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EVALUATION OF THE GENETIC AND NUTRITIONAL CONTROL OF OBESITY AND TYPE 2 DIABETES IN A NOVEL MOUSE MODEL ON CHROMOSOME 7: AN INSIGHT INTO INSULIN SIGNALING AND GLUCOSE HOMEOSTASIS

SARAH NELSON AND MADHU DHAR

ABSTRACT

Obesity is the main cause of type 2 diabetes, accounting for 90-95% of all diabetes cases in the US. Human obesity is a complex trait and can be studied using appropriate mouse models. A novel polygenic mouse model for studying the genetic and environmental contributions to and the physiological ramifications of obesity and related phenotypes is found in specific lines of mice bred and maintained at Oak Ridge National Laboratory. Heterozygous mice with a maternally inherited copy of two radiation-induced deletions in the *p* region of mouse chromosome 7, $p^{23DFioD}$ and p^{30PUb}, have significantly greater body fat and show hyperinsulinemia compared to the wild-type. A single gene, *Atp10c*, maps to this critical region and codes for a putative aminophospholipid translocase. Biochemical and molecular studies were initiated to gain insight into obesity and glucose homeostasis in these animals and to study the biological role of *Atp10c* in creating these phenotypes. Glucose and insulin tolerance tests were standardized for the heterozygous $p^{23DFioD}$ and control mice on a custom-made diet containing 20% protein, 70% carbohydrate, and 10% fat (kcal). *Atp10c* expression profiles were also generated using Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR). Heterozygous $p^{23DFioD}$ animals showed insulin resistance after receiving a dose of either 0.375 or 0.75 U/kg Illetin R insulin. RT-PCR data also shows differences in *Atp10c* expression in the mutants versus control mice. Using these standardized biochemical assays, future studies will further the understanding of genetic and nutritional controls of glucose homeostasis and obesity in animal models and subsequently in human populations.

INTRODUCTION

Obesity is a growing health concern for today's society, due partly to its causal relationship to non-insulin dependent type 2 diabetes and cardiovascular disease. Recently, the U.S. Surgeon General reported that 61% percent of Americans are obese (Berger 2002). In addition, there are over 16 million cases of diabetes in the United States, 90-95% of which are type 2 diabetes (McLaughlin 2001). As with other complex traits, obesity and diabetes in humans are due to complex interactions between genetic and environmental factors. Despite the polygenic nature of these disorders, genetic assays related to quantitative trait loci (QTL's- specific regions of chromosomes containing a gene or genes that are involved in a complex phenotype without being solely responsible for that phenotype) are helpful in determining their cause, course, and affect (Dhar 2002).

Since 1949, the Mammalian Genetics Section at Oak Ridge National Lab (ORNL) has created and maintained a large number of mouse strains carrying radiation-induced, chromosomal deletions. To date, over 100 mutations have

been isolated in the *p* region of chromosome 7, shown through QTL's to be a critical region for affecting body fat in mice. Dhar et al. have identified and mapped a novel gene to this region, *Atp10c*, that codes for a putative aminophospholipid translocase (ATPase). This protein product is involved in the modulation of body fat through its role in transporting phospholipids across cell membranes (Herzing et al. 2001). Two specific homozygous lethal deletions studied by Dhar et al., $p^{23DFiOD}$ and p^{30PUb} (deletions of the promoter and first two exons and of the entire *Atp10c* gene, respectively- see Figure 1), have been shown to lead to obesity and diabetes-related phenotypes in affected mice. The maternal inheritance of either the $p^{23DFiOD}$ or p^{30PUb} mutation causes heterozygous mice to become visibly fatter as they age and exhibit hyperinsulinemia and hyperglycemia (Dhar 2002). The paternal inheritance of the same deletions renders the pups null for obesity and diabetes-related phenotypes, suggesting paternal imprinting (maternal expression), or "gene silencing."

Unlike other mouse models for obesity, these deletions correspond to a maternally expressed human orthologue on chromosome 15 related to Angelman Syndrome, a neurobehavioral disease (Meguro et al. 2001). A subset of those affected by AS also exhibit obesity, leading researchers to hypothesize that a lack of *ATP10C* expression in brain tissues may be involved in obesity. Dhar et al. have suggested that in both mice and humans, the expression of this novel aminophospholipid translocase plays a role in the metabolism of fatty acids and is therefore relevant to obesity and diabetes.

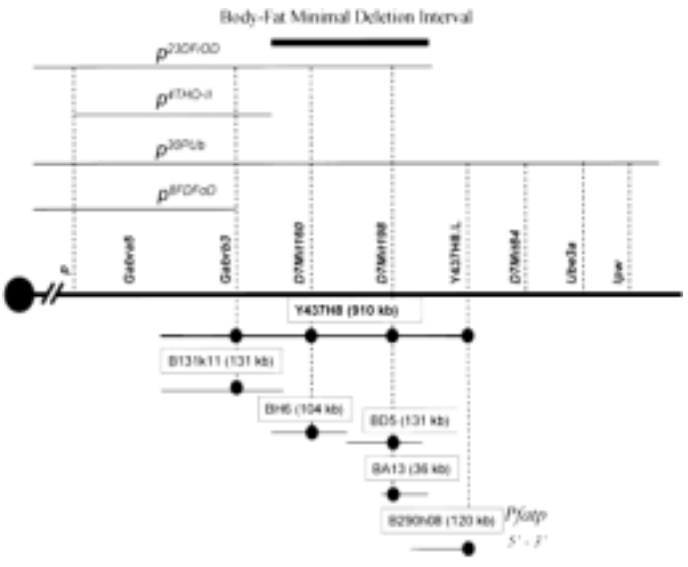


Figure 1. A genetic and physical map of the $p^{23DFiOD}$ and p^{30PUb} deletions in the *p* region of murine chromosome 7. Yeast and bacteria artificial chromosomes (YACs and BACs, respectively) mapped to this area are also shown. Adapted from:
Dhar, Madhu, Webb, L., Smith, L., Hauser, L. Johnson, D., West, D.B (2000). A novel ATPase on mouse chromosome 7 is a candidate gene for increased body fat. *Physiological Genomics*, 4, 93-100.

Product Number:	LabDiet 5001*		ResearchDiet D12450B	
Kcal/gm:	3.04		3.85	
Nutritional Info	gm %	kcal %	gm %	kcal %
Protein	23.4	28.049	19.2	20
Carbohydrate	49.9	59.814	67.3	70
Corn Starch	31.9		33.2	34.5
Sucrose	3.68		33.2	34.5
Fat**	4.5	12.137	4.3	10
-lard/animal fat			1.896	4.44
-soybean oil			2.37	5.55
Nitrogen Free Extract	49.9		75.1	
Fiber	5-20		4.7 (cellulose)	0

	Saturated fat	Monosaturated fat	Polyunsaturated fat
Lard (animal)	37%	46%	11%
Soybean (vegetable)	14%	24%	61%

*Lab Diet is a closed formula that can vary from batch to batch. Fiber can range from 5-20% and the fiber is from digestible and non-digestible sources. Protein, fat, carbohydrates, ash, added and mineral can also vary from batch to batch.

** Lab Diet 5001 (regular chow) has animal fat as main fat source. By wt. %age, regular chow is 1.50% saturated fatty acids, 1.58% monounsaturated fatty acids, and 0.26 % Omega-3 fatty acids Research Diet D12450B gives following breakdown of fatty acids:

Table 1. Nutritional components of regular lab chow vs. custom made diet.

METHODS

Mice containing the radiation-induced, Oak Ridge $p^{23DFiOD}$ deletion (notated pxpl, indicating a maternally inherited deletion unless notated pxpl –pdel, meaning a paternal deletion) were monitored and weighed every two weeks, along with control mice (notated pxpx). Thirty-four mice (males: 6 pxpx, 8 pxpl, 2 pxpl-pdel; females: 10 pxpx, 5 pxpl, 3 pxpl-pdel) were maintained on the regular chow fed to all mice in the facility, LabDiet 5001. Others (males: 8 pxpx, 15 pxpl; females: 5 pxpx, 5 pxpl), after reaching 5-7 weeks, were placed on custom made, Research Diet D12450B (referred to as the yellow diet) containing slightly less fat and more carbohydrate. (For specific nutritional breakdowns of these two diets, see Table 1). The weight gain of pxpx vs. pxpl on both the regular and the special diet was monitored and recorded.

Mice from both experimental and control groups were sacrificed and dissected. Their tissues (heart, liver, kidney, spleen, pancreas, inguinal fat, epididymal fat, cerebellum, hippocampus, and the rest of the brain) were removed and snap frozen with liquid N_2, collected for RNA expression analysis. Immediately following CO_2 asphyxiation, blood was collected from the heart to be sent for off-site lipid analyses (HDLs, LDLs, etc). The fat depots (see Figure 2) were collected and used to calculate the adiposity index (AI)- a crude measure of body fat. (See Dhar et al. 2000)

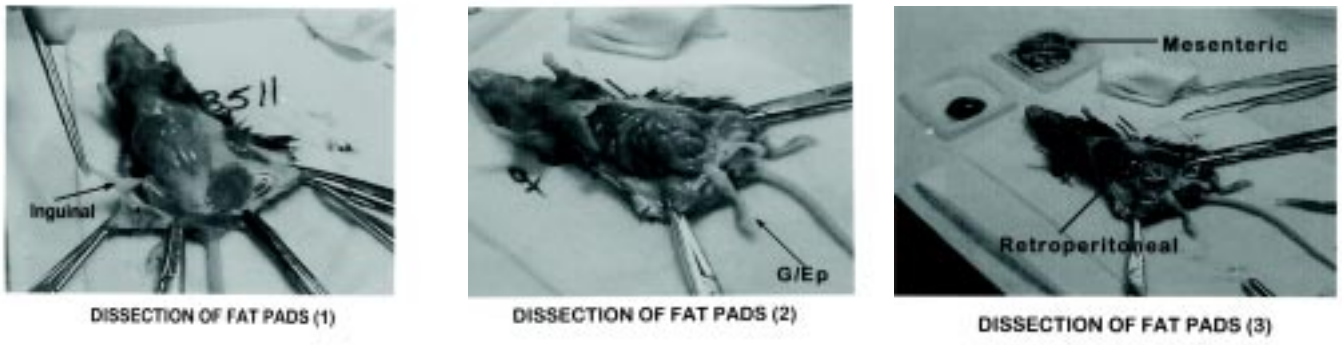


Figure 2. Fat depots collected and weighed during dissection. A) inguinal (subcutaneous) fat. B) epididymal (gonadal) fat. C) mesenteric and retroperitoneal fat depots.

Molecular Analysis

RNA was extracted from mouse tissues and quantitated using the protocol for Preparation of Total RNA Using the GIT CsCl Step-Gradient technique (Bultman 1990).

RT–PCR was used to analyze the level of expression of *Atp10c* in different mouse tissues, using b- actin and *Ube3a* as controls (See Dhar et al. 2000). ORNL primers 827/829 were used for Atp10C, 559/560 for b- actin, and 605/606 for *Ube3a*.

Biochemical Analysis

Glucose tolerance tests and insulin tolerance tests were used to determine the presence of diabetic phenotypes in the mice. The protocol for glucose tolerance testing was standardized as follows: mice were fasted overnight (at least 16 hours), after which a tail bleed was induced using a surgical blade. Blood glucose was measured (time 0) using the commercially available OneTouch Ultra Brand meter and FastTake test strips. Mice were then subcutaneously injected with a glucose solution (2 g/kg) and blood glucose readings were taken again at each of the following time points: 30, 60, 90, 120 and/or 150 min (dependent on whether glucose had returned to within the normal range). Data was entered into Microsoft Excel and line graphs were plotted of the average responses. Results were entered into StatView, undergoing the ANOVA Factorial test to determine statistical significance between pxpx and pxpl responses at each time point.

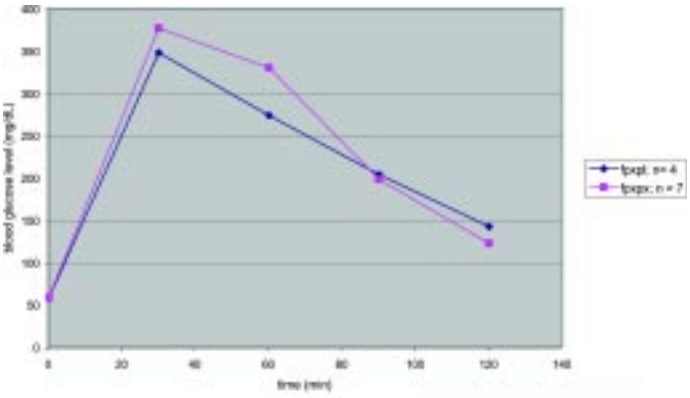


Figure 4. Glucose Tolerance Test (2 g/kg) in overnight fasted, 4-6 mo. 23D females on regular chow, pxpx vs. pxpl.

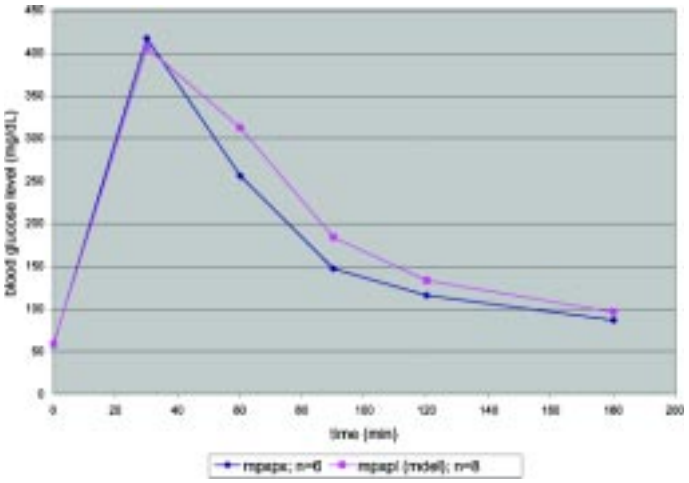


Figure 3. Glucose Tolerance (2 g/kg) Test in overnight fasted 10-12 mo. Old 23 D males on regular chow, pxpx vs. pxpl.

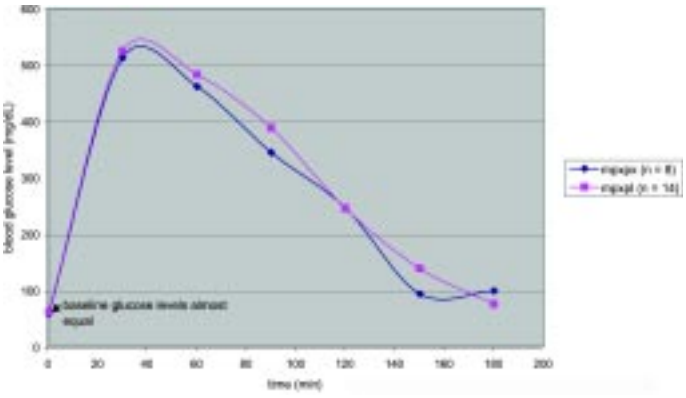


Figure 5. Glucose Tolerance Test (2 g/kg) in overnight fasted, 4-6 mo. 23D males on yellow diet, pxpx vs. pxpl.

RESULTS

This research initiated and standardized biochemical analyses for the study of insulin signaling and glucose homeostasis in $p^{23DFioD}$ mice on a custom-made diet. Figure 3 shows results of the glucose tolerance test (2 g/kg) in 10-12 month pxpx and pxpl males on LabDiet 5001; results for 4-6 month females on the same diet is shown in Figure 4. Figure 5 shows the glucose tolerance test in 4-6 month males on ResearchDiet D12450B, and Figure 6 shows results for the same test in 4-6 month females. No statistical significance exists between pxpx and pxpl averages at any time point in the glucose tolerance tests for males or females on either diet.

Insulin tolerance test results (0.75 U/kg, fasted 2 hours) for 10-12 month males on LabDiet 5001 are shown in Figure 7. The average blood glucose level in the mutants is significantly higher at 120 minutes ($p < 0.5$). Figure 8 shows the same test in 4-6 month old males on the custom-made diet, with no statistical significance at any time point. Insulin tolerance test results for 4-6 month females on the custom-made diet is shown in Figure 9. At 90 minutes, 3 out of 4 pxpx animals went into insulin shock and had to be injected subcutaneously with a glucose solution. For these animals, blood glucose level was not measured at 120 minutes. A lower dosage of insulin (0.375 U/kg) was used for the insulin tolerance test in 4-6 month males on the custom diet, shown in Figure 10. Here, the pxpl animals were significantly higher ($p = 0.0362$) at 120 minutes.

The RT-PCR expression profile for *Atp10c*, as compared to b- actin *Ube3a*, in a pxpx and pxpl mouse is shown in Figure 11.

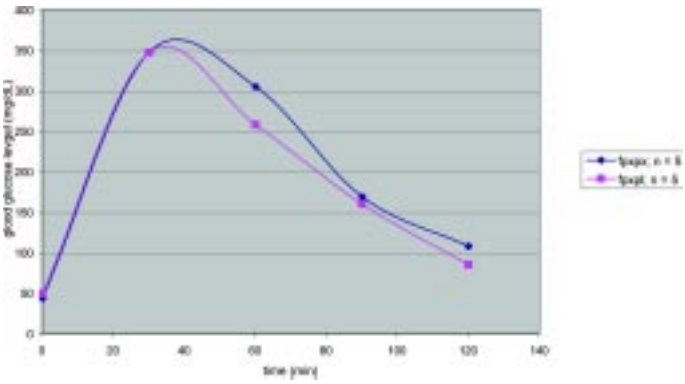


Figure 6. Glucose Tolerance Test (2 g/kg) in overnight-fasted, 4-6 mo. 23D females on yellow diet, pxpx vs. pxpl.

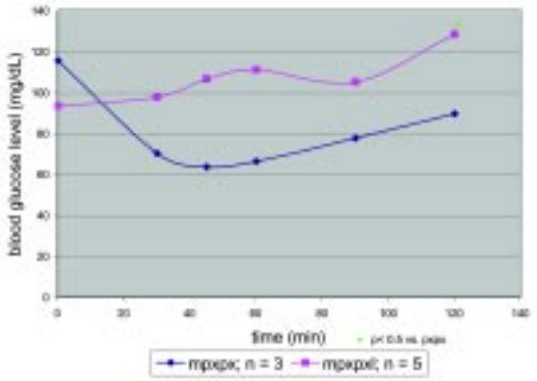


Figure 7. Insulin Tolerance Test (.075U/kg) in 2 hour fasted, 10-12 mo. 23D males on regular chow, pxpx vs. pxpl.

CONCLUSIONS

TESTS WERE INITIATED AND STANDARDIZED

Mice carrying a maternally inherited copy of the $p^{23DFioD}$ deletion have been shown to have significantly greater body fat than either the wild type or mice with a paternally inherited copy of the deletion. These studies were the first step towards looking at the effects of nutritional variables in the physiology of these mice. The experiments standardized in this work will further the investigation of a possible obesity-related, type 2 diabetic phenotype in this mouse model already recognized for polygenic obesity. Insulin and glucose tolerance tests were standardized for animals on the custom made diet because laboratory chow is not consistent enough from batch to batch to use in a study of nutritional variables. ResearchDiet D12450B contains a fixed amount of fat, carbohydrate, and protein, making it a better diet to use for nutritional comparisons.

Glucose tolerance tests appear normal while insulin tolerance tests suggest a trend of insulin resistance in $p^{23DFioD}$ mice.

In the glucose tolerance test, mutant and control mice were virtually indistinguishable in their responses, indicating that there is no apparent defect in glucose metabolism in these mice. The insulin tolerance tests, however, suggest that there may be some defect in the insulin response pathway related to the $p^{23DFioD}$ deletion. Males 10-12 months old on the regular chow show statistical significance ($p = .04$) as compared to the pxpx animals at 120 minutes in the insulin tolerance test. Their increased blood glucose levels at these latter time points could suggest insulin resistance- a phenotype associated with type 2 diabetes. Younger males on the custom-made diet showed significance at 120 minutes when tested with a lower dosage of insulin (0.375 U/kg), but did not exhibit the phenotype on the higher dosage (0.75 U/kg). This phenomenon may be understood by considering the use of insulin injections as a treatment for insulin resistance in type 2 diabetics. A decreased efficiency in insulin response is treated by essentially overwhelming the system

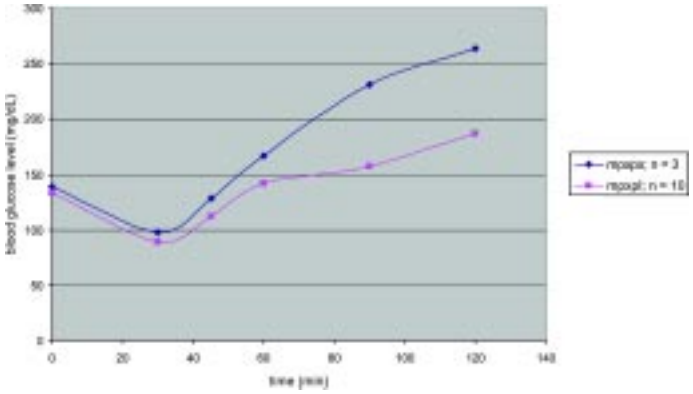


Figure 8. Insulin Tolerance Test (0.75U/kg) in 2 hour fasted, 4-6 mo. 23D males on yellow diet, pxpx vs. pxpl.

with insulin, maximizing the insulin induced transport of glucose from the bloodstream into the body cells by "occupying" every available insulin receptor with a steady supply of insulin. At the higher dosage, the younger mice may have been receiving a high enough concentration of insulin to mask any insulin resistance. In humans with type 2 diabetes, it is known that insulin resistance often increases with age- rendering lower dosages of insulin less effective in older patients. It may be for this reason that the 10-12 month males showed a trend of insulin resistance at the higher dosage.

Figure 7 illustrates the trend of insulin resistance seen in the 10-12 month old mice. Thirty minutes after the insulin injection, the blood sugar of the control mice dropped to an average of 65 mg/dL, while the mutants were maintaining levels near 100 mg/dL. A particularly interesting trend was found in females on the custom diet, shown in Figure 9. Three out of 4 controls were in danger of hypoglycemic shock 90 minutes after the insulin injection (0.75 U/kg) and had to be given glucose. All 4 mutants were still visibly active at this time, and while their blood sugar level was still lower than normal fasting level, they showed a signifisignificantly

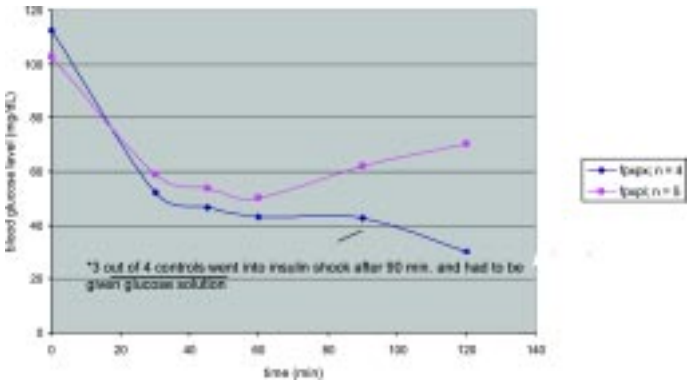


Figure 9. Insulin Tolerance Test (0.75U/kg) in 2 hour fasted, 4-6 mo. 23D females on yellow diet, pxpx vs. pxpl.

diminished response to the insulin. Although not statistically significant, the results from these females suggest also suggest a trend of insulin resistance. This data is preliminary; however, and more animals need to be added to these studies before conclusions can be made in regard to the role of the $p^{23DFioD}$ deletion in the glucose-insulin pathway.

Little is known about the biological role of translocases, such as the one coded for by *Atp10c*, other than that they somehow alter the orientation of receptor molecules in the cell membrane. Based on the knowledge of the cellular signaling involved in the insulin pathway, lipolysis, and general lipid metabolism, it seems reasonable to suggest that this class V putative aminophospholipid translocase may be involved in creating a diabetes-related insulin resistance, in addition to obesity. Kahn et al. (2000) suggest that even though insulin resistance is characteristic of obesity and type 2 diabetes, it may not be that all of insulin's actions are

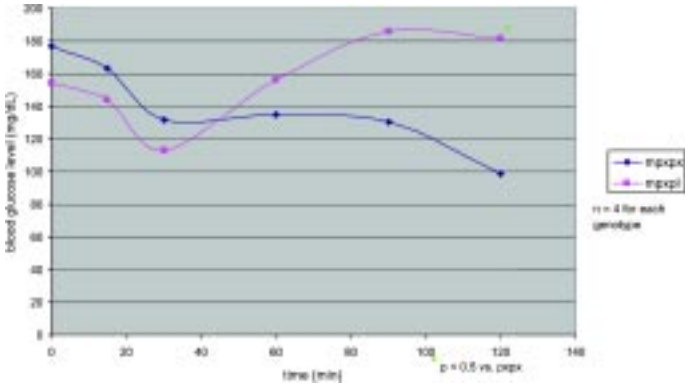


Figure 10. Insulin Tolerance Test (0.037U/kg) in 2 hours fasted, 4-6 mo. 23D males on yellow diet, pxpx vs. pxpl.

affected in people with these conditions. The varied roles of insulin in the body may explain why these mice show impaired insulin response in insulin tolerance tests while exhibiting a normal profile in the glucose tolerance tests. Glucose homeostasis involves complex pathways, and although these mice do not exhibit all the symptoms associated with type 2 diabetes, their abnormal responses in the insulin tolerance test may elucidate the role of *Atp10c* in physiological responses characteristic of obesity–related diabetes.

Mutant shows decreased Atp10c expression.

The RT-PCR results of the pxpl animal on the custom diet show decreased expression of *Atp10c* in liver, cerebellum, and inguinal and epididymal fat depots. Based on this pxpl animal, the observation can be made that a decreased amount of *Atp10c* in the fat depots is associated with increased body fat (larger fat depots). The inverse relationship seen between expression in the fat tissue and the size of the

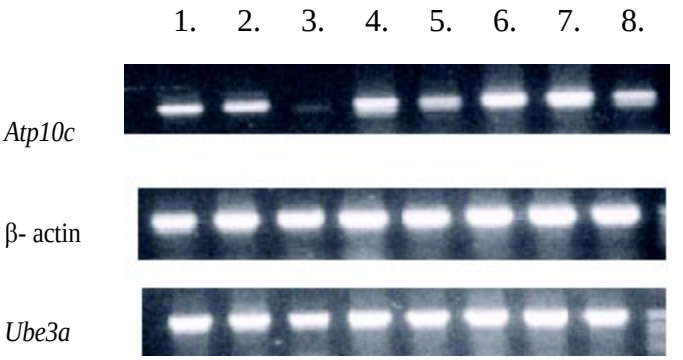
fat depots seems logical in light of *Atp10c*'s role in lipid metabolism.

These results, however, compare only one pxpx animal to one pxpl. These results need to be repeated before any conclusions are drawn. For example, the pxpx shows decreased expression in the pancreas as compared to the pxpl. It is debatable whether this decreased band intensity is truly due to lower expression or rather to degradation of the sample.

Future Directions

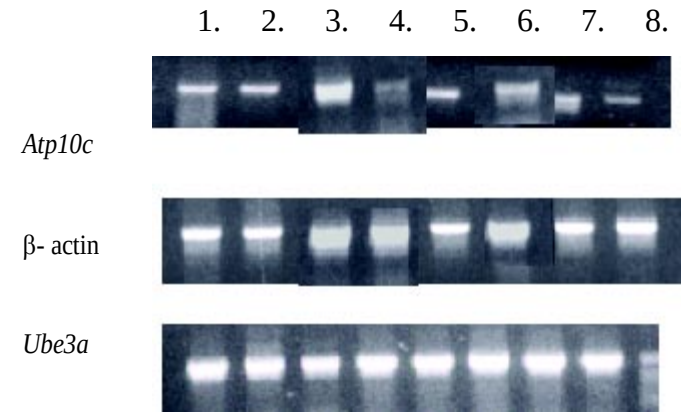
Now that the actions of glucose and insulin in relation to *Atp10c* for $p^{23DFioD}$ mice on a custom made diet have been studied, experiments are underway to observe the effects of nutritional variables. Several $p^{23DFioD}$ mice have been placed on a high fat (45% kcal) diet. Glucose and insulin tolerance tests are in progress for these animals, whose results will be compared to mice on ResearchDiet D12450B (10% kcal). High fat diets have been shown to increase adiposity and are known to aggravate symptoms of type 2 diabetes in humans. Preliminary tests in $p^{23DFioD}$ mice on the high fat diet for 4 weeks already show significant insulin resistance (from 30 minutes onward) and an abnormal profile in the glucose tolerance test. The hypothesis for these experiments is that the high fat diet will induce more severe diabetes-related phenotypes and increased fat depots in $p^{23DFioD}$ mice.

Until recently it has been difficult to generate many pups containing the longer deletion, p^{30PUb}- shown to cause a more severe form of obesity than that seen in the $p^{23DFioD}$ animals. These pups are now being bred more successfully and included in the high-fat diet study. Preliminary data from 2 p^{30PUb} animals (as compared to $p^{23DFioD}$ controls) show an abnormal glucose tolerance test, suggesting trends of hyperglycemia (p= 0.0014 at 0 min.) and glucose intolerance ($p < 0.5$ at 30 and 60 min.) in these mice. This is in addition to the insulin resistance and hyperinsulinemia also seen in the shorter deletion. Preliminary insulin tolerance tests in the p^{30PUb} animals show significant insulin resistance from 60 minutes onward (p = 0.00200 at 60 min., 0.0243 at 90 min., and 0.0021 at 120 min.). With the presence of p^{30PUb} controls and more animals carrying the deletion, future glucose and insulin tolerance tests results may show that these animals provide a better mouse model for obesity-induced type 2 diabetes in humans.



1. kidney
2. liver
3. pancreas
4. hippocampus
5. rest of brain
6. cerebellum
7. epididymal fat
8. inguinal fat

Expression profile from pxpx male on yellow diet.



1. pancreas
2. heart
3. rest of brain
4. inguinal fat
5. cerebellum
6. epididymal fat
7. hippocampus
8. liver

Expression profile from pxpl male on yellow diet.

Figure 11. RT-PCR Atp10c expression profiles for pxpx and pxpl mice on yellow Research Diet.

ACKNOWLEDGEMENTS

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Much credit is due to Carla Sommardahl and Erica Halterman for their expert veterinary advice and indispensable role in these experiments.

My gratitude also goes to the US Department of Energy and the National Science Foundation for funding the Energy Research Undergraduate Laboratory Fellowship (ERULF)- a program in which I have been honored to take part. I would also like to thank the staff of Oak Ridge Institute for Science and Education (ORISE)- especially Linda Holmes and Cheryl Terry- for all the work they have put into this summer's program.

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