Methionine Sulfoxide Reductase Expression in Response to Anoxic Stress Conditions in D. *melanogaster*

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Anoxia is condition of oxygen deprivation. Thousands of human deaths each year are attributed to events of induced anoxic conditions – ischemic strokes and heart attacks being the most numerous. But whereas mammals poorly tolerate anoxic stress, D. melanogaster endures hours of anoxia with no apparent problems. In response to anoxia, flies suppress overall energy levels and enter protective comma - spreading depression. Animals recover from comma after being returned to normal oxygen levels. Period following reintroduction of oxygen is characterized by overabundance of Reactive Oxygen Species (ROS), which oxidize vital molecules in cells. Methionine is exceptionally susceptible to oxidation by ROS, but can be catalytically restored by enzyme Methionine Sulfoxide Reductase (Msr). Currently, little is known about the relationship between Msr activity and recovery from anoxic stress in Drosophila. Expression of Msr genes in response to anoxia is the subject of this study. We use anoxia chamber to induce comma in flies, followed by Drosophila Activity Monitor to record recovery times. Our results show, that single mutant knockout flies do not take significantly longer than wildtype flies to recover from spreading depression. However, double mutants take significantly longer to recover and a greater number of Msr-deficient flies die as a result of anoxia. Failure to recover from anoxia becomes more pronounced as animals approach senescence. Protein oxidation levels are significantly increased in post-anoxic Msr double-mutant flies in comparison to wildtype animals. These studies offer insight into the role of oxidative damage during reperfusion period following cardiac stroke.

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BACKGROUND AND SIGNIFICANCE

Oxygen plays a dual role in all life: in one regard, this molecule is critical for the sustenance of life, while conversely, it can be a highly toxic substance. Nonetheless, every aerobic organism requires oxygen for life, growth, and development. But as a shortcoming of extensive oxygen-use, all organisms have to deal with ROS (Reactive Oxygen Species). Oxidative stress presents an imbalance between ROS and an organism's ability to detoxify them and repair the damage. Lipids, nucleic acids and proteins are sensitive to damage by ROS (Radak 307). Most protein damage has adverse consequences for its structure and function, and the accumulation of oxidized proteins may promote a wide range of human pathologies (Davies 94). ROS-induced damage increases with aging, which accelerates the progression of neurodegenerative diseases. Due to the high rate of metabolism, brain tissue is specifically sensitive to oxidative stress. It is well-established that accumulation of ROS and misfolded proteins in neuronal tissue is strongly-linked to the onset of a variety of neurodegenerative diseases, including age-related disorders, such

as Parkinson's disease (Jellinger 1114). In this respect studying redox homeostasis is important in understanding aging and the onset of neurodegenerative diseases (Labunskyy 1369).

Anoxia or oxygen deprivation is one of the major stressors for most organisms, and is characterized by the high rate of redox reactions. Anoxia can compromise viability of the tissues subjected to inadequate oxygen supply and thereby threaten the life of the organism as a whole. In humans, Anoxic conditions such as ischemia in neuronal or cardiac tissues leading to stroke and heart attacks are one of the leading causes of death worldwide (Woorons 27). Although extremely damaging in humans, anoxia can be tolerated relatively well by some animals, specifically turtles and fruit flies. The common fruit fly, Drosophila *melanogaster*, has evolved a special mechanism – *spreading depression*, which allows it to withstand hours of anoxia (Stadtman 235). Spreading depression can be characterized by an abrupt increase in [K+] in the neuropil, which leads to the arrest of Central Nervous System function during anoxia. In this way, animals protect themselves during certain environmental occurrences, such as flash floods and monsoons. Recovery from anoxia occurs very quickly once normoxic conditions are reestablished (Armstrong 8229). Both humans and the D. *melanogaster* experience the greatest anoxic stress during the period of re-oxygenation, when oxygen is introduced back into the cells, leading to an outburst of ROS that rapidly damages cellular components (Blokhina 181).

Under normal conditions, ROS can be produced within the organism by a variety of processes, ranging from highly controlled radicals, such as oxygen species produced by neutrophils that are used in host defense, to the formation of oxidants, such as such as peroxide and superoxide which are produced in cells as by-products of cellular metabolism (Davies 95). Formation of most ROS occurs in mitochondria, where the majority of the oxygen is utilized within most cells (Boveris 707). A series of oxidative reactions accomplished by protein complexes on the inner membrane of the mitochondria produce energy for the cellular metabolism in the form of the ATP. Leakage of electrons from these complexes promote single-electron reduction of oxygen to form superoxide – a precursor to most ROS. Further dismutation of superoxide results in hydrogen peroxide production (Krivoruchko 187). Consequently, interactions between hydrogen peroxide and superoxide produce hydroxyl radicals

(either through Haber-Weiss reaction or Fenton reaction) (Hermes-Lima 322).The latter are key players in cysteine (Cys) and methionine (Met) oxidation – two essential amino-acids with an extreme degree of vulnerability to oxidation (Drazic 1369). Recent studies confirm remarkable similarity between the functions of Cys and Met – most importantly, both are subject to reversible oxidation and reduction (Kim 901).

Met is readily oxidized by ROS to methionine sulfoxide (MetO) and consequently reduced back to its functional form by methionine sulfoxide reductases (Msr) which is universally conserved throughout aerobic organisms. Oxidizing Met to MetO renders two forms - S and R, which are diastereomers of one another. Thus, organisms have two types of Msr: Msr A, encoded by the *MsrA* gene that specifically reduces the S-form of MetO, as well as MsrB, encoded by *MsrB* gene that specifically reduces the R-form of MetO (Kim 901). Currently it is accepted that both MsrA and MsrB are effective endogenous antioxidants in reducing MetO to its functional form – Met, as well as scavenging ROS before they have a chance to damage the cells (Stadtman 235). Proposed functions of these enzymes involve repair of oxidized proteins, regulation of protein function, and elimination of oxidants through reversible formation of methionine sulfoxides (Levine 304). In particular, MsrB proteins were identified and characterized in many organisms including bacteria (Grimaud 48916), yeast (Kryukov 4246), fruit fly (Kumar 37528), and mammals (Jung 93). Thus far, two mammalian MsrB proteins were identified: selenocysteine (Sec)-containing protein or selenoprotein R and its homolog CBS-1, in which Cys is present in place of Sec (Kim 1058). Although Msr enzymes have been extensively studied in recent years, *Msr* gene activity and its relation to anoxia tolerance has remained largely uncharacterized.

Using *D. melanogaster* as a model, we began conducting studies on *Msr* with overexpression of genes involved in anoxia recovery. The results of these studies have been inconclusive and no significant phenotype was found (Ruan 2752 and Shchedrina 430). However, with classical genetic mutations – particularly gene deletions– we could better assess the activity of *Msr* genes. A series of such mutations have been created in order to produce strains of flies with *MsrA* loss-of-function (LOF) allele, *MsrB* LOF allele, and *MsrA/MsrB* LOF alleles. At this time it is possible to use further molecular testing, such as Western blotting and Protein carbonyl formation to

understand how anoxic conditions effect the expression and activity of *Msr* gene family and if there is a link between the absence of functional *Msr* genes and anoxia tolerance in *D. melanogaster*. Previous studies have shown that *MsrA/MsrB* double LOF flies do not recover as well from anoxic stress conditions in comparison to the wildtype individuals (Howard 5). Further studies confirmed former findings in regards to MsrA/MsrB LOF but also showed that single LOF *MsrA* and *MsrB* mutants experience a difference in recovery time during middle age, but not near senescence (Suthakaran 7).

The objective of this project is to establish the relationship between anoxic conditions and the expression of the *Msr* genes in the model organism – *D. melanogaster*. By using *MsrA* and *MsrB* deletion lines of the flies at different stages of their lifespan, we foundthat *Msr* single LOF genetic mutants are more susceptible to oxidative stress induced by anoxic conditions. We hypothesize that the lack of *Msr* gene activity will compromise *D. melanogaster's* ability to recover from anoxic stress conditions when both of the *Msr*-encoding genes are deleted from the genome of the organism, leading to the accumulation of ROS and damaged proteins in cells. The results of these experiments have the potential to explain how oxidative stress induced by anoxic conditions contribute to aging and the onset of age-related pathologies. Further, these findings suggest a possible mechanism by which oxidative stress response pathways are being regulated. The results of this study will allow for the development of novel *Msr*-targeted drugs, which could become therapeutic tools to slow the progression of age-related diseases, as well as hypoxia-induced tissue damage.

MATERIALS AND METHODS

Genetic lines and experimental design.

For this study four genotypes of D. *melanogaster* were used: wildtype line (WT31), *MsrA* LOF line (A90), *MsrB* LOF line (B54), and *MsrAB* LOF (AB46). These genetic lines were used to calculate % recovery from the anoxic stress conditions, average recovery time, anoxia induction, as well as measurement of protein carbonyl levels in post-anoxic flies.

Examining the effect of anoxic stress conditions in single and double

mutant D. melanogaster – Anoxia Chamber Experiments.

Flies of each genotype were obtained from a stock maintained in lab. To produce an F1 generation, flies were raised in separate stock bottles on standard cornmeal agar media (Genessee) for 10 days at 25°C, then P (Parental) generation was carefully cleared with CO_2 gas to anesthetize and take them out of the bottles. This marked day 1 of the F1 generation. After five more days in the incubator (25°C), male flies of F1 generation were selected, their age was determined and female flies were sacrificed. These flies were placed in vials (10-15 flies per each vial) and aged to 5-10 days, 20-25 days, 30-35 days, 40-45 days (early senescence for the double mutant flies – AB46); 50-55 days and 60-65 days (senescence). Flies were maintained in the vials, and placed to the fresh vial every two weeks. Upon approaching late middle age senescence, vials were replaced every week to ensure survivability. One group of flies was used as a control line and exposed to normal level of atmospheric oxygen (normoxia). Remaining groups of flies were exposed to anoxic an environment for 1 hour, immediately after exposure the flies were loaded into a Drosophila Activity Monitor (DAM System) to record their recovery times. The recording was continuous for 5 hours after re-oxygenation.

The baseline movement of each age group of flies was first analyzed with DAM (Drosophila Activity Monitor) for 10 minutes to calibrate the system. DAM System consists of MAN2 gas distribution manifold with 32 holes, each one having a transparent tube with small perforations on the sides to allow for the gases to flow freely in and out of the tubes. Single flies were placed in each DAM tube and were immediately covered to begin acclimation. Tubes were linked to the DAM System with an infrared beam moving down the center to record the movement of the animal. After the initial DAM run, flies were positioned (remaining inside the tubes) in the anoxic tank, where they were subjected to 1 hour of anoxic stress with continuous flow of 100% nitrogen gas. Nitrogen gas is flowing under pressure and effectively displaces air originally present in the tank introducing anoxic conditions. The countdown began at the time when all of the flies fell into coma (spreading depression). The tank used for anoxia experiments was a sealed glass chamber (Anoxia Chamber) with tubing connected to 100%

Nitrogen gas that was constantly flowing inside to ensure complete absence of oxygen.

Flies were taken back to the DAM System after spending one hour in the chamber. DAM holders with flies were connected to the computer with the DAM software. The system was set up to record flies' activity every minute. This is done through the infrared beam of light that is triggered and is being displaced by flies' movement. DAM counted each beam displacement as a movement. DAM was continuously recording beam displacements over a period of 5 hours to determine the recovery time, % recovery was registered by the DAM computer software.

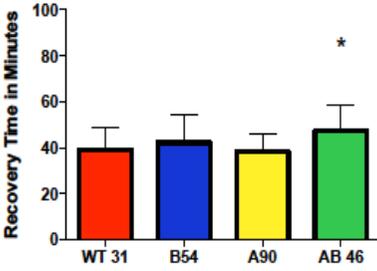## Examining the potential for MsrA and/or MsrB to be induced in anoxic conditions – Western Blot Analysis.

Western Blot Assay was employed to determine, if *Msr* enzymes leveel was upregulatedas a result of anoxic stress. Wildtype (WT31), Msr single mutants (A90; B54) , and Msr double mutants (AB46) at 5-10 days old were exposed to normoxic conditions (normal atmospheric oxygen level) post-anoxic conditions (flies just removed from the anoxic tank) and post-recuperation conditions (5 hours post-anoxic stress experience). 20 flies per genotype were frozen at -80°C after the exposure to the described conditions (post-anoxic stress flies were frozen right away following their removal from the anoxia tank). Upon freezing, each group of 20 flies was homogenized (with 2.5mM Tris-HCl/150mM NaCl buffer) and centrifuged. Afterwards, the supernatant was removed, from which the protein sample was analyzed. Following this procedure, Bradford assay was used to determine protein sample concentrations, as compared to the standard. The Bradford protein assay is a spectroscopic analytical procedure used to measure the concentration of protein in a solution. It is dependent on the amino acid composition of the measured protein. Bradford assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. Samples for the Western blotting each contained 20ug of protein. In preparation for the Western blotting, we made our own PAGE gels (15%), utilizing the standard lab protocol. We used 30ug of each of the protein sample and compared it to a ladder Novex SeeBlue. Gels were run for 1.5-2 hours on 140V. Utilizing semi-dry blotting method, samples were transferred at 15V for an hour. As one of the final steps, gels were blocked with 5% dry-milk solution and TTBS (Tris-buffered saline with 0.1% tween 20) and left overnight in the refrigerator. Gels then were washed and subjected to primary MsrB rabbit polyclonal antibody. GoatXRabbit secondary antibody detection system (Millipore) and Biorad was used to visualize the MsrB protein. GeneSys software (without light or filter) detected the detection reagent mentioned earlier applied to our Western blots. ImageJ was used to analyze the images.
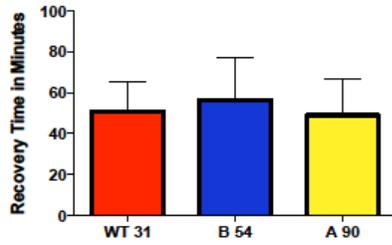
Determining the protein oxidation levels in Msr-deficient flies in comparison to the wildtype post-anoxia.

Two genotypes were selected for this study – wildtype and Msr double mutant flies. Flies were first subjected to 1 Hour of anoxia (Anoxia Chamber – 100% Nitrogen gas), and then left untreated for another Hour post-anoxic. Samples were kept in -80 °C. Protein was extracted from approximately 100 whole flies (per genotype / per treatment) in 1.2 ml of 2.5mM Tris-HCl/150mM NaCl buffer. Samples were pestle homogenized and centrifuged at 16,000 x g for 20 minutes at 4°C to obtain 1 ml of the supernatant. The supernatant was divided into two 500 µl samples. Each of these samples were treated with equal volumes of 20% trichloroacetic acid (TCA) vortexed, incubated on ice for 10 minutes, and centrifuged at 2,500 x g for 5 minutes at 4°C. The supernatant was discarded and previously-described process was repeated again. Next the supernatant was discarded and one portion of the sample was resuspended in 600 µl 10mM 2,4-Dinitrophenylydrozine in 2M HCl, while the other portion was resuspended in 600 µl 2M HCl. The samples were sonicated (Misonix XL2000 Series) for 5 seconds and kept in the dark at room temperature for 1 hour, vortexing every 15 minutes. After incubation, another TCA precipitation was performed. 600 µl of 20% TCA was added to the samples, and were incubated on ice for 10 minutes and then centrifuged at 2,500 x g for 10 minutes at 4°C. The supernatant was discarded and the protein pellet was washed with 1 ml of 1:1 ethanol/ethyl acetate. The sample was thensonicated and centrifuged at 1000 x g for 10 minutes at room temperature.
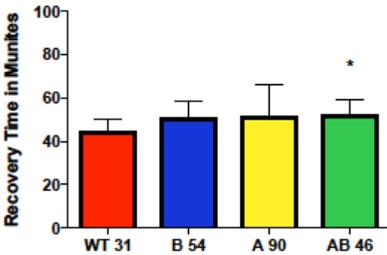
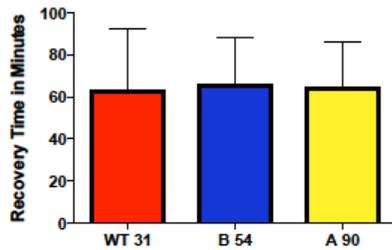## 10-15 Days Old Average Recovery Time

25-30 Days Old Average Recovery Time

40-45 Days Old Average Recovery Time

Fig 1. Recovery times of wildtype, MsrA LOF, MsrB LOF, and Msr A/B LOF flies ages 10-15, 25-40, and 40-45 days old. (Error bars represent SEM; * indicates p-value ≤ 0.05 – analyzed with 1-way ANOVA; ** indicates p-value ≤ 0.01 – analyzed with 1-way ANOVA; data calculated from three runs' average (32 flies per run/per genotype).

50-55 Days Old Average Recovery Time
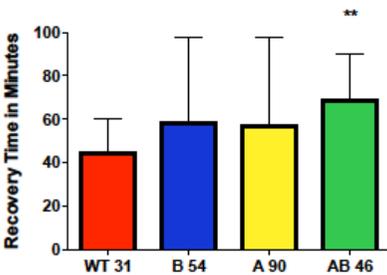

55-60 Days Old Average Recovery Time

Fig 2. Recovery times of wildtype, MsrA LOF, and MsrB LOF flies aged 50-55 and 55-60 days old. No significance in recovery times is observed between single mutant flies and wildtype flies approaching senescence. (Error bars represent SEM; * indicates p-value ≤ 0.05 – analyzed with 1-way ANOVA).

This wash-sonication-centrifugation protocol was repeated three more times. The protein pellet was then resuspended in 650 μl of 6M Guanidine- HCl. Following this, the samples were vortexed and incubated at 37°C for 5 minutes to allow the pellet to fully go into solution. The samples were centrifuged at 1000 x g for 15 minutes at room temperature and the majority of the supernatant was removed without touching the bottom or sides of the microcentrifuge tube. The absorbance of both the DNPH treated and HCl control samples was read at 370nm using multi-place reader. A BSA sample was run through this process as well to ensure that the procedure was working properly. A Bradford assay was used to normalize protein concentrations. The Bradford assay was completed using Bio Rad's Protein Assay Dye Reagent (Catalog #500-0006) and the SpectraMax plate reader (Molecular Devices) with 39 bovine serum albumin (BSA) used to create a standard curve.

Statistical Analysis

All results were evaluated by the Prism Software, where comparisons were analyzed with t-test and 1-way ANOVA (p-value of ≤ 0.05 were deemed significant).

RESULTS

Anoxia assays were done with 10-15, 25-30, and 40-45 day old single mutant, double mutant, and wildtype flies. There were no significant difference in the recovery times between Msr single mutant and wildtype flies. Whereas Msr double mutant flies (AB 46) take significantly longer to recover than wildtype (WT 31) or Msr single mutant LOF (B 54 and A 90) for all three age groups.

There was a significant difference in observed recovery times between Wildtype and Msr A/B LOF, whereas MsrA LOF and MsrB LOF do not display a significant difference in recovery in comparison to the Wildtype for all age groups (Fig 2). Msr single mutant LOF flies (B 54 and A 90) did not show a difference in recovery times in comparison to the wildtype flies (WT 31) upon approaching senescence (Fig 2).

Double mutants could not be tested as all the flies died at around 50 days of age. Furthermore, Msr double mutant flies (AB 46) have significantly lower percent survival than wildtype flies (WT 31)

immediately after exposure to anoxic stress conditions (Fig 3).

Msr Double Mutant flies (AB 46) have significantly higher levels of protein carbonyls 1 Hour post-anoxia in comparison to the wildtype, which is indicative of higher levels of protein oxidation in the organism (Fig 4).

DISCUSSION

Results of Anoxia Chamber Experiments confirmed the hypothesis that recovery from anoxia is affected by the presence of *Msr* genes – Double Mutant flies displayed significant increase in recovery time in comparison to the Wildtype for all three age groups (10-15, 20-25, and 40-45 days old). Double Mutant flies also showed significantly lower percentages of survival immediately following anoxic stress in comparison to the Wildtype. This could be explained with the over-accumulation of ROS during the re-oxygenation period following anoxic stress, which causes rapid and poorly-controlled oxidation of methionine (and other molecules) in cells. Effects of ROS oxidation become more pronounced as flies approach senescence – this suggests overall protective role of Msr in an animal's ability to withstand anoxic stress and its potential contribution to aging processes. Single Mutant flies do not show any significance in either recovery times, nor in ability to survive anoxic stress. This may suggest that a third component is present in cells – epimerase of Msr which might help to promote the conversion of *MsrA* to *MsrB* (and vice versa).

To drive our inquiry forward, we decided to look further into the results of Single Mutant vs Wildtype recovery and understand why Single Mutant flies don't show significant differences in recovery and survival from anoxia. As an experimental assay of choice, we ran Western Blot to ascertain the hypothesis that *MsrA* will be overexpressed in the absence of *MsrB*, and *MsrB* will be overexpressed in the absence of *MsrA*. This overexpression pattern should be consistent with the observed behavior of *MsrA* LOF and *MsrB* LOF, which don't differ in their overall tolerance of anoxia in comparison to the Wildtype flies, suggesting the presence of the compensatory mechanism. Msr enzymes act in stereospecific manner, therefore the induction of either form of Msr in a Single Mutant fly alone would not suffice to explain the results of anoxia chamber experiments. This is why we propose the presence of an epimerase, which interconverts one
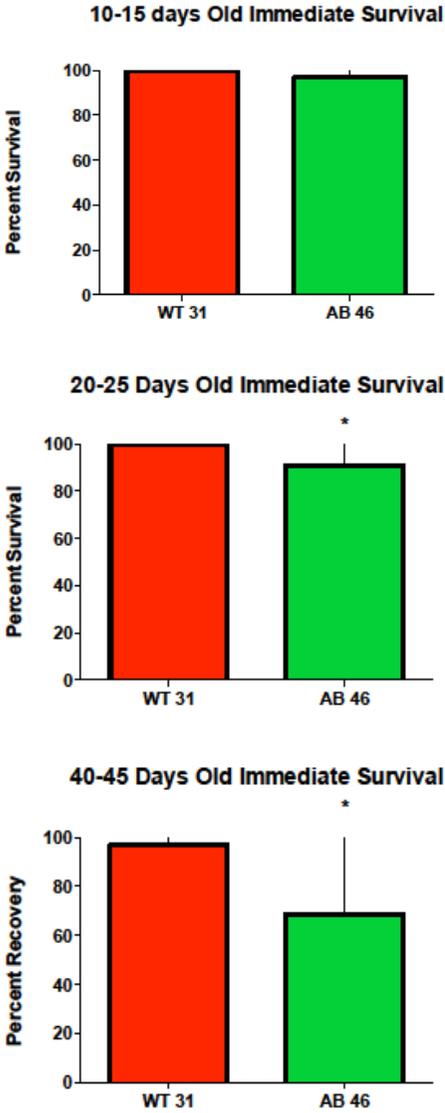
10-15 days Old Immediate Survival

20-25 Days Old Immediate Survival

40-45 Days Old Immediate Survival

Protein Carbonyl Formation

Fig. 4 **Protein carbonyls' formation are affected by loss of Msr in post-anoxic Msr double mutant flies.** Protein extracts from each genotype were treated with 2,4-Dinitrophenylhydrozine and analyzed via spectrophotometry. Double Mutant Flies (AB 46) have significantly higher levels of protein carbonyls 1 Hour post-anoxia in comparison to the Wildtype (WT 31). (Error bars represent SEM; * indicates p-value ≤ 0.05 – analyzed with 1-way ANOVA)

Fig 3. **Percent survival of Wildtype and Msr A/B LOF flies.** Both genotypes tend to survive anoxic stress relatively well in their young age. Although, as flies get older, they become more susceptible to oxidative damage, and fail to withstand anoxia. Failure to survive anoxia becomes more pronounced as flies approach senescence. (Error bars represent SEM; * indicates p-value ≤ 05 – analyzed with 1-way ANOVA). Wildtype flies do survive until 60 days but Double Mutants die at or before 50 days old.
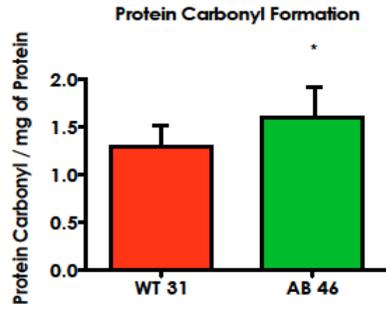
form of Msr into another. This hypothesis is yet to be tested further.

Additional experiments are currently being carried out in order to better understand the mechanism of Msr activity and the basis of the spreading depression. It is well-understood that mitochondria are both the source and target of the vast majority of ROS. Oxidative damage to the methionine residues in essential mitochondrial proteins could lead to mitochondrial dysfunction. If an organism lacks its Msr repair system, these problems should be exacerbated. Given our previous observation of the increased recovery times from spreading depression in Msr double mutants, and their lower survivability immediately post-anoxia, it becomes rationalized to analyze levels of protein oxidation in post-anoxic flies.

It is widely accepted that almost all kinds of amino acid residues in proteins can be oxidized by ROS (Stadtman 1252). Oxidation of some amino acid residues (methionine being one of the most-readily oxidized) cam lead to the formation of carbonyl derivatives and they are considered to be the most significant products of protein oxidation by free

radicals (Amici 3343). Thus, presence of the carbonyl proteins in samples and tissues can be accepted as a marker for oxidative stress (Chevion 34).

The most recent analysis to determine protein oxidation levels in flies post-anoxia revealed that in the absence of Msr-encoding genes (*Msr*A/*Msr*B LOF flies) the levels of protein carbonyls following the recovery from spreading depression is significantly higher in comparison to the wildtype flies. These results suggest a greater level of oxidative damage experienced by mutant flies after anoxia (during reperfusion period), which is consistent with the previously-postulated hypothesis rationalizing the overabundance of ROS in cells and their action upon methionine residues in proteins. Based on these results we may conclude that Msr is indeed involved in protection of flies post-anoxia from the consequences of reperfusion. Further studies are still needed to include both pre- and post-anoxic fly groups for comparison. These studies have an incredible potential to help in discovering novel therapeutic tools that may be efficient in protection against damaging effects of induced anoxic environments in humans, as in the case of strokes and myocardial infarction.

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