that lacked *MsrA* and *MsrB* (AB46). (b) Rate of failure comparisons between wild type (WT31) with *MsrA* and *MsrB*, lack of *MsrA* and *MsrB* (AB46), lack of *MsrA* (A90), and lack of *MsrB* (B54).

Figure 1. n=79: 31 AB46 and 48 WT31 flies aged between 35 and 39 days were stressed for 35 minutes at 38.5 ^{o}C. AB46 was found to have a much slower failure rate than WT31. A Log-rank test was performed and the resulting $p < 0.0001$(a). n=80: 32 AB46, 16 WT31, 16 A90, and 16. AB46 was found to have a much slower failure rate than WT31 followed by B54 and A90. A Log-rank test was performed and the resulting p-values were $p = 0.7076$ for WT31 vs. A90, $p = 0.0005$ for WT31 vs. B54, $p = 0.0002$ for WT31 vs. AB46, $p = 0.0069$ for A90 vs. AB46, $p = 0.0242$ for A90 vs. B54, and $p = 0.9400$ for B54 vs. AB46 (b).

Recent research has hinted the role of *MsrB* in other organisms. In organisms like *E. faecalis* and *Galleria mellonella* it seems that the *Drosophila* have the same oxidative role[11]. The study proved that the Msr enzymes were playing some role against ROS, which was H_2O_2 in the study. Another study used the mammalian MsrB3 enzyme, which is targeted for the endoplasmic reticulum in *Drosophila* to show that overexpression of this enzyme in the central nervous system lead to a higher tolerance against thermic stress and prolonged the lifespan[10]. The study suggested when using a mammalian MsrB3 enzyme it is possible that the endoplasmic reticulum pathway is the

molecular basis *MsrB*. However, the study has not concluded the true molecular basis of Drosophila *MsrB*. Better understanding of this process is important since hyperthermia is both an environmental stress and it is being applied in some human health situations.

2.Materials and Methods

2.1 Generation Of *Drosophila*:

We confirmed the deletions of MsrA and MsrB were done correctly by using assays like RT-PCR, and West Blot. The lab coordinator conducted the assays: Lindsay Bruce. The flies were then kept in stock bottles for 14 days. Next, the bottles were cleared for virgin females. The virgin females were collected into test tubes where they were moved into a test tube with males that had a knockdown version of *MsrB*; this is referred to as the genetic crossing stage. The flies were kept in the bottles for 10 days. The flies were then cleared again and the offspring or F1 generations were collected 5 days later. The male yellow-eyed non-curly flies were collected to be tested.

2.2 Activity Monitoring System:

The *Drosophila* Activity Monitoring system is a computerized system that measures the movement of individual flies by recording every time they pass an infrared beam in a minute. The system has two monitors. Monitor 8 was held at room temperature (25° C) and Monitor 7 was placed in an micro hybridization incubator at a temperature of 38.5° C. Each monitor has 32 slots that contain 32 three-inch glass tubes; this is all attached to a DAM system holder. 32 flies can be tested at a time.

2.3 Micro Hybridization Incubator:

A micro hybridization incubator was used to mimic hyperthermic conditions. The incubator was set to 38.5° C for every experiment conducted.

2.4 Thermal Stress:

For both experiments, flies were moved into monitor 8 and were left at room temperature for 15 minutes. This is referred to as the stabilization period. The flies were then moved to monitor 7 and placed in the incubator at 38.5° C for 30 minutes. Only male flies were tested in the study. More specifically, all male non-curly red-eyed flies were tested. The minute they failed during the experiment was then recorded.

2.5 Statistics:

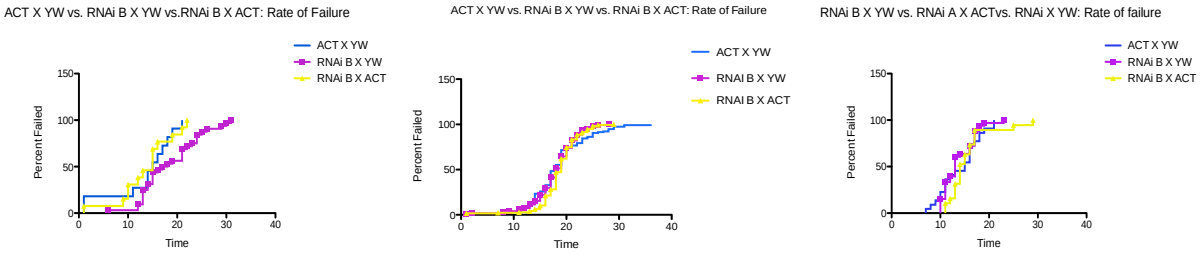
In this study, Graph Pad Prism was used. Flies that failed were labeled as 1 and the flies that were outside of the experimental methods were labeled as 0. Log-rank tests were used to analyze the resulting times to failure. A p value of less than 5% was used in the experiment resulting in a confidence interval of 95%.

3. Results

3.1 *MsrB's* role is more profound as flies' age past 35

The aim of the knockdown assay was to validate the results from the previous genetic deletion assays (Figures 1A and 1B). Once the assay was confirmed, we could manipulate the RNAi interference to knockdown *MsrB* in a specific tissue. Actin was the first driver used because it is a ubiquitous driver that helped mimic the results from the deletion experiment. The knockdown expression of *MsrB* was paired with Actin. Results indicated that knockdown expressions of *MsrB* in the young age (days 5-24) animals showed no difference between the parental and

experimental groups (Figures 2B and 2C). However, once the flies reached day 35 it failure rates increased in animals having MsrB expression knocked down. The RNAi interference exposed that fly life spans were relative to the assay. As deletion assay flies could not live past day 35, where as RNAi interference could live up to day 60. *Drosophila* lacking both *MsrA* and *MsrB* died by day 40. The genetic deletions of just MsrA or just MsrB lived almost as long as wild type, at least 60-65 days. The next aim of this study is to age flies beyond day 60 to achieve confirmation that RNAi interference is truly working in the flies.



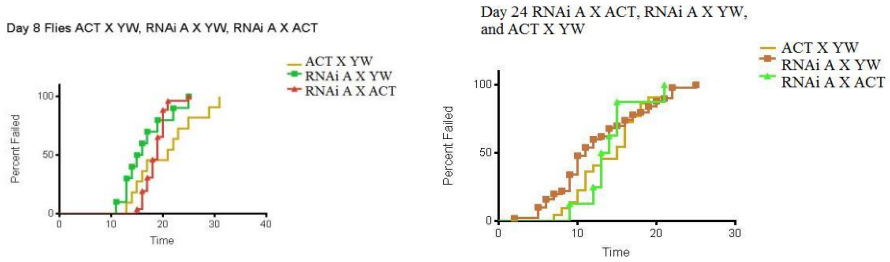
(a) (b) (c)

Figure 2. (a) Rate of failure of flies that were aged to 5 to 9 days: ACT X YW, RNAi B X YW, and RNAi B X ACT. (b) Rate of failure of flies that were aged to 21 to 24 days: ACT X YW, RNAi B X YW, and RNAi B X ACT. (c) Rate of failure of flies that were aged to 30 to 35 days: ACT X YW, RNAi B X YW, and RNAi B X ACT.

Figure 2. RNAi B X YW (n=13), RNAi B X ACT (n=31), and ACT X YW (n=11) flies were stressed for 30 minutes at 38.5 ^{o}C using the Drosophila Activity Monitor (DAM) as described in the Methods. A Log-rank test was performed for RNAi B x YW vs. RNAi B X ACT was 0.6153 and RNAi B X ACT vs. RNAi B X YW was 0.5759(a). RNAi B X YW (n=270), RNAi B X ACT (n=173), and ACT X YW (n=116) flies were stressed for 30 minutes at 38.5 ^{o}C using the Drosophila Activity Monitor (DAM) as described in the Methods. A Log-rank test was performed and the resulting p-values for RNAi B X ACT vs. ACT X YW was 0.6238 and RNAi B X ACT vs. RNAi B X YW was 0.3270(b). RNAi B X YW (n=32), RNAi B X ACT (n=33), and ACT X YW (n=20) flies were stressed for 30 minutes at 38.5 ^{o}C using the Drosophila Activity Monitor (DAM) as described in the Methods. A Log-rank test was performed for RNAi B X YW vs. RNAi B X ACT was 0.1408 and RNAi B X ACT vs. ACT X YW was 0.0111(c).

3.2 Knockdown Expressions Of *Msra* Showed No Significant Difference In Young Age Flies, Failure Rates Were Same As The Parental Lines

The deletion experiments proved that *MsrA* did not play much of a role against the hyperthermic stress. It seemed that these findings were consistent in the knockdown experiments. Results indicated failure rates were the same compared to the parental lines. When a log rank test was conducted the p value was not significant.



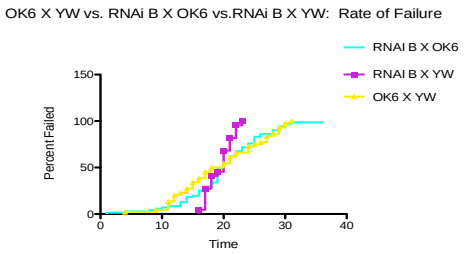
(a) (b)

Figure 3. (a) Rate of failure of flies that were aged to 8 days: ACT X YW, RNAi A X YW, and RNAi A X ACT. (b) Rate of failure of flies that were aged to 24 days: ACT X YW, RNAi A X YW, and RNAi A X ACT.

Figure 3. RNAi A X YW (n=11) RNAi A X ACT (n=10), and ACT X YW (n=26) flies were stressed for 30 minutes at 38.5 ^{o}C using the Drosophila Activity Monitor (DAM) as described in the Methods. A Log-rank test was performed for ACT x YW vs. RNAi A X ACT was 0.0696 and RNAi A X ACT vs. RNAi A X YW was 0.3359(a). RNAi A X YW (n=51), RNAi A X ACT (n=11), and ACT X YW (n=24) flies were stressed for 30 minutes at 38.5 ^{o}C using the Drosophila Activity Monitor (DAM) as described in the Methods. A Log-rank test was performed and the resulting p-values for RNAi A X ACT vs. ACT X YW was 0.7253 and RNAi A X ACT vs. RNAi A X YW was 0.8341(b).

3.3 Knockdown Expressions Of *Msrb* In The Motor Neurons Displayed No Significant Difference, As Failure Rates Were The Same For Parental Lines

The first aim of the study was to confirm that RNAi interference was truly knocking down *MsrB.* The next aim of the study was to manipulate the system to knockdown *MsrB* in a specific tissue. The first area was the OK6 motor neurons. When *MsrB* was knockdown in the motor neurons, the flies did not have much of a difference in failure rates (Figure 3A). Currently, only young age flies were tested. The next aim of the study is to age the flies up to day 60 to truly see if knocking *MsrB* is detrimental to the fly.



(a)

Figure 4: (a) Rate of failure of flies that were aged 5 to 9 days: OK6 X YW, RNAi B X OK6, and RNAi B X YW.

Figure 4. RNAi B X OK6 (n=71), RNAi B X YW (n=20), and OK6 X YW (n=43) flies were stressed for 30 minutes at 38.5 ^{o}C using the Drosophila Activity Monitor (DAM) as described in the Methods. A Log-rank test was performed for RNAi B X OK6 vs. OK6 X YW was 0.9198 and RNAi B X OK6 vs. RNAi B X YW was 0.0689.

4.Discussion

The deletion of Msr proved that flies failed faster compared to their wild type counterpart (Figure 1A). Further experiments indicated that *MsrB* played a bigger role in thermotolerance (Figure 1B). These experiments proved that Msr is playing some role in thermotolerance. The molecular basis of the process is unknown. RNAi interference was the assay used to help investigate what the molecular basis of *MsrB* was. The first aim of the knockdown experiment was to confirm that the results were consistent with deletion assay. This was done with the aid of the actin driver. If the results proved to be consistent with this assay then it is possible to manipulate this system to knockdown *MsrB* in a specific tissue.

It is apparent that knockdown of *MsrB* expression in all tissues in young and middle age flies shows no statistical difference (Figure 2A and 2B). However, as the flies age to day 35 some statistical difference is starting to show (Figure 2C). Therefore, it seems that *MsrB* plays a role later in the life of *Drosophila.* The RNAi interference knockdown assay also proved that the lifespan of the flies were relative to the assay. Comparing 35 days in deletion to 35 days knockdown was not an accurate comparison (Figures 1A and 2A). The flies in deletion only lived to day 40, where as in knockdown the flies lived to day 60. Therefore, to really receive similar results flies have to be aged to day 60. Currently, studies are being conducted with flies aged to 60 days. Recent research is proving that Msr is playing roles in oxidative, cold and heat stresses, and even life spans. A previous study conducted showed that overexpressing endoplasmic reticulum targeted human *MsrB* lead to higher tolerance against heat and cold stresses[10]. The study investigated a cytosolic form of ER targeted *MsrB* in the *Drosophila.* The results indicated that the flies had lower failure rates when overexpression of ER targeted human *MsrB* was incorporated into the ER. Thereby, suggesting that the methionine-R-sulfoxide reduction pathway could be the molecular basis of *MsrB.* Other studies have concluded that Msr repair enzymes have oxidative stress roles in other organisms like *E. faecalis* and *Galleria mellonella*[11]. It seems that the role of Msr has been accurately stated, however, research hasn't fully concluded the molecular basis of the role. It is possible in this study, that the molecular basis of the *MsrB* lies in areas of the *Drosophila* where they are more prone to oxidative stress.

The first attempt was the motor neurons or OK6. Early results with the OK6 driver have suggested that specific tissue knockdown of *MsrB* does not affect the motor neurons (Figure 4A). Future direction of this study is to age the flies past 35 days and to examine what the *MsrB* role is at a later stage of the *Drosophila*. In addition, new drivers such as GawB and MHC are going to be introduced to help explain the biochemical pathway.

The statistical difference in one parental line in 35-day-old flies can be attributed to the limitations of the RNAi knockdown system. RNAi interference is not doing a full knockdown. The assay does leave some residual amount of *MsrB.* Western blots are being conducted to confirm the residual amount is negligible in this study. On the other, deletion assays completely get rid of *MsrB* leaving no residual amount left.

5. Acknowledgements

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6.References

1. Harvey B. Simon and others. "Hyperthermia." New England Journal of Medicine 329, no. 7 (August1993): 483-487,
http://ezproxy.fau.edu/login?url=http://search.proquest.com/docview/223988135?accountid=10902.

2. Andreas Gallmetzer and others, "Functional characterization of the Aspergillus nidulans methionine sulfoxide reductases (MsrA and MsrB)." Frugal Genetics and Biology 46, no.5 (May 2009): 410-417,
http://www.sciencedirect.com.ezproxy.fau.edu/science/article/pii/S1087184509000255.

3. Rodney Levine and others, “Methionine oxidation and aging”. Biochimica et Biophysica Acta 1703, no.17 (January2005): 135-140,
http://www.sciencedirect.com.ezproxy.fau.edu/science/article/pii/S1570963904002213.
4. Fukumura Hidenobu and others, "Effect of ascorbic acid on reactive oxygen species production in chemotherapy and hyperthermia in prostate cancer cells." Journal of Physiological Sciences 62, no.3 (March 2012): 251-257, http://link.springer.com.ezproxy.fau.edu/article/10.1007%2Fs12576-012-0204-0.
5. Gary Armstrong and others, "Suppression of Spreading Depression-Like Events in Locusts by Inhibition of the NO/cGMP/PKGPathway.” Journal of Neuroscience 33, no.22 (June 2009): 8225-8235,
http://www.jneurosci.org.ezproxy.fau.edu/content/29/25/8225.full.
6. V.Yu Shilova and others. "Small Heat Shock Proteins and Adaptation of Various Drosophila Species to Hyperthermia." Russian Journal of Developmental Biology 40, no.2 (March 2006): 271-276,
http://search.proquest.com.ezproxy.fau.edu/docview/760116366?pq-origsite=summon.
7. M.B. Evgen'ev and D.G. Garbuz. "Heat Shock Proteins: Functions and Role in Adaptation to Hyperthermia."Russian Journal of Developmental Biology 36, no.4 (July 2005): 218-224,
http://link.springer.com.ezproxy.fau.edu/article/10.1007%2Fs11174-005-0036-4.
8. Sanei Nader. "Hyperthermia and Cancer Treatment." Heat Transfer Engineering 30, no.12 (2009): 915-917, http://www.tandfonline.com.ezproxy.fau.edu/doi/abs/10.1080/01457630902854371#.VK7O94rF-4Q.
9. Hua-Gang, Zhang and others. "Hyperthermia on immune regulation: A temperature’s story." Cancer Letters 271,no.2 (November2008): 191-204,
http://www.sciencedirect.com.ezproxy.fau.edu/science/article/pii/S0304383508003674.
10. Hwa-Young Kim. "Methionine sulfoxide reductase B in the endoplasmic reticulum is critical for stress resistance and aging in Drosophila.” Biochemical and Biophysical Research Communications 419, no.1 (March 2012): 20-26, http://www.sciencedirect.com.ezproxy.fau.edu/science/article/pii/S0006291X12001465.
11. M Sanguinetti. "Role of Methionine Sulfoxide Reductases A and B of Enterococcus faecalis in Oxidative Stress and Virulence." infection and immunity 78, no.9 (September 2009): 3889-3897,
http://ezproxy.fau.edu/login?url=http://search.proquest.com/docview/811069878?accountid=10902.