

OBESITY STUDIES: OVEREXPRESSION OF ENZYMES IN THE SPHINGOLIPID METABOLIC PATHWAY IN *DROSOPHILA* MUTANTS

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Obesity is a multi-factorial metabolic disorder that is associated with many related pathologies. This study's aim is to begin elucidating the relationships between obesity and the sphingolipid metabolic pathway. Over-expressing several key enzymes on the degradative portion of the pathway will enlighten some of the mechanism at work and how these mechanism effect body weight, fat body distribution and morphology, triglyceride and cholesterol levels, and lipid droplet formation.

Obesity is an epidemic human disorder that is associated with the accumulation of excessive body fat, increased weight, and elevated triglyceride and cholesterol levels, all of which are established elements of obesity. Obesity is associated with related pathologies including type II diabetes, heart disease, high blood pressure, and certain cancers in both males and females. Although little is known about the genetic components of obesity, this disease is considered to be polygenetic. The aims of this study are to begin to elucidate the relationships between obesity and sphingolipid metabolism by over-expressing enzymes in the sphingolipid metabolic pathway that regulate the levels of key metabolites within the pathway.

The use of *Drosophila* as a model organism for studying the human condition of obesity has been demonstrated by prior studies undertaken in the Harris Laboratory (SDSU) with the characterization of three genes: *Sk1*, *Sk2*, and *Sply*. Additionally, *Drosophila* has been a classical model used in research due to the large amount of conserved sequences shared between humans and *Drosophila* through evolution.

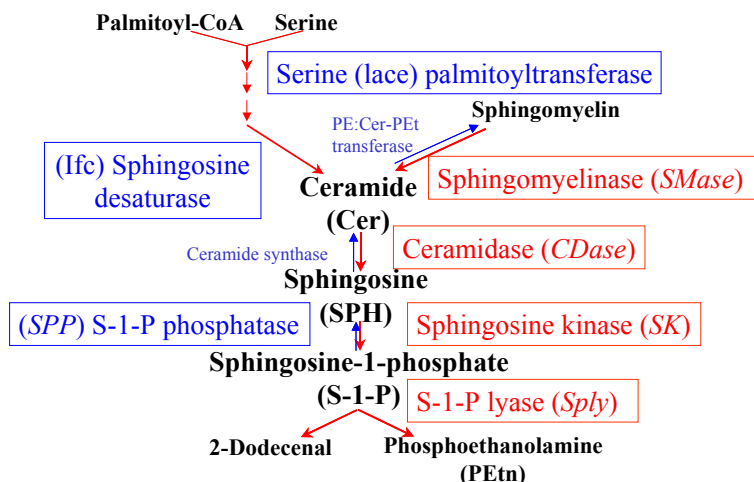


Figure 1. Sphingolipid biosynthetic pathway. Key enzymes shown in red (*Drosophila* homolog in parenthesis).

Sphingolipids are a ubiquitous class of lipids found in the plasma membrane of most eukaryotic cells. The major bioactive polar sphingolipid metabolites, **ceramide**, **sphingosine**, and **S-1-P** are regulated by the anabolic and catabolic activities of the following enzymes in the sphingolipid metabolic pathway (**Fig 1**): serine palmitoyltransferase (*lace*), sphingosine desaturase (*Ifc*), sphingomyelinase (*SMase*), ceramidase (*CDase*), S-1-P phosphatase (*SPP*), sphingosine kinase (*SK 1 and 2*), and S-1-P lyase (*Sply*). This study will investigate the over-expression of the degradative enzymes, *SMase*, *CDase*, *SK2*, and *Sply*.

Ceramide can be formed via de novo synthesis and by the degradation of sphingomyelin by sphingomyelinase (*SMase*). Ceramide is a second messenger and a known pro-apoptotic mediator. In a recent study evidence has been found that suggests that elevated levels of the intramyocellular lipid ceramide leads to insulin resistant skeletal

muscle in subjects exhibiting obesity and type II diabetes by possibly inhibiting insulin receptor signaling^{1,2,3}. The degradation of ceramide by ceramidase (*CDase*) gives rise to sphingosine, a possible growth inhibitor. Sphingosine is broken down to sphingosine-1-phosphate by sphingosine kinase (*Sk2*), this enzyme's strategic location is important to cell fate. Metabolites upstream of this enzyme function in the pro-apoptotic capacity while the sphingolipid intermediate S-1-P pushes the cell fate toward mitogenesis. Sphingosine-1-Phosphate (S-1-P), a second messenger, is generated by the degradation of sphingosine by sphingosine kinase. S-1-P is known to be anti-apoptotic, promoting cell proliferation and survival⁴. This major metabolite is degraded in the only irreversible step in the pathway by S-1-P lyase (*Sply*). Recent studies have shown that *Sply* regulation of sphingolipid signaling is critical for *Drosophila* development. *Sply* mis-expression mutants exhibit musculature defects, semi-lethality, and larval lethality, in addition to male and female reproductive structural defects⁵.

Methods

Drosophila Husbandry and Genetics

Flies were reared on standard yeast/sucrose media at 21°C. A12 (2nd and 3rd chromosome balancers), 1799 (3rd chromosome hs-Gal4 driver), 2077 (2nd chromosome hs-Gal4 driver) was obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN). The following fly lines consist of a random insertion of an extra copy of the gene of interest: *SMase*, *CDase 1A*, *Sk2 12A*, *Sply 13A*, and *HSpl 8/HSpl k-l*. These genes are controlled under the UAS-Gal4 system. Over-expression transgenics were generated by first introducing selectable markers (A12 balancers) to the following genes of interest: *SMase*, *CDase*, *Sk2*, *Sply*, *HSpl*, and *HSpl k-l* for a single generation and screening for selected phenotypes. Simultaneously, Selectable markers (A12) were introduced to transgenic lines containing the hs-Gal4 construct on either the 2nd or 3rd chromosome for a single generation. Selected progeny from these two crosses were crossed to yield heterozygous transgenic over-expression mutants. The resulting progeny were self-crossed to obtain the desired homozygous transgenic mutants carrying the hs-Gal4 construct.

Over-expression

Gene over-expression was induced in the heterozygous and homozygous progeny by heat shock treatment. Heat shock treatment was performed in an Integrated Separation System Enprotech hybridization oven at 37°C for one hour/day on day zero of embryogenesis after the removal of parents.

Future Tests

Flight Analysis

1-7 day old flies will be released in a top-lit, Plexiglas chamber. Flight performance will be scored as follows: upward flight=3, lateral flight=2, downward flight=1, flightless=0⁵.

Larval Locomotion

Individual larvae will be placed on a 3% clear agar substrate containing a millimeter grid. The number of squares entered within 15s time intervals will be recorded over a test period of five minutes.

Feeding Behavior

Food intake will be assessed based upon the consumption and rate of consumption of small fixed quantities of normal laboratory culture media given to small groups of flies in glass culture vials. Red dye will be added to the media so that it will be possible to determine, at least quantitatively, whether there are changes in the rate of appearance of excrement from the test diet.

Body Weight

Induced and non-induced flies will be weighed in groups of ten using a Mettler laboratory balance.

Fat Body Distribution, Morphology, and Cytology

Heamtoxylin and/or Sudan black stained 15 µm cryosections of non-induced and induced mutant flies will be examined using light microscopy. A morphometric analysis of fat body tissue and cells including the size of lipid

droplets will be analyzed using NIH Image software. Ultrathin sections of adult fat body tissue will be prepared and examined using TEM.

Longevity

The lifespan of over-expression transgenic flies will be determined by establishing cultures (10 flies/vial) that will be maintained at constant temperature (24°C), changed weekly, and scored for the flies left alive until all have died.

Triglyceride and cholesterol

One larva for each induced and non-induced transgenic line will be homogenized for thirty seconds in separate 1.7 ml eppendorf tubes, each containing 50µl of reagent (Infinity Triglyceride or Infinity Cholesterol reagent, respectively; Sigma). The reactions are incubated for 5 minutes at 37°C and read on a spectrophotometer (Beckman DU 640) and the resulting absorbances plotted against an absorbance curve of varying concentrations of lipid or cholesterol standard. From each of the triglyceride or cholesterol reactions 4µl will be transferred to cuvettes containing 1ml of Bradford solution. The cuvettes will then be vortexed and incubated for 10-15 minutes at room temperature, and then read on a spectrophotometer. Measurements will be calculated against standard curves.

Discussion

Due to the ubiquitous nature of sphingolipids, the regulation of sphingolipid intermediates is critical. Sphingolipid regulation is important to the functioning of several biochemical processes including cell signaling, cell fate, cell membrane architecture, lipid raft signaling complexes, motility as well as fatty acid synthesis and cholesterol regulation via SREBP (Sterol Regulatory Elements Binding Proteins) by sphingomyelin and ceramide⁷. (Fig. 2).

Mutations as a result of mis-expression in *Drosophila* result in a variety of related and non-related obesity defects discussed previously. Additionally, musculature abnormalities, hypertrophied tissue, and increases in lipid droplets have been observed as well as defects in reproductive structures and decreased fecundity.

The over expression of some of the genes in this study under control of hs-Gal4 promoter should not only alleviate many of the observed defects of the knock out mutants, but may cause other deficiencies within the sphingolipid metabolic pathway as a result of the over expression of specific enzymes.

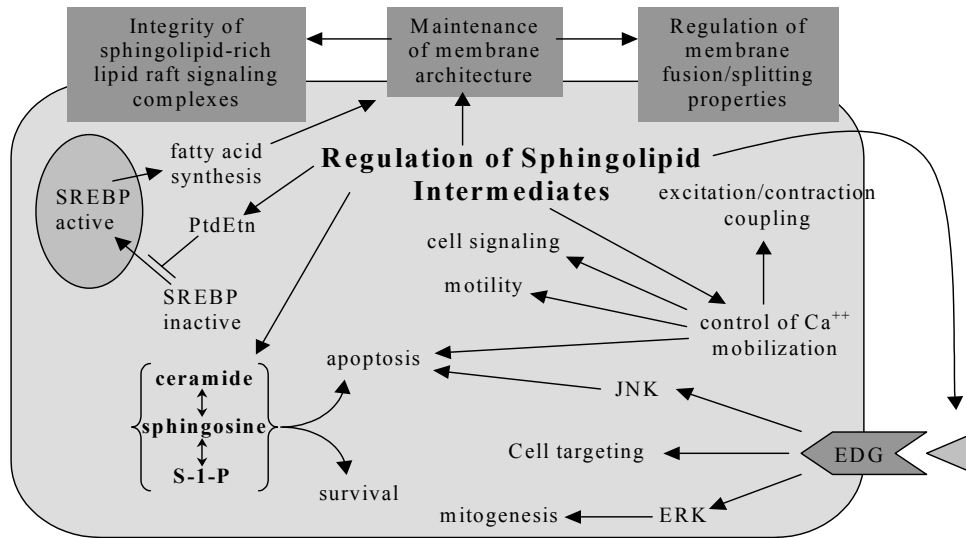


Figure 2. Possible mechanisms of sphingolipid action during *Drosophila* development⁵.

Due to time constraints beyond the researcher's control many of the physiological, morphological and biochemical assays were not performed. However, this study is ongoing and therefore, generalized hypotheses will be given as to the expected results of over expressing *SMase*, *CDase*, *Sk2*, *Sply*, and *HSpl* (see Fig. 1 to follow pathway).

SMase

The over-expression of *SMase* (sphingomyelinase) will deplete sphingomyelin and increase downstream intermediates- primarily ceramide. The depletion of sphingomyelin and increase in ceramide has been reported by Worgall et al. to affect SREBP. Lowered sphingomyelin and elevated ceramide levels contribute to the regulation of (Sterol Regulatory Elements) SRE-mediated gene transcription⁷. Additionally, increases in ceramide levels, which is an apoptotic mediator, may cause an increase in cell death in the organism.

CDase

Over expressing *CDase* (ceramidase) should lower incidences of cell death and increase sphingosine.

Sk2

The over-expression of *Sk2* (sphingosine kinase 2) will cause a decrease in sphingosine and an increase in sphingosine-1-phosphate, a mitogen. A recent study involving mis-expression mutants showed evidence of an obese phenotype of excessive weight gain, hypertrophied fat bodies and an increase in the fatty tissue surrounding the gonads⁴. One phenotype that will be interesting to observe after over-expressing this gene will be weight and fat body content and morphology. Will the fat bodies be atrophied? Will these flies appear emaciated?

Sply/HSpl/HSpl k-1

The mis-expression of this gene causes severe defects in musculature, and adult reproductive structures, and addition to cell death⁵. Over-expressing *Sply* (sphingosine-1-phosphate lyase) will cause a decrease in sphingosine-1-phosphate (S-1-P) levels. *HSpl* is the human homolog to *Sply*. *HSpl k-1* is the human mutant form. This over-expression study has included these homologs to compare whether the over-expression of these genes will act upon the sphingolipid metabolites in the same capacity as the *Drosophila* form of the gene.

By over-expressing *Sply* as well as the other genes we hope to elucidate the mechanisms at work in the sphingolipids metabolic pathway related to obesity. Further studies include creating double mutants where each of the genes in this study will be over-expressed in a *Sply* background, to investigate the relationship between sphingosine-1-phosphate lyase and *SMase*, *CDase*, and *Sk2*.

References

1. Adams II JM, Pratipanawatr T, Berria R, Wang E, DeFronzo RA, Sullards C C, & Mandarino LJ (2004). Ceramide Content Is Increased in Skeletal Muscle From Obese Insulin-Resistant Humans. *Diabetes*. 2003 October, 6; 53 (1): 25-31.
2. Kanety H, Hemi R, Papa MZ, Karasik A. Sphingomyelinase and ceramide suppress insulin-induced tyrosine phosphorylation of the insulin receptor substrate-1. (1996). *J Biol Chem*. 271: 9895-9897
3. Colberg S, Simoneau J-A, Thaete FL, Kelley DE. (1995) Impaired FFA utilization by skeletal muscle in women with visceral obesity. *J Clin Invest*. 95: 1846-1853.
4. Herr DR, Fyrst H, Creason MB, Phan VH, Sabas JD, & Harris GL (2004). Characterization of the *Drosophila* Sphingosine Kinases and Requirement for *Sk2* in Normal Reproductive Function. *Journal of Biological Chemistry*. 2004 March, 26; 279 (13): 12685-12694.
5. Herr DR, Fyrst H, Phan V, Heinecke K, Georges R, Harris GL, Saba JD (2003). *Sply* Regulation of sphingolipid molecules is essential for *Drosophila* development. *Development*. 2003 February, 24; 130 (11): 2443-2453.
6. Warren JT, et al., (2002) Molecular and biochemical characterization of two P450 enzymes in the ecdysteriodogenic pathway of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A*. Epub 2002 August, 12; 99 (17):9. 11043-8.
7. Worgall TS, Johnson RA, Seo T, Gierens H, Deckelbaum, RJ. (2002). Unsaturated Fatty Acid-mediated Decreases in Sterol regulator element-mediated Gene transcription are Linked to Cellular sphingolipids metabolism. *J Biol Chem*. 2002 Feb 8; 277 (6):3878-85. Epub 2001 Nov 13.