Trichostatin A enhances catalase activity in *Drosophila melanogaster*^{ap56f}

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In an aerobic environment, catalase plays an important role in the defense of cells and organisms against the toxic effects of oxygen. Possible consequences of the production of reactive oxygen species (ROS) from the metabolism of oxygen include damage to biological macromolecules such as lipids, proteins, and nucleic acids. The detrimental effects on cellular metabolism can result in the loss of viability. Catalase, an antioxidant enzyme, prevents the accumulation of hydrogen peroxide, a reactive oxygen species, by catalyzing conversion of the substrate to water and molecular oxygen. In this inquiry, the role of an epigenetic agent on catalase levels was investigated to ascertain the potential of this approach for bolstering antioxidant defense systems in aerobic organisms. Trichostatin A (TSA) is a histone deacetylase inhibitor that targets the class I and class II histone deacetylases (HDAC) and histones H3 and H4 (Huidobro *et al*., 2013). Eukaryotic DNA is arranged into chromatin in which histone components of nucleosomes can be regulated by reversible acetylation. Histone acetylation is regulated by histone acetyltransferases and histone deacetylases (HDACs), which play important roles in transcription, DNA replication, and cell cycle progression (Benayoun *et al*., 2015). Trichostatin A, which inhibits HDAC, has been shown to stop cell cycling, induce differentiation, and reverse morphological changes seen in the cell cycle arrest (Santos *et al*., 2018). In this study, the activity of catalase in response to TSA was determined in four-day old adult flies of a mutant *Drosophila melanogaster* strain.

Drosophila melanogaster strain ap^{56f} flies used in this study were obtained from Carolina Biological Supply Company in Burlington, North Carolina. Flies were grown in vials containing 15 ml of Formula 4-24® Instant *Drosophila* medium (Carolina Biological Supply Company, Burlington, NC) mixed with 15 ml deionized water supplemented with yeast. *D. melanogaster*^{ap56f} were transferred to vials and maintained at room temperature (25°C±1). Every three weeks, the *D. melanogaster*^{ap56f} were transferred to fresh vials. Four-day old *D. melanogaster*^{ap56f} were separated into two groups with 25 males in each vial. Both groups were dehydrated for two hours, then transferred to vials containing 5 ml of 2% sucrose on a saturated Kimwipe. Group one (control) adults were incubated at 25^{o}C for 24 hours in the absence of TSA and group two (treated) adults were incubated at 25^{o}C for 24 hours in the presence of 50 µM TSA. The flies were collected and stored in microcentrifuge tubes at -80^{o}C until used for further experimentation. A homogenizing solution was prepared by combining 1.5 ml of 50 mM potassium phosphate buffer (pH 7) and 1.5 µl of protease inhibitor cocktail (Sigma cat. no. P8340). Twenty-five *D. melanogaster*^{ap56f} were homogenized in 0.5 ml of homogenizing buffer (4^{o}C), using chilled pestles and mortars. The homogenized solution was centrifuged at 16,000 x g (4^{o}C) for 30 minutes. Supernatants were concentrated using Amicon® 30K Ultra 0.5 ml Centrifugal Filters and were centrifuged at 5,000 x g (4^{o}C) until the retentate was reduced to a final volume of 50 µl for each sample**.** The concentrated samples were used for protein concentration determinations, enzyme assays and non-denaturing polyacrylamide gel electrophoresis. The Pierce 660 nm Protein Assay Kit (ThermoFisher Scientific Inc., Rockford, IL) was used to ascertain protein concentrations. A modified version of the Beers and Sizer catalase assay was performed to determine the enzymatic activity of the protein

samples (Beers and Sizer, 1952). Each reaction mixture had a total volume of 3 ml. The assay was prepared by mixing 1.9 ml of deionized water, 1 ml of 0.059 M hydrogen peroxide (diluted in 0.05M potassium phosphate, pH 7) and 100 µl of diluted protein sample. Assays were performed at 25°C. The absorbance for the reaction mixture was read at a wavelength of 240 nm on a Genesys 5 UV-visible spectrophotometer and the readings were recorded every ten seconds for one minute. Five assay repetitions were performed for each sample. The enzyme unit was defined as µmol/min/mg. Novex™ WedgeWell™ 4-12% tris-glycine gradient gels were used (Invitrogen, Carlsbad, CA) for gel electrophoresis. Tris-glycine native running buffer was used for runs at 100 volts for 30 minutes and at 200 volts (4°C) for an additional 30 minutes with 25 micrograms of protein per sample mixed with 2X Novex™ tris-glycine native sample buffer. Gels were soaked in 100 ml of 0.03% hydrogen peroxide for 15 minutes with gentle agitation. The staining solution of 50 ml of two percent iron chloride ($FeCl_3$) and 50 ml of two percent potassium ferricyanide ($K_3Fe(CN)_6$) was poured on each gel to create a blue pigment in areas lacking catalase activity. The yellowish catalase bands were viewed and photographed using Bio-Rad ChemiDoc™ Imaging system.

Figure 1 is the image of a typical native 4-12% polyacrylamide gel with 25 µg of protein sample in each lane. A single major isoform was detected in samples from flies exposed to zero TSA and those exposed to 50 µM TSA. The gel results suggest an up-regulation of catalase in *D. melanogaster*^{ap56f} incubated for 24 hours in TSA where a larger and more intense band of catalase activity (lane 2) is discernible when compared to that of the [no TSA] controls.

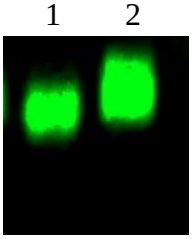

Fig. 1- Catalase from four-day-old *D. melanogaster*^{ap56f} treated with 50 µM TSA. Samples were resolved on a native 4-12% polyacrylamide gel. lane 1, -- TSA; lane 2, 50 µM TSA.

Figure 2 represents the quantitative results from the enzyme assays of three independent experiments. Each experiment consisted of ten total vials: five vials each contained 25 flies for the control group and five vials each of which contained 25 flies per TSA-treated group. The data indicate significantly higher enzyme activities (mean µmol/min/mg ± std. dev.) in the samples from the TSA-treated flies (3624.33±146.58) [*$p<0.05$] than in the samples from control flies (no TSA) (2749.00±274.61). Catalase is an antioxidant enzyme that plays an important role in the defense of cells and organisms against the toxic effects of oxygen free radicals. For aerobic organisms, this is an essential function since damage to biological macromolecules can lead to aberrant biochemical and physiological functions and may ultimately result in death of the affected

cells and organisms. This study suggests that apterous^{56f} mutants of *Drosophila melanogaster* may moderate the deleterious effects of a stress induced oxygen free radical surge by a process which exposes them to a histone deacetylase inhibitor agent like TSA to upregulate catalase levels. Future studies will be conducted to clarify the relationship between TSA concentration and catalase activity increases.

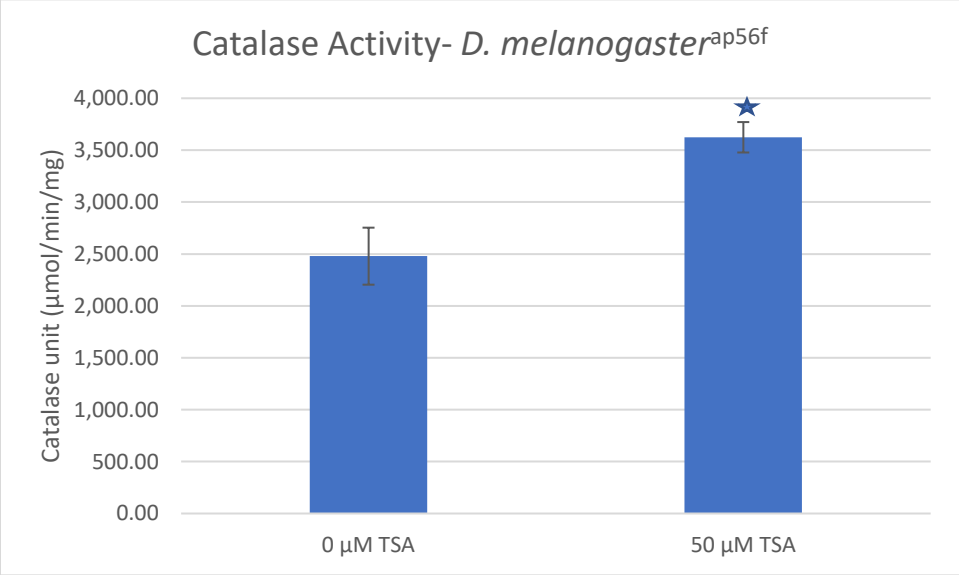

Fig. 2 - Mean catalase units (µmol/min/mg ptn). Four day old *D. melanogaster*^{ap56f} treated with 50 µM TSA. [★$p<0.05$]

References: Huidobro, C., Fernandez, A. F., and Fraga, M. F. (2013). Molecular Aspects of Medicine, 34(4): 765-781; Benayoun, B., Pollina, E.A., and Brunet, A. (2015) Nature Reviews Molecular Cell Biology 16:593-610; Santos, J., Zuma, A. A., de Luna Vitorino, F. N., da Cunha, J.P.C., deSouza, W., & Motta, M. C. M. (2018). Parasitology, *146*(4): 543-552; Beers, R.F., and Sizer, I.W. (1952). J. Biol. Chem, 195(1): 133-140; Woodbury, W., Spencer, A. K., and Stahmann, M.A. (1971). Analytical Biochemistry, *44*(1): 301-305.