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The Role of Methionine Sulfoxide Reductase in the Thermal Stress Response of *Drosophila melanogaster*

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Abstract. Methionine sulfoxide reductase (Msr) is an enzyme that is used by the majority of organisms to reverse oxidative damage caused by reactive oxygen species (ROS). ROS are free radicals which react with electron-rich molecules such as nucleotides, carbohydrates, or proteins. The highly electronegative amino acid methionine is frequently oxidized to methionine sulfoxide (met-(o)) by ROS; Msr can repair the damaged methionine by reducing met-(o) to functional methionine. There are two distinct enzymes, MsrA and MsrB, which reduce the two enantiomers of met-(o), met-S-(o) and met-R-(o), respectively.

Since additional ROS are produced during an organism's exposure to thermal stress, we investigated whether MsrA and/or MsrB play a role in the response to thermal stress. Drosophila melanogaster lacking both MsrA and MsrB were found to be less efficient in their thermal stress response when compared to wild-type, suggesting that MsrA and MsrB may play a role in thermal stress response. In addition to MsrA and MsrB, some heat shock proteins (HSPs), especially Hsp70, help mitigate the damaging effects of thermal stress. HSPs are upregulated during periods of hyperthermia and help to maintain protein integrity and provide neuronal protection. Thus, we also examined the possibility that HSP upregulation prior to exposure to hyperthermic conditions improves thermal stress response and survival in Drosophila lacking MsrA and MsrB. Hyperthermic preconditioning was shown to worsen the thermal stress response of D. melanogaster lacking MsrA or MsrB, as well as the wild-type. Future studies include examining the biochemical mechanisms that govern the effect of MsrA and MsrB on hyperthermia tolerance. By determining the roles of MsrA and MsrB in hyperthermia tolerance, we should obtain insight into the multiple functions of MsrA and MsrB throughout an organism.

Introduction

Methionine sulfoxide reductase (Msr) is an antioxidant protein that can restore methionine sulfoxide (met-o), the oxidized form of methionine, to functional methionine. Methionine residues are easily oxidized because they contain sulfur, a highly electronegative atom [7]. Methionine is oxidized into two enantiomers designated met-o-S and met-o-R, and MsrA and MsrB can restore each form of met-(o), respectively [8]. MsrA was discovered in 1979 and shown to be a 25kD protein, while the structurally distinct MsrB was discovered in 2001 and it is a 17kD protein [9] [10]. Amino acid sequence analysis of the MsrA gene in multiple species has revealed a conserved sequence in the N-terminal portion of the protein at position 72: GCFWG. The cysteine contained in this conserved sequence (Cys-72) has been found to be absolutely required for enzymatic activity [20]. MsrB has a distinct, but also highly conserved amino acid sequence. MsrA and MsrB do not share similar amino acid sequences but are, nevertheless, related because their active sites are mirror images of each other, reflecting the fact that they are epimer-specifc [20].

MsrA and MsrB were first identified in E. coli [9][10] but have since been found to be present in a wide range of species [5]. However, different organisms contain different isoforms of MsrA and MsrB, which are targeted to specific cellular compartments. In mammals, for example, one gene encodes MsrA and two different isoforms are produced: a long form and a short form. The long form is targeted to the cytosol, mitochondria, and nucleus, while the short form is localized to the nucleus and cytosol [18]. In mammals, three genes encode three different forms of MsrB: MsrB1, MsrB2, and MsrB3. MsrB1 is targeted to the nucleus and cytosol, MsrB2 is targeted to the mitochondria, and MsrB3

encodes two splice variants, MsrB3A and MsrB3B, which are localized to the endoplasmic reticulum (ER) and the mitochondria, respectively [19].

ROS are free radicals that are highly reactive because of the presence of unpaired electrons, and are most commonly the superoxide ion or hydrogen peroxide [5]. Thus, ROS usually react with highly electronegative molecules such as nucleotides, carbohydrates, or amino acids. This can lead to a variety of mutations, which have been found to lead to a faster aging rate [5]. ROS production has also been found to increase as an organism ages [15]. ROS are byproducts of normal aerobic metabolism inside a cell [6]. Additionally, ROS are produced during periods of hyperthermia [4].

Hyperthermia, also known as thermal stress, is the condition where an organism is exposed to physiologically elevated temperatures [1]. Thermal stress affects a wide range of plants and animals and occurs under many environmental conditions. For example, Drosophila melanogaster larvae are exposed to extreme temperature fluctuations in the fermenting fruit where they mature [2]. Organisms respond to thermal stress in different ways. For example, mammals often undergo stroke when exposed to hyperthermic conditions, while most invertebrates, such as D. melanogaster, enter a reversible protective coma called spreading depression [3]. Spreading depression is characterized by a loss of both neural and motor function and has been well studied in literature as a possible means of conserving energy under stressful conditions [3]. Spreading depression is often reversed when the organism is returned to normal conditions [1].

Most organisms also contain heat shock proteins (Hsp's) to help respond to thermal stress. HSPs are a family of proteins that serve as molecular chaperones and aid in protein homeostasis when an organism experiences heat shock [12]. HSPs are upregulated during thermal stress and they provide neuronal protection during hyperthermia [13, 14]. Given that hyperthermia increases ROS production, we proposed that the antioxidant proteins, MsrA and MsrB, may play a role in an organism's thermal stress response. In addition, since HSPs are involved in the response of organisms to thermal stress, we investigated whether HSP upregulation prior to exposure to hyperthermic conditions may improve an organism's thermal stress response.

Table 1 - Designation of Genotypes

	Strain:	Size of Deletion:
WT ((MsrA$^{+/+}$ MsrB$^{+/+}$)	WT77, WT31, WT60	
MsrA LOF (MsrA$^{LOF/LOF}$ MsrB$^{+/+)}$	A90	1.5 kb
MsrB LOF (MsrA$^{+/+}$ MsrB$^{LOF/LOF}$)	B54	2.5 kb
MsrA/MsrB LOF (MsrA$^{LOF/LOF}$ MsrB$^{LOF/LOF}$)	AB113, AB46	

Invertebrates enter a protective coma known as spreading depression when exposed to hyperthermic conditions. In this study, we measured the time required for Drosophila mutants, lacking either MsrA or MsrB, or both MsrA and MsrB, to undergo spreading depression, commonly termed failure. Young (5-9 day old) and old (35-39 day old) flies were examined to test the effects of increased ROS production due to aging on an organism's thermal stress response. Flies lacking both MsrA and MsrB are referred to as old between 35-39 days of age since they reach senescence in this age range, although wild-type and flies lacking either MsrA or MsrB do not reach senescence until much later. The animals were also pre-conditioned to hyperthermia by exposure to sub-lethal thermal conditions before exposure to standard thermal stress to induce HSP upregulation. The thermal stress response of flies pre-conditioned to thermal stress was then compared with the response of

flies exposed only to thermal stress conditions without preconditioning.

Materials and Methods

Genotypes of *Drosophila*

All experiments used male flies and the thermal stress response of 5-9 day old flies and 35-39 day old flies was examined.

Drosophila Activity Monitoring System

The Drosophila Activity Monitoring (DAM) system is a computerized system that measures the movement of individual flies by recording the number of times a fly crosses an infrared beam over the course of a minute [17]. The DAM system consists of two monitors: one monitor was held at room temperature, while the other was placed inside an incubator set at 36.0 °C or 38.5 °C. Each monitor contains 32 slots that hold 32 three-inch glass tubes. A DAM system holder is used to hold the glass tubes in place in the DAM system monitors and one fly is placed into each tube (Figure 1).

A Micro Hybridization Incubator was used in these experiments to create hyperthemic conditions. The incubator was held at two different temperatures, 36.0 °C or 38.5 °C. We used 36.0 °C as the preconditioning temperature, while 38.5 °C was used to impose thermal stress.

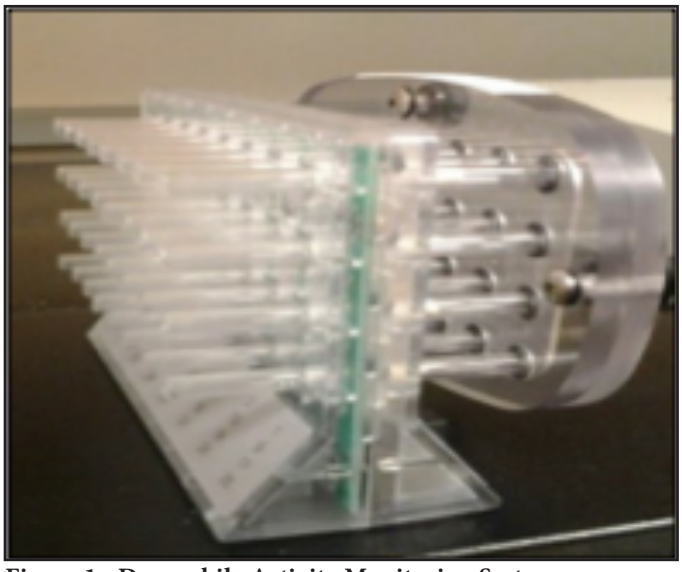

Figure 1 - Drosophila Activity Monitoring System

Thermal Stress

One male fly was placed into each tube at room temperature and allowed to acclimate with their surroundings. The DAM system holder was then placed into the incubator at 38.5 °C for 30 minutes and the time to failure was recorded.

Preconditioning

To examine the effect of preconditioning on the thermal stress response of Drosophila, one male fly was placed into each tube at room temperature to allow the flies to become acclimated with their surroundings. The DAM system holder was then placed into an incubator held at 36.0 °C for 60 minutes to precondition the flies to hyperthermic conditions. Following preconditioning, the flies were exposed to hyperthermic conditions, as described above, and their times to failure were recorded.

Statistics

Using Graph Pad Prism, failed flies were scored as a 1, while flies whose response was outside of the experimental methods was scored as a 0. Log-rank tests were then performed in order to analyze the resulting times to failure. A p-value less than 5% was used in this experiment resulting in a confidence interval of 95%.

Experimental Results

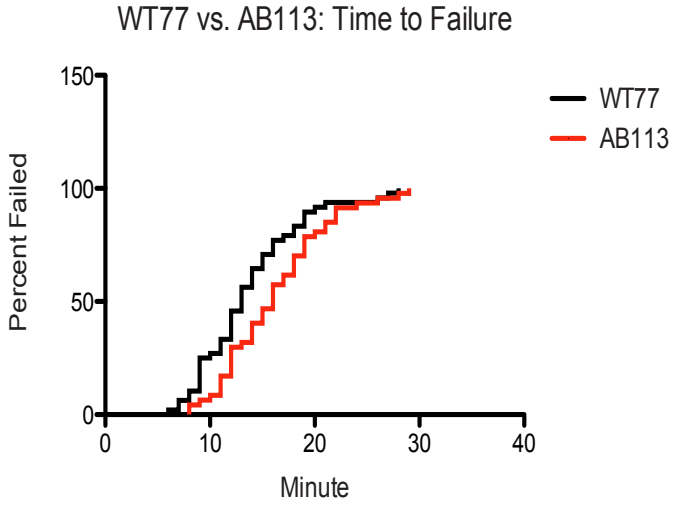

Figure 2 - Failure Times of Young Wild-Type and Msr-Deficient Animals. 5-9 days old AB113 (n=48) and WT77 (n=47) flies were stressed for 30 minutes at 38.5 °C using the Drosophila Activity Monitor (DAM) as described in the Methods. A Log-rank test was performed and the resulting p-value was p = 0.0329.

A larger percentage of young wild-type flies fail when compared to flies lacking Msr

Initial experiments used a wild-type (WT77; MsrA+/+ MsrB+/+) and MsrA/MsrB LOF line (AB113; MsrALOF/LOF MsrBLOF/LOF). Young (5-9 day old) male flies were exposed to hyperthermic conditions (38.5 °C for a maximum of 30 minutes) and the times to failure (absence of movement) were determined. A larger percentage of WT77 individuals were found to fail at any given minute when compared to AB113 ($p = 0.0329$) (Fig. 2).

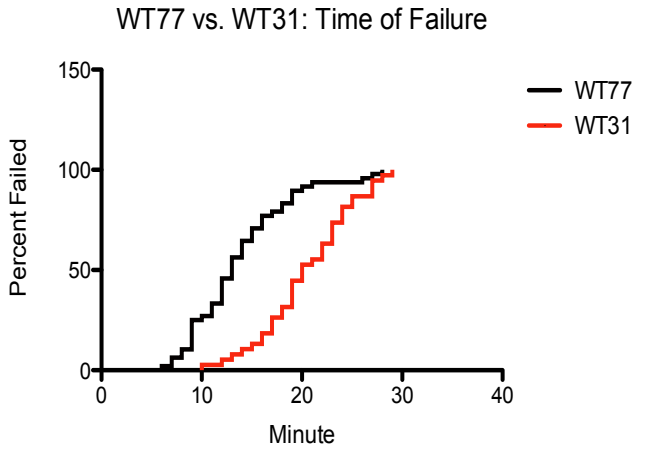

Figure 3 - Failure Times of Two Young Sibling Wild-Type Lines. 5-9 days old WT77 (n=48) and WT31 (n=38) flies were stressed for 30 minutes at 38.5 °C using the Drosophila Activity Monitor (DAM) as described in the Methods. A Log-rank test was performed and the r resulting $p < 0.0001$.

A larger percentage of wild-type flies fail when compared to that of a sibling fly line

The observation that the wild-type line (WT77) failed sooner than the MsrA/MsrB LOF line (AB113) was unexpected. Therefore, we compared WT77 to a sibling wild-type line (WT31). Young (5-9 day old) male flies were exposed to hyperthermic stress (38.5 °C for a maximum of 30 minutes) and the time to failure for each animal was determined using the DAM system. The genotypes used in this experiment consisted of the initial wild-type tested above (WT77; MsrA+/+ MsrB+/+) and a sibling wild-type line (WT31; MsrA+/+ MsrB+/+). The sibling wild-type line was found to have a significantly lower percentage of failing flies over the course of the experiment when compared to the initial wild-type line ($p < 0.0001$) (Fig. 3). Additional experiments using WT77 has shown that it is not a representative wild-type line and it was not used in subsequent experiments (data not shown).

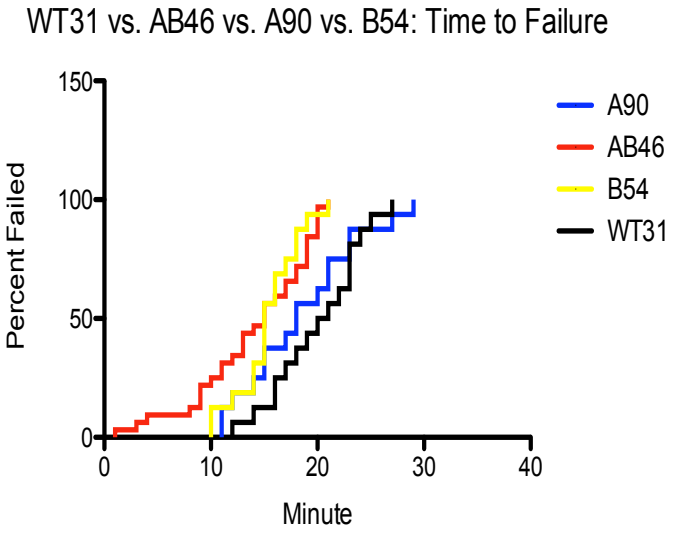

Figure 4 - Failure Times of Young Drosophila Lacking MsrA, MsrB or Both Activities. The following lines were tested for resistance to thermal stress as described in Figure 1 and the Methods: 5-9 days old AB46 (n=32), WT31 (n=16), A90 (n=16), and B54 (n=16) were stressed for 30 minutes at 38.5 C using the Drosophila Activity Monitor (DAM) as described in the Methods. 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.

A larger percentage of younger flies fail in the absence of MsrB regardless of the presence or absence of MsrA

Young (5-9 day old) male flies were exposed to hyperthermic stress (38.5 °C for a maximum of 30 minutes) and the time to failure for each animal was determined using the DAM system. The genotypes used were wild-type (WT31), MsrA LOF (A90) MsrB LOF (B54), and MsrA/MsrB LOF (AB46) (Table 1). A larger percentage of flies lacking MsrB were found to fail at any given minute when compared to wild-type ($p=0.0005$), but the percentage of failing MsrB LOF and MsrA/MsrB LOF flies was similar ($p=0.9400$). Additionally, the percentage of failing MsrA LOF flies was not different from that of wild-type flies ($p=0.7076$) (Fig. 4).

A larger percentage of older flies with no known Msr activity fail when compared to wild-type

To test the effect of age on thermal stress response, old (35-39 day old) male flies were exposed to hyperthermic conditions as described above. This age was select-

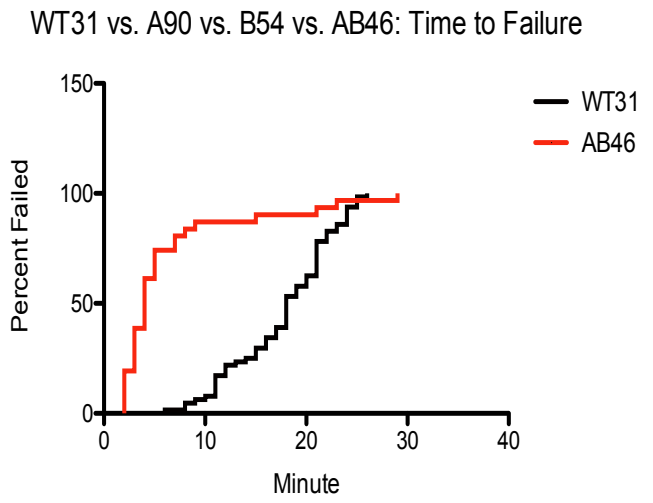

Figure 5 - Failure Times of Old Drosophila Lacking MsrA and MsrB Activity. The following strains were tested for resistance to thermal stress as described in Figure 1 and the Methods except the animals were 35-39 days old: AB46 (n=31) and WT31 (n=48) flies aged between 35 and 39 days and were stressed for 30 minutes at 38.5 °C using the Drosophila Activity Monitor (DAM) as described in the Methods. A Log-rank test was performed and the resulting $p < 0.0001$.

ed since it is close to the maximum lifespan for the lines lacking both MsrA and MsrB (data not shown). The percentage of flies lacking any known Msr activity (AB46) failing under thermal stress was significantly higher than that of wild-type flies ($p<0.0001$; Fig. 5). Additionally, a significantly larger percentage of older animals failed under thermal stress when compared to younger animals (Compare Fig. 4 and 5).

Following preconditioning, an animal's thermal stress response is not affected by the presence or absence of MsrA or MsrB and a shorter time to failure is observed

Published studies have shown that exposure to sub-lethal hyperthermic conditions prior to exposure to heat stress extends the time to failure for wild-type flies [14]. We investigated whether flies with mutant MsrA or MsrB genes would also show an extended time to failure in response to thermal stress when preconditioned. Three genotypes of 5–9 day old male flies were tested: wild-type (WT60), MsrA LOF (A90), and MsrB LOF (B54) (Table 1). We found that wild-type and MsrA/MsrB LOF flies preconditioned to thermal stress failed sooner than flies that had not been preconditioned (Compare Fig. 4 and 6). We did not observe any significant difference between the percentage of failing wild-type, MsrA LOF, and MsrB LOF animals (WT60 vs. A90 $p = 0.6205$; WT60 vs. B54 $p = 0.4913$; A90 vs. B54 $p = 0.7572$) (Fig. 6).

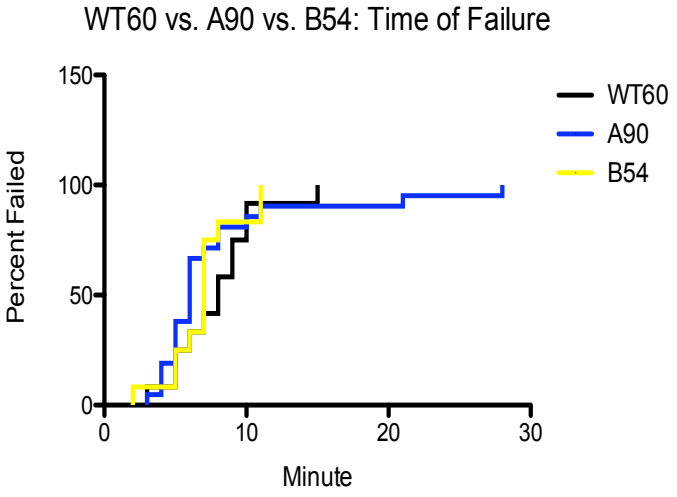

Figure 6 - Failure Times of Young Drosophila Lacking MsrA or MsrB Activity. The following lines were tested for resistance to thermal stress following preconditioning as described in the Methods: WT31 (n=12), A90 (n=21), and B54 (n=12) flies were pre-conditioned for 60 minutes at 36.0 °C. Following pre-conditioning, flies were stressed for 30 minutes at 38.5 °C. A Log-rank test was performed and the resulting p-values were, $p = 0.6205$ for WT60 vs. A90, $p = 0.4913$ for WT60 vs. B54, and $p = 0.7572$ for A90 vs. B54.

Discussion

MsrA and MsrB have been found to reduce the effects of ROS-related conditions [11]. This suggests that Msr might play a role in an organism's response to thermal stress, since hyperthermic exposure also produces ROS [4]. Our findings suggest that the presence of MsrB alone may be sufficient to influence the response of flies to thermal stress.

Initial experiments demonstrated that a larger percentage of wild-type (WT77) animals failed when compared to young (5-9 day old) animals lacking both MsrA and MsrB (AB113) (Fig. 2). These results suggested that the presence of MsrA and MsrB reduces the ability of animals to adequately respond to thermal stress. However, experiments with additional wild-type lines revealed that these results are significantly contrasting with those obtained from utilizing other wild-type lines (data not shown). Thus, we concluded that WT77 may not be representative of a wild-type. As a result, young flies of a sibling line (WT31) were also tested, compared to WT77 and

were found to take longer to fail (Fig. 3), which was consistent with data obtained from other wild-type lines (data not shown). WT77 was not used in any subsequent experiments. As a result, we investigated whether genotypes derived from the sibling wild-type line (WT31) would yield results that were consistent with other experiments being done in our lab. The following sibling lines (WT31; MsrA+/+ MsrB+/+, A90; MsrALOF/LOF MsrB+/+, B54; MsrA+/+ MsrBLOF/LOF, AB46; MsrALOF/LOF MsrBLOF/LOF) were exposed to hyperthermic conditions (38.5 °C for a maximum of 30 minutes) and the times to failure (absence of movement) were recorded. The results of these experiments demonstrated that a higher percentage of organisms lacking both MsrA and MsrB failed under thermal stress when compared to wild-type (Fig. 4). This finding suggests that MsrA and MsrB may play a role in an animal's response to thermal stress. Interestingly, we also found that a larger percentage of flies lacking only MsrB were found to fail under thermal stress when compared to wild-type, while the percentage that failed was approximately the same as that of flies lacking both MsrA and MsrB. In contrast, approximately the same percentage of flies lacking only MsrA failed when compared to wild-type. This suggests that MsrB may play a more important role in an organism's response to thermal stress than MsrA.

The initial experiments showed that WT77 failed from thermal stress sooner than AB113. This unexpected finding is probably due to unknown genetic mutations in WT77. Additional experiments using WT31, WT 60 and another wild-type sibling line not used in these experiments also found that WT77 shows significant differences when compared to the other wild-type lines. WT60 and WT31 also benefited from numerous backcrosses to a lab wild-type line (YW) to provide a more consistent genetic background.

In order to test the effect of age on thermal stress response, we exposed flies aged between 35–39 days old to hyperthermic conditions and found that a higher percentage of organisms lacking both MsrA and MsrB failed when compared to wild-type (Fig. 5). However, it was also observed that a significantly larger percentage of older animals failed under thermal stress when compared to younger animals (Fig. 4 and 5). This further suggests that MsrA and MsrB may play a role in an organism's thermal stress response. In the future, we will test the thermal stress response of old flies lacking either MsrA or MsrB. We anticipate that a higher percentage of flies lacking MsrB will fail sooner under hyperthermic conditions when compared to wild-type, while approximately the same percentage of MsrB LOF and MsrA/MsrB LOF will fail. In addition, we expect that flies lacking MsrA will fail at a rate that is not significantly different than the wild-type. If this hypothesis is correct, this will further suggest the importance of MsrB in response to thermal stress. We can explore this hypothesis regarding the significance of MsrB in response to thermal stress by performing transgenic rescue experiments on all of our mutant genotypes (MsrA LOF, MsrB LOF, and MsrA/MsrB LOF). If MsrB does play a more important role in an animal's thermal stress response, then transgenic expression of MsrA in animals lacking any Msr activity should not significantly slow the time of failure. However, similar transgenic rescue experiments using MsrB should show increased protection from failure induced by thermal stress.

Thermal stress preconditioning has been shown to provide neuronal protection to animals during thermal stress exposure by the upregulation of heat shock proteins, particularly Hsp70 [13, 14]. As a result, we investigated whether thermal stress preconditioning would increase the time to failure of MsrA LOF, MsrB LOF, and MsrA/MsrB LOF mutants. However, our findings suggest that preconditioning may have a detrimental effect on the thermal stress response of these animals. These experiments are being repeated because the protective effect of preconditioning is a well-documented phenomenon [14]. However, identification of the conditions necessary to obtain this effect is very challenging since a slight variation in temperature can overstress the animal, resulting in a detrimental rather than protective effect. As a result, preconditioning experiments using only the wild-type lines are underway at the time of this writing in order to determine the conditions that are required so that the wild-type strain behaves in a predictable manner compared to that of the literature results. Then, under those conditions, we can compare the various Msr mutant lines.

Although specific biochemical functions are well established for the Msr enzymes, there are many functions of these proteins in various physiological processes that are not yet clear. From this study we have observed that the antioxidant, MsrB, may play a role that is distinct from that of MsrA in aiding an organism's response to thermal stress. The results of these experiments as well as other experiments currently underway in the lab suggest that MsrA and MsrB have important biochemical functions that are unknown at this time.

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