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Early mitochondrial dysfunction leads to altered redox chemistry underlying pathogenesis of TPI deficiency

Stacy L. Hrizo^{1,2,*}, Isaac J. Fisher², Daniel R. Long², Joshua A. Hutton², Zhaohui Liu¹, and Michael J. Palladino¹

¹Department of Pharmacology & Chemical Biology, University of Pittsburgh Medical School, Pittsburgh, PA 15261

²Department of Biology, Slippery Rock University of Pennsylvania, Slippery Rock, PA 16057

Abstract

Triose phosphate isomerase (TPI) is responsible for the interconversion of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate in glycolysis. Point mutations in this gene are associated with a glycolytic enzymopathy called TPI deficiency. This study utilizes a *Drosophila melanogaster* model of TPI deficiency; *TPF^{sugarkill}* is a mutant allele with a missense mutation (M80T) that causes phenotypes similar to human TPI deficiency. In this study, the redox status of *TPF^{sugarkill}* flies was examined and manipulated to provide insight into the pathogenesis of this disease. Our data show that *TPF^{sugarkill}* animals exhibit higher levels of the oxidized forms of NAD⁺, NADP⁺ and glutathione in an age-dependent manner. Additionally, we demonstrate that mitochondrial redox state is significantly more oxidized in *TPF^{sugarkill}* animals. We hypothesized that *TPF^{sugarkill}* animals may be more sensitive to oxidative stress and that this may underlie the progressive nature of disease pathogenesis. The effect of oxidizing and reducing stressors on behavioral phenotypes of the *TPF^{sugarkill}* animals was tested. As predicted, oxidative stress worsened these phenotypes. Importantly, we discovered that reducing stress improved the behavioral and longevity phenotypes of the mutant organism without having an effect on *TPF^{sugarkill}* protein levels. Overall, these data suggest that reduced activity of TPI leads to an oxidized redox state in these mutants and that the alleviation of this stress using reducing compounds can improve the mutant phenotypes.

Keywords

Oxidative Stress; Redox; TPI deficiency; Triosephosphate Isomerase; Glycolytic enzymopathy; *Drosophila*

Introduction

Triose phosphate isomerase (TPI) is responsible for the conversion of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate in glycolysis. Point mutations in *TPI* are associated with a glycolytic enzymopathy called TPI deficiency in humans (Ahmed et al.,

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*Corresponding Author: Stacy L. Hrizo, 300J Vincent Science Center 1 Morrow Way, Slippery Rock, PA 16057, Phone: 724-738-4561. Fax: 724-738-4782. stacy.hrizo@sru.edu.

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2003; Arya et al., 1997; Celotto et al., 2006; Karg et al., 2000; Olah et al., 2002; Orosz et al., 2001; Orosz et al., 2006; Schneider et al., 1965). Individuals with TPI deficiency exhibit age dependent neurodegeneration, hemolytic anemia and susceptibility to infection that ultimately results in a significantly shortened lifespan. A previously identified missense mutation (M80T) in *Drosophila melanogaster*, referred to as *TPF^{sugarkill}*, leads to progressive neurodegeneration, shortened lifespan and conditional paralysis resulting from mechanical stress and temperature (Celotto et al., 2006; Hrizo and Palladino, 2010; Seigle et al., 2008). Data collected on the *TPF^{sugarkill}* flies suggest that there is similarity between the pathogenesis of TPI deficiency in *Drosophila* and humans, for example the temperature sensitivity observed in the fly model correlates well with research on human TPI alleles which demonstrated that some TPI deficiency mutations result in heat sensitive enzymes (Chang et al., 1993; Daar et al., 1986; Orosz et al., 2001). In addition, the *TPF^{sugarkill}* allele is fully recessive as has also been observed in humans with TPI alleles that result in TPI deficiency (Celotto et al., 2006). Furthermore, the *TPF^{sugarkill}* allele does not result in bioenergetic impairment in the flies, which is consistent with the normal ATP levels observed in TPI deficient patients and in other neurodegenerative *Drosophila* with mutant *TPI* alleles (Eber et al., 1991; Gnerer et al., 2006). Therefore, this *TPF^{sugarkill}* fly model has been used as an amenable model to study the poorly understood pathology of TPI deficiency

In addition to the focus on enzyme activity and biogenesis, previous studies of *TPF^{sugarkill}* flies demonstrated that the mutant protein is unstable and degraded by the proteasome in a chaperone-dependent manner (Hrizo and Palladino, 2010; Seigle et al., 2008). Increasing *TPF^{sugarkill}* protein levels results in improvements in the behavioral phenotypes, but the rescue of the mutant phenotypes was incomplete suggesting other factors may influence the pathogenesis of TPI deficiency in *TPF^{sugarkill}* animals. One factor commonly observed in pathogenesis of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, is oxidative stress (Alberio et al., 2012; Collins and Neafsey, 2012; de la Torre, 2011; Fischer et al., 2012; Guix et al., 2009; Kulic et al., 2011). In many cases, an increase in oxidative stress correlates with neurodegeneration and decreased lifespan.

The metabolism of glucose and other sugars via aerobic metabolic pathways results in the production high-energy electrons that are often utilized in the production of ATP through oxidative phosphorylation. The major carrier of these high-energy electrons is nicotinamide adenine dinucleotide (NAD⁺). This molecule accepts electrons produced through the metabolism of sugar and is converted to the reduced form of the molecule (NADH) which then shuttles the electrons to NADH dehydrogenase or complex I of the electron transport chain. NAD⁺ is critical in the formation of another important redox molecule nicotinamide adenine dinucleotide phosphate (NADP⁺) (Lerner et al., 2001; Pollak et al., 2007b; Ying, 2008). During cellular biosynthesis reactants are typically more oxidized than the products; therefore electron donors are necessary to support these chemical reactions. The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) often fulfills this critical role (Pollak et al., 2007a; Ying, 2008). A third important redox molecule in the cell is glutathione. Glutathione is a small hydrophobic tripeptide that alternates between its oxidized (GSSG) and reduced (GSH) forms. The coupling of oxidation and reduction of glutathione is important to many cellular processes such as protein folding and maintaining a reducing environment within the mitochondria. GSSG relies on NADPH to be reduced back to GSH in its redox cycle in the cell (Ying, 2008). As a result, the redox status of NAD⁺, NADP⁺ and glutathione are all intimately linked. The redox ratios of these molecules affect cellular processes from transcription, protein modification, mitochondrial function, and protein folding. Overall, the flow of electrons to and from NAD⁺, NADP⁺ and glutathione are critical to cellular health and the ratios of these molecules are a measure of the metabolic activity (Belenky et al., 2007; Pollak et al., 2007a; Ying, 2008).

As metabolic pathways, including glycolysis, are involved in producing electrons that are carried by NAD^+ and NADP^+ , perhaps the redox status of *TPF^{sugarkill}* animals has been altered due to changes in metabolic activity in the animals. *Drosophila* often exhibit behavioral changes when challenged with oxidative stress. These responses include exacerbated startle response, increased locomotor activity, and decreased lifespan (Grover et al., 2009; Weber et al., 2012; Zhong et al., 1998). Therefore, it is expected that *TPF^{sugarkill}* flies may exhibit altered behavior in response to changes in redox status. These data will provide new insight into the role of oxidative stress due to altered metabolism on progressive neurodegeneration in a multi-cellular animal model with a complex nervous system.

In this study, the redox status of flies with the *TPF^{sugarkill}* mutant allele was examined to better understand the role of metabolism on redox state and organism health. It was hypothesized that *TPF^{sugarkill}* animals would have altered sensitivity to oxidative stress due to changes in metabolic activity. In order to test this hypothesis, the ratios of the reduced and oxidized forms of the nicotinamide nucleotides and glutathione were examined in head extracts. It was determined that *TPF^{sugarkill}* animals exhibit higher levels of the oxidized forms of these molecules and the increase in the oxidative stress occurs in an age dependent manner. Furthermore, when the mitochondria in *TPF^{sugarkill}* animals were examined and found to have increased oxidative stress suggesting that altered metabolism may be contributing to the change in redox status. In addition, the effect of oxidizing and reducing stressors on the behavioral phenotypes of the *TPF^{sugarkill}* animals was tested. It was found that reducing stress improves the phenotypes of the mutant organism while oxidative stress worsens these phenotypes. Finally, as all previous examples of behavioral improvement correlated with increase *TPI^{sugarkill}* stability, we examined the stability of mutant protein when animals were treated with oxidizing and reducing stressors and determined that *TPI^{sugarkill}* protein levels were unaffected by redox stressors. Overall, these data suggest that altered metabolism in *TPF^{sugarkill}* flies causes an imbalance in redox maintenance that contributes to *TPI* deficiency pathogenesis in *D. melanogaster*.

Methods and Materials

Drosophila Stocks and Culture

Bloomington stock media was used to maintain the fly cultures (Genessee Scientific). Flies were maintained at 23°C, unless otherwise noted. The *TPF^{sugarkill}* mutation is maintained as a homozygous viable *ve e sgk* strain (Celotto et al., 2006). Wildtype controls are *ve e* homozygotes unless otherwise noted. For measurement of redox molecules the JS10 *TPI* deletion strain was used to exacerbate the phenotype and is described in (Celotto et al., 2006). For lifespan analyses, *TPF^{sugarkill}* homozygotes lacking *ebony* were utilized with the corresponding Canton S wildtype control.

Pharmacology

Hydrogen peroxide (Sigma) and beta-mercaptoethanol (Sigma) were diluted in distilled water to the required concentrations and used to hydrate instant *Drosophila* media (Formula 4-24, Carolina Biological Supply). For hydrogen peroxide 0.5, 5, and 162.5mM concentrations were used. Beta-mercaptoethanol was used at 0.005, 0.05, and 4mM concentrations. Animals were maintained on media containing hydrogen peroxide or beta-mercaptoethanol for the indicated period of time. For long-term analyses, stress-sensitive paralysis and life span, fresh media and drug/compound were provided every day.

Locomotor Function and Lifespan

Mechanical stress sensitivity (a.k.a., bang sensitivity) was assayed by vortexing homozygous *ve,e TPF^{sugarkill}* or the *ve,e* wildtype control flies in a standard media vial for 20 sec and measuring the length of time each animal remained paralyzed (Seigle et al., 2008). Temperature sensitivity was assayed by acutely shifting homozygous *ve,e TPF^{sugarkill}* or the *ve,e* wildtype control animals to 38°C using an empty food vial submerged in a temperature controlled water bath and recording time to paralysis (Seigle et al., 2008). For lifespan, 30 females were maintained at 25°C on Formula 4-24 media containing the appropriate concentration of each drug/compound tested. The animals were moved onto fresh food with drug/compound every day, deaths were recorded and the percent survival was analyzed using Prism software and statistical significance was determined using log-rank (Mantel-Cox) analysis.

Molecular Analysis of Redox Molecules

Ratios of reduced to oxidized NADH and NADPH were quantified using spectrophotometric reactions (AbCam). Twenty fly heads were harvested from the wildtype control (*ve e*), homozygous *TPF^{sugarkill}* (*ve e TPF^{sugarkill}*) and heterozygous *TPF^{sugarkill}/JS10* (TPI deletion) flies aged to day 4 or 20 at 25°C on standard yeast molasses media. Flies were anesthetized on ice and then homogenized into 400ul of the extraction buffer provided by AbCam using a mechanical homogenizer. Cell debris was pelleted at 10 000×g for 5min at 4°C. The supernatant was then filtered through a Amicon Ultra 10kDa filter to remove interfering enzymes. The resultant flow thru was then utilized as the extract in the enzymatic reactions to determine molecule concentrations. The absorbance at 450nm was determined in 10ul of each extract following 1 hour incubation at room temperature in the enzyme mix (AbCam) provided the total NADP/NADPH (NADPHt) or NAD/NADH (NADHt). To decompose NAD and NADP and determine the amount of NADH or NADPH only, 10ul of the cell extract was heated at 60°C for 30min prior to addition to the enzyme mix for 1 hour at room temperature. The absorbance at 450nm of the decomposed extracts provided the measurement of the total NADH or NADPH in the extract. A standard curve for the absorbance of NADPH or NADH at 450nm was conducted with 0, 20, 40, 60, 80 and 100pmole of each molecule in the enzyme reaction. The measurements were then applied to the standard curve to detect the concentration of NADHt, NADPHt, NADH, NADPH. The ratio of NAD/NADH was calculated at (NADt-NADH)/NADH. The ratio of NADP/NADPH was calculated at (NADPt-NADPH)/NADPH. Each experiment was performed in triplicate and then averaged. All measurements of absorbance were conducted using the Spectramax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA).

Glutathione (GSH and GSSG) measurements were obtained using a fluorometric assay provided by AbCam. Twenty fly heads were harvested from the wildtype control (*ve e*), homozygous *TPF^{sugarkill}* (*ve e TPF^{sugarkill}*) and heterozygous *TPF^{sugarkill}/JS10* (TPI deletion) flies aged to the day 4 or day 20 at 25°C on standard yeast molasses media. Flies were anesthetized on ice and then homogenized into 100ul the extraction buffer provided by AbCam using a mechanical homogenizer and treated with 20ul of 6N Perchloric Acid to preserve the sample. Cell debris was pelleted at 13 000×g for 2min at 4°C. The supernatant was then neutralized with 20ul of ice cold 3N KOH. To detect the GSH in the extract, 5uL of the sample was added into a 90ul enzyme reaction mix (AbCam). To detect GSSG in the extract, a 90ul enzyme reaction mix was set up that included a GSH Quencher for 10min, subsequently a reducing agent was added to destroy the excess GSH quencher and convert GSSG to GSH for detection. A standard curve was generated with 90ul enzyme reactions containing 0, 0.4, 0.8, 1.2, 1.6, and 2.0 ug GSH per well. An o-phthalaldehyde probe was added into all of the reactions and incubated for 10min at RT. The fluorescence of the samples and standards were read using the Spectramax Gemini FM plate reader (Molecular

Devices, Sunnyvale, CA) with 340/405nm excitation and emission settings. Following background subtraction from the blank standard well the relative fluorescent units of the sample reactions were compared to the standard curve to determine GSH in each reaction. The concentration of GSSG was divided by the concentration of GSH to provide the ration of GSSG/GSH in each extract. All reactions were performed in triplicate and averaged.

Western Blot Analysis of TPI Protein

Ten fly heads were ground by pestle in 50 μ L 2X SDS PAGE sample buffer (4%SDS, 4% β -mercaptoethanol, 130mM Tris HCl pH 6.8, 20% glycerol). Proteins were resolved on a 12% SDS polyacrylamide gel and transferred onto nitrocellulose membrane. Following blocking in 1% milk Phosphate Buffered Saline with 0.1% Tween 20 (PBST), the membranes were incubated with anti-TPI antibody (1:5000; rabbit polyclonal FL-249; Santa Cruz Biotechnology) or anti-Beta-tubulin antibody (used as the loading control-1:1000; mouse monoclonal D-140; Santa Cruz Biotechnology). The membranes were then washed in PBST, incubated in the appropriate HRP- conjugated secondary antibody, and developed using the ECL Western Blotting Substrate (Pierce). Quantification of the scanned films was performed digitally using ImageJ software (National Institutes of Health).

Redox state analysis on adult brain

Mitochondrial redox state was examined using the mitochondrial targeted redox-sensitive fluorescent protein MTSroGFP2, as previously described (Celotto et al., 2012; Liu et al., 2011). Brains from elav-Gal4; UAS-MTSroGFP2/+; SGK/SGK and ve e control animals were dissected in PBS on day 3, 15 and 30 at 25C. After dissection, brains were placed in mounting medium (Victor, H-1000) on a thin cover slip attached to a Petri dish. An Olympus IX 81 inverted laser scanning Fluoview 1000 confocal microscope (Olympus, Tokyo, Japan) was used for imaging. Images of fluorescence related to roGFP were acquired by using a BA 510 540 filter following excitation at 405 and 488 nm. The 20 $\times$ objective lens and Z-scan by 10um stepsize was used to obtain the whole brain image. Six brains were dissected in every group and 8 regions in images of every brain were measured. The ratios of 405nm/488nm fluorescence were evaluated using FV10-ASW2.0 software.

Statistics

Statistics were performed using Prism software to conduct a Student's t-test- for all experiments except lifespan analysis which used log rank (Mantel Cox) analysis. For all graphs * is p<0.05, ** is p<0.01, *** is p<0.001. For pharmacological studies statistical comparisons were made between the various treatment groups and vehicle (water) controls. For all graphs error given is SEM.

Results

***TPF^{sugarkill}* Flies Exhibit Increased Levels of the Oxidized Forms of NADH, NADPH, and Glutathione**

As a glycolytic enzyme, TPI contributes to the metabolism of sugars yielding energy that is utilized by the cell. A key component of glycolysis and aerobic respiration is the production of high energy electrons. The cell has several key carriers of high energy electrons and the ratio of reduced and oxidized forms of these carriers is critical to cellular health. When the ratio between reduced and oxidized becomes unbalanced it triggers oxidative stress pathways that can alter cellular health and viability (Alberio et al., 2012; Collins and Neafsey, 2012; de la Torre, 2011; Fischer et al., 2012; Guix et al., 2009; Kulic et al., 2011; Wang et al., 2008). We examined the oxidized and reduced ratios of three key electron carriers (NAD⁺, NADP⁺ and Glutathione) in cell extracts from wildtype and *TPF^{sugarkill}*

animals (Figure 1). In all cases we observed an increase in the oxidized form of these molecules in an age dependent manner. It has been reported that wildtype animals exhibit increased levels of the oxidized molecules as they age and that this stress contributes to senescence associated with aging (Hirano et al., 2012; Weber et al., 2012; Ying, 2008). Therefore the increase in oxidized levels of NAD⁺, NADP⁺ and Glutathione in wildtype flies was expected. However, when the ratios of the electron carriers was examined in homozygous *TPF^{sugarkill}* flies the oxidized forms significantly increased in an age dependent manner compared to the wildtype control. This indicates that while at a young age *TPF^{sugarkill}* exhibit modest oxidative stress, this is greatly exacerbated in older animals. Furthermore, this increase was more severe in *TPF^{sugarkill}/JS10* (null) animals. This increase is consistent with *TPF^{sugarkill}* being a hypomorphic loss-of-function allele and with previously described studies (Celotto et al., 2006; Seigle et al., 2008). It should be noted that only the ratios of reduced to oxidized glutathione changed, the total cellular levels of glutathione (GSH+GSSG) were similar in all samples (data not shown).

Mitochondria In *TPF^{sugarkill}* Flies Are Oxidatively Stressed

The reduction in efficiency of glycolysis may affect other metabolic pathways resulting in altered mitochondrial function leading to oxidative stress (Jeong et al., 2004; Schulz et al., 2007). Thus, we examined the oxidative status of mitochondria in homozygous *TPF^{sugarkill}* animals. In order to measure the redox status of mitochondria a mitochondrial targeted redox-sensitive GFP was utilized (MTSroGFP) allowing ratio-metric imaging of the redox status of the mitochondrial environment (Liu et al., 2011). When the mitochondria of *TPF^{sugarkill}* animals were examined the mutant animals exhibited a higher degree of oxidative stress even at an early age (Figure 2). This suggests that mitochondrial stress may be contributing to the phenotypes of the *TPF^{sugarkill}* animals. Again, as expected oxidative stress increased with age even in the wildtype animals, however, the increase in oxidative stress was more severe in *TPF^{sugarkill}* animals (Figure 2). Importantly, mitochondrial oxidative stress is evident at a much earlier time point than was seen using our assays of NADH, NADPH, and Glutathione. These data suggest that mitochondrial dysfunction may be the source of pathogenic ROS and that cellular mechanisms to buffer ROS become overwhelmed as the animals age under conditions of chronic dysfunction.

Oxidative Stressor Treatment Worsens Behavioral Phenotypes in *TPF^{sugarkill}* Flies

TPF^{sugarkill} animals exhibit paralysis in response to stress conditions such as mechanical stress and high temperature (Celotto et al., 2006; Seigle et al., 2008). We tested these behavioral phenotypes in animals that were challenged with chronic treatment of the oxidative stressor, hydrogen peroxide. Hydrogen peroxide is a general oxidative stressor that has been shown to be effective for mediating general oxidative stress when provided to flies for ingestion (Grover et al., 2009; Wang et al., 2008). Following treatment with increasing concentrations of hydrogen peroxide both the mechanical stress sensitivity (Figure 3A) and temperature sensitivity (Figure 3B) of *TPF^{sugarkill}* animals worsens in a dose-dependent manner. It should be noted that neither mechanical stress nor temperature sensitivity were observed in the wildtype animals following hydrogen peroxide treatment, suggesting it is not a general stress response and that *TPF^{sugarkill}* animals are hyper-sensitive to this oxidative stressor.

Reducing Agent Treatment Improves Behavioral Phenotypes in *TPF^{sugarkill}* Flies

As oxidative stressor treatment worsened behavioral phenotypes it was hypothesized that treatment with a reducing agent may mitigate some of the oxidative stress and reduce the severity of the behavioral phenotypes. Beta-mercaptoethanol is a potent reductant and has been utilized previously in flies for redox studies (Wang et al., 2008). As expected, treatment with sub-lethal levels of beta-mercaptoethanol improved the mechanical stress

sensitivity (Figure 4A) and temperature sensitive paralysis (Figure 4B) in the mutant animals. Again, treatment with beta-mercaptoethanol did not cause the appearance of the mutant phenotypes in the wildtype animals.

Pharmacologic modulation of behavior does not affect *TPI^{sugarkill}* levels

TPI^{sugarkill} has been shown to have increased degradation in a proteasomal and chaperone-dependent manner and previous improvements in behavioral phenotypes have correlated with an increase in the amount of cellular *TPI^{sugarkill}* protein (Hrizo and Palladino, 2010; Seigle et al., 2008). In order to determine whether the treatments with the redox stressors altered cellular levels of *TPI^{sugarkill}* and thus altered the behavioral phenotypes, a western blot was performed on cellular extracts from animals treated with hydrogen peroxide and beta-mercaptoethanol at 25°C. At this temperature, the flies exhibit a consistent level of *TPI* protein over time, but the overall level is reduced compared to the wildtype flies (Hrizo and Palladino, 2010; Seigle et al., 2008). Therefore, if the compounds are altering protein stability a decrease in protein levels following treatment should be observed. Interestingly, there was no change in the levels of *TPI^{sugarkill}* in response to treatment with the compounds suggesting that the behavioral changes were mitigated by a different cellular mechanism (Figure 5).

Aged *TPF^{sugarkill}* Flies Exhibit Hypersensitivity to Lethal Doses of Hydrogen Peroxide and Resistance to Lethal Doses of Beta-Mercaptoethanol

High doses of hydrogen peroxide have been shown to be lethal to wildtype animals in a short period of time (Grover et al., 2009; Wang et al., 2008). Therefore, we used similar doses of hydrogen peroxide and examined longevity of *TPF^{sugarkill}* and control animals. As expected, young (Day 3 at the start of the experiment) *TPF^{sugarkill}* animals exhibited increased mortality when treated with 162.5mM hydrogen peroxide demonstrating that they are hypersensitive to hydrogen peroxide-mediated oxidative stress (Figure 6A). However this sensitivity is modest when compared to animals aged 15 days prior to treatment. In this case, the *TPF^{sugarkill}* animals exhibited even greater sensitivity to the same dose (162.5mM) of hydrogen peroxide (Figure 6B). The aging reduced the median lifespan on 162.5mM hydrogen peroxide from 5.0 days for young animals to 3.0 days for aged *TPF^{sugarkill}* animals. Treatment with 162.5mM hydrogen peroxide reduced the median lifespan of wildtype to 5.5 days (day 3 treatment) and 4.0 days (day 15 treatment). In addition, treatment with a higher dose (650mM hydrogen peroxide) yielded a similar increased sensitivity in aged *TPF^{sugarkill}* animals (data not shown). Overall, this suggests that the increase in the ratio of oxidized molecules in the aged wildtype and *TPF^{sugarkill}* animals exacerbates the sensitivity to an oxidative stressor.

It has been shown that beta-mercaptoethanol is toxic to wildtype flies (Wang et al., 2008). Therefore, for chronic treatment we used a higher dose (4mM) of beta-mercaptoethanol than was used for our acute experiments of locomotor function. We again examined two treatment onsets young (Day 3) and aged (Day 15) with wildtype and *TPF^{sugarkill}* flies. While young animals of both types exhibited increased rates of death when exposed to 4mM beta-mercaptoethanol, the *TPF^{sugarkill}* flies died slightly faster than the wildtype control (Figure 6C). However, when the animals were aged for 15 days prior to exposure to 4mM beta-mercaptoethanol the *TPF^{sugarkill}* flies now exhibited more resistance to the treatment than the wildtype control (Figure 6D). It should be noted that the effect of beta-mercaptoethanol is more modest when administered to young flies than to aged animals. The median lifespan of the young wildtype and *TPF^{sugarkill}* flies on 4mM beta-mercaptoethanol was 5 days and 4 days, respectively. However the median lifespan of the aged wildtype flies on the 4mM beta-mercaptoethanol was reduced to 3 days while the *TPF^{sugarkill}* flies median lifespan was extended to 5 days following aging. Since beta-mercaptoethanol is a potent

toxin the finding that longevity is improved in *TPF^{sugarkill}* flies compared to wildtype is surprising. These data demonstrate *TPF^{sugarkill}* flies are resistant to the toxic reductant compared to wild type and that this resistance increases with the age of the animal akin to the increases seen in oxidative stress. Overall the data demonstrate ROS is critical to the pathogenesis of locomotor and longevity phenotypes in *TPF^{sugarkill}* flies.

Discussion

Alterations in glycolysis have been suggested to influence the redox status of cells and changes in redox can have consequences for cellular function and viability (Grover et al., 2009; Gruning et al., 2011; Jeong et al., 2004; Karg et al., 2000; Kruger et al., 2011; Liu et al., 2011; Thorpe et al., 2004; Weber et al., 2012; Ying, 2008). In this study we examined the effects of a mutation in a glycolytic enzyme on the redox status of *Drosophila melanogaster*. We determined that *TPF^{sugarkill}* flies are oxidatively stressed and that treatment with compounds that affect redox alter the behavioral phenotypes of the mutant animals as expected. Cumulatively, this suggests that redox does play a role in the disease pathogenesis in this model of TPI deficiency.

There have been several previously proposed mechanisms into TPI deficiency including dimer instability, TPI protein aggregation and enzyme inactivity. Previous studies indicate that the *TPF^{sugarkill}* protein can form a dimer and when overexpressed can rescue the mutant phenotypes in the flies suggesting the enzyme retains some activity (Celotto et al., 2006; Seigle et al., 2008). In addition, the mutant TPI protein does not appear to be aggregation prone and stays in solution in cell fractionation experiments (Hrizo and Palladino, 2010). Furthermore, lactic acid levels are decreased in *TPF^{sugarkill}* flies suggesting that there has been an overall decrease in glycolysis in the animals (Celotto et al., 2006).

However, there are several reasons why animals with a mutation in TPI may exhibit oxidative stress which may contribute to the disease pathology. One cause may be the formation of a compound in the cells called methylglyoxal. TPI is responsible for the conversion of dihydroxyacetone phosphate (DHAP) to glyceraldehyde-3-phosphate in the cell. With reduced TPI function, DHAP will accumulate in the cell and it can be converted to methylglyoxal (MG) through the function of methylglyoxal synthase (Ahmed et al., 2003; Guix et al., 2009; Karg et al., 2000). If a reduction in TPI activity observed in the animals as indicated by previous research, this would result in an accumulation of DHAP that is converted into MG, it may be the source of oxidative stress. MG is electrophilic and can cause oxidative stress. The catabolism of MG to an inert product, D-lactate, utilizes cellular GSH. This can alter the balance of oxidized (GSSG) to reduced forms (GSH) of glutathione in the cell and has been shown to deplete the cell of GSH, thus making the cells more sensitive to oxidative stress. MG is also implicated in mitochondrial function. MG has been shown to help to form advanced glycation end products (AGEs) which are implicated in the aging process (Ahmed et al., 2003). Lastly, cells treated with MG have been shown to increase mitochondrial oxidative stress (Miyazawa et al., 2010; Wang et al., 2009). This last observation is especially important since we observed an increase in mitochondrial oxidative stress as an early event in our *TPF^{sugarkill}* animals, suggesting this may be a significant aspect of pathogenesis. Overall, MG formation may be a key step in the development of oxidative stress in our *TPF^{sugarkill}* animals and contribute to the disease pathogenesis in this model of TPI deficiency.

Interestingly, work by the Krobisch lab with a yeast model system has shown that expression of one human TPI mutant variant (I170V) in yeast results in increased resistance to the oxidative stressor diamide. The authors have postulated that this change in redox status is due to an increase in pentose phosphate pathway activity (Ralser et al., 2006; Ralser

et al., 2007). However, the other human TPI mutant variants, including the best studied mutant allele (E104D), exhibited no change in the susceptibility toward diamide in their unicellular system. *TPF^{sugarkill}* animals exhibit early mitochondrial dysfunction and a shift in mitochondrial redox status toward oxidization and later elevated cellular oxidative stress. Thus, this allele may function similarly to the several human disease causing mutations that have been shown to have increased MG formation and elevated oxidative stress (Ahmed et al., 2003; Guix et al., 2009; Karg et al., 2000). When we examined *TPF^{sugarkill}* flies treated with diamide no change in bang sensitivity or temperature sensitivity phenotypes were observed (data not shown). However, diamide is a thiol specific oxidizing agent that will target glutathione while hydrogen peroxide will increase cellular peroxide levels. Therefore, it should be noted that oxidative stressors such as hydrogen peroxide and diamide have been shown to activate different cell stress response pathways and differentially affect various oxidative stress sensitive mutants (Pocsi et al., 2005; Thorpe et al., 2004; Wemmie et al., 1997). Furthermore, when aged *TPF^{sugarkill}* flies were administered with moderate concentrations of hydrogen peroxide (5mM) and beta mercaptoethanol (0.5mM) in the food we did observe modest changes in the ratios of NAD⁺/NADH as expected (data not shown). This does suggest ingestion of these compounds can alter global redox ratios modestly and thus may have multiple downstream cellular targets that effect behavioral changes.

In addition to providing insight into the pathogenesis of TPI deficiency, studying the mechanism and consequence of oxidative stress formation in the brain may yield insight into the critical role of metabolism and brain health. Mutations in genes involved in metabolism and energy production have been linked to the formation of oxidative stress that has consequences for brain function and health in the fly (Grover et al., 2009; Wang et al., 2008; Weber et al., 2012; Zhong et al., 1998). It is becoming increasingly apparent that diet and exercise play critical roles in the development and progression of neurodegenerative disorders in humans (Butterfield et al., 2010; Fischer et al., 2012; Fujikake et al., 2008; Kulic et al., 2011; Wang et al., 2011). However, the pathways regulating the contribution of metabolic activity to neuronal health have not been clearly elucidated. The data presented in this paper demonstrate that altered metabolism does have consequences for oxidative stress in flies and that these changes in oxidative stress can change behavior and longevity in response to stressors, in accord with previous observations (Grover et al., 2009; Wang et al., 2008; Weber et al., 2012; Zhong et al., 1998). Therefore this study and others that utilize mutant *Drosophila* to examine the development and consequences of oxidative stress due to altered metabolism are providing key insight into the pathogenesis of TPI deficiency and other neurodegenerative diseases in humans.

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Highlights

- Early mitochondrial dysfunction is observed in a glycolytic mutant.
- The early mitochondrial dysfunction contributes to progressive oxidative stress.
- Pharmacological alteration of redox chemistry modifies phenotypes in mutant flies.

Figure 1. Ratios of redox molecules are significantly shifted toward the oxidized form in an age-dependent manner in the *TPF^{sugarkill}* animals

A) The ratio of NAD⁺ to NADH in the control animals (white) is not significantly different from the mutant TPI samples (homozygous sgk black, sgk/ JS10 grey) at day 4. However, by day 20 there is a significant increase in NAD⁺/NADH ratio in mutant samples compared to the control samples, indicating that the animals are experiencing oxidative stress. B) The ratio of NADP⁺ to NADPH in the control animals (white) is not significantly different from the mutant homozygous sgk samples (black) at day 4, but a slight increase is seen in the sgk over the JS10 deficiency samples (grey) when compared to wildtype. By day 20 there is a significant increase in NADP⁺/NADPH ratio in both mutant samples compared to the control samples. C) The ratio of oxidized glutathione (GSSG) to reduced glutathione (GSH) is significantly increased in both homozygous sgk (black) and sgk over the JS10 deficiency (grey) compared to wildtype (white) at day 4. By day 20 there is a non-significant increase in the GSSG/GSH ratio in homozygous sgk samples compared to wildtype and a significant increase in this ratio in sgk/JS10 samples compared to wildtype. Cumulatively, the data in all three panels indicate that the ratios of multiple cellular redox molecules are shifted significantly toward the oxidized form in aged animals and that aged *TPF^{sugarkill}* animals are exhibiting oxidative stress. Data shown are the average from 3 independent homogenates and error bars represent SEM.

Figure 2. The mitochondria in *TPF^{sugarkill}* flies are oxidatively stressed, even at a young age *TPF^{sugarkill}* flies exhibit an age-dependent increase in neural ROS by measuring MTSroGFP2. At day 3, there is a significant difference between homozygous *TPF^{sugarkill}* mutant (black) and the wildtype control (white) brains (***P<0.001). At day 15 and day 30 the ROS levels showed a significant difference and a progressive increase in aged flies. N = 6

Figure 3. Treatment with hydrogen peroxide worsens behavioral phenotypes in *TPI^{sugarkill}* flies
A) The mechanical stress sensitivity of *TPI^{sugarkill}* flies (black) was significantly worsened by treatment with 5.0 mM hydrogen peroxide compared to the vehicle only control (-). Wildtype flies (white) did not exhibit stress sensitivity with similar treatments. All animals were tested at day 20 when mutants exhibit a highly-penetrant stress phenotype. B) Temperature induced paralysis of day 3 *TPI^{sugarkill}* flies (black) was significantly worsened by treatment with 5.0mM hydrogen peroxide compared to the water only treated control (-). Day 3 wildtype flies did not exhibit any temperature sensitive paralysis even when treated with hydrogen peroxide and therefore are not denoted on the graph. N = 30 for each treatment.

Figure 4. Treatment with beta-mercaptoethanol improves behavioral phenotypes in *TPI^{Sugarkill}* flies

A) The mechanical stress sensitivity of *TPI^{Sugarkill}* flies (black) was significantly improved by treatment with 0.05mM beta-mercaptoethanol compared to the water only treated control (-). Treatment of wildtype flies (white) did not cause significant mechanical stress sensitivity. All animals were tested at day 20. B) Temperature induced paralysis of day 3 *TPI^{Sugarkill}* flies (black) was significantly improved by treatment with 0.005 and 0.05mM beta-mercaptoethanol compared to the vehicle only treated control (-). Day 3 wildtype flies did not exhibit any temperature sensitive paralysis when treated with the same dosages of beta-mercaptoethanol and therefore are not denoted on the graph. N = 30 for each treatment

Figure 5. Treatment with Hydrogen Peroxide and Beta-Mercaptoethanol do not affect *TPI^{sugarkill}* protein stability

Western blots were conducted on fly extracts from homozygous *TPI^{sugarkill}* animals 48 hours after treatment with the indicated compounds at 25C. A) The average relative amount of TPI in each extract type is denoted. The levels of protein were compared to the vehical only control (-). Data were not found to be significantly different between experimental and control samples (Student's t-test, N = 6). B) A representative western blot demonstrating the similar levels of *TPI^{sugarkill}* protein in the flies treated with hydrogen peroxide and beta-mercaptoethanol. Beta-tubulin was included as a loading control in the experiment.

Figure 6. Aged *TPFSugarkill* flies are sensitized to high doses of hydrogen peroxide and resistant to high doses of beta-mercaptoethanol

A) Treatment with toxic levels of hydrogen peroxide (162.5mM) shortens the lifespan of young (Day 3) homozygous *TPFSugarkill* flies (□) compared to the wildtype flies (●) treated with the same concentration of hydrogen peroxide and incubated at 25°C. B) Aged (Day 15) *TPFSugarkill* animals (□) exhibit even greater sensitivity to treatment with 162.5mM hydrogen peroxide compared to the wildtype flies (●) treated with the same concentration of hydrogen peroxide and incubated at 25°C. C) Treatment with toxic levels of beta-mercaptoethanol (4mM) kills young (Day 3) homozygous *TPFSugarkill* flies (□) faster than the wildtype control treated with the same dose of beta-mercaptoethanol (●). D) Aged (Day 15) *TPFSugarkill* animals (□) exhibit greater resistance to treatment with toxic level (4mM) of beta-mercaptoethanol compared to the wildtype flies (●) treated with the same concentration of compound and incubated at 25°C. All graphs represent the percent living animals over time. For all panels the curves were found to be significantly different by Log Rank (Mantel Cox) Analyses with Prism software. *** $p < 0.0005$ for curve comparisons in panels A, B, C, and D. $N = 90$ for each genotype and condition. Error bars represent SEM.