The Role of Punicic Acid (c9t11c13-CLNA) in Lipid and Energy Metabolism of Mice

A Senior Honors Research Thesis

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ABSTRACT

Punicic acid (c9t11c13-CLNA) is a conjugated linolenic fatty acid found at 72% concentration in pomegranate seed oil. While research has elucidated the positive health benefits of punicic acid’s metabolite, c9t11-CLA, little is known about the physiological effects of punicic acid (PA). This senior honors research thesis aims to elucidate how punicic acid alters fat and energy metabolism in a mouse model. Male C57Bl6 mice were fed diets of 0% PA, 1% PA, or 2% PA for 4 or 16 weeks. We measured the extent to which punicic acid affected weight gain, food intake, food efficiency, insulin sensitivity, and fatty acid composition in mice. Diets containing increased levels of punicic acid resulted in a dose-responsive accumulation of conjugated linolenic acid in skeletal muscle, liver, and epididymal adipose tissues. In an effort to determine if punicic acid altered genes responsive to peroxisome proliferator-activated receptor-gamma (PPARγ), I investigated how punicic acid alters the expression of genes involved in lipid and energy metabolism. Levels of mRNA of AP2 (FABP4), UCP1, and PPARγ were not significantly altered in epididymal fat by dietary treatments during the 16-week feeding study, while expression of UCP2 was significantly increased in mice fed the 2% PA diet. It remains to be determined if punicic acid serves as a weak ligand for PPARγ. Understanding the mechanism of punicic acid and its effects on energy metabolism may ultimately improve recommendations for those battling obesity or the numerous diseases associated with it.

INTRODUCTION

An epidemic of obesity is threatening to undermine the health of people living in industrialized societies. Excess accumulation of adipose tissue is a major risk factor for insulin resistance, hyperglycemia, abnormal blood lipid profiles, and hypertension. All of these conditions contribute to cardiovascular disease, the leading cause of death in the Western world (Lehrke & Lazar, 2005). Obvious choices for reducing obesity include decreasing food intake and increasing physical activity. Rational
intervention for obesity requires an understanding of external factors, such as diet, as well as internal factors, such as adipocyte genes, development, and function. The overall goal of this senior honors research thesis is to characterize the ability of a dietary bioactive lipid, punicic acid, to influence diet, weight, and lipid metabolism in mice. Understanding the mechanism of punicic acid and its effects on energy metabolism may ultimately improve recommendations for those battling obesity or the numerous diseases associated with it.

The term, conjugated fatty acid, is a generic name for positional and geometric isomers of polyunsaturated fatty acids with conjugated double bonds (Arao et al., 2004). One such fatty acid with potentially beneficial biological effects is conjugated linoleic acid (c9t11-CLA), existing naturally in ruminant fats such as beef, lamb, and dairy products. The fatty acid c9t11-CLA has been found to have many favorable physiological benefits, such as anti-atherosclerosis, anti-obesity, and anti-tumor effects, as well as a role in regulating lipid metabolism (Belury, 2002). Conjugated fatty acids also exist in some plant seed oils. One such fatty acid is punicic acid (c9t11c13-CLNA), a conjugated linolenic fatty acid found at 72% concentration in pomegranate seed oil (Arao et al., 2004). Interestingly, punicic acid (PA) is metabolized to c9t11-CLA via a saturase mechanism, as shown in Figure 1 (Tsuzuki et al., 2006).

Studies have shown c9t11-CLA's ability to reduce adipose tissue in several animal models of obesity, as well as its ability to positively affect muscle mass (Steck et al., 2007). Mice with cancer cachexia

![Diagram](image)

**Figure 1. Pathway of Conversion of Punicic Acid (c9t11c13-CLNA) to c9t11-CLA**
(symptoms include weight loss, skeletal muscle wasting, and fatigue) were fed a diet supplemented with 0.5 % c9t11-CLA. Mice experienced reduced muscle wasting, indicating that c9t11-CNA has the ability to protect against muscle atrophy in mice (Graves, Hitt, Pariza, Cook, & McCarthy, 2005).

The physiological effects and mechanisms of action of punicic acid have not been well characterized. This study illuminates the metabolic effects of punicic acid on body weight, food intake, insulin sensitivity, and adipocyte differentiation. Mice were fed diets of 0% PA, 1% PA, or 2% PA for 4 or 16 weeks. In addition to in-vivo measurements, analysis of gene expression was conducted to further understand punicic acid's effect on lipid metabolism.

**MATERIALS AND METHODS**

**Animals and Diets.** Male C57Bl6 mice were purchased from Charles River Laboratories (Wilmington, MA) at 4 weeks of age. Mice were housed in the OSU vivarium 6 mice/cage, 1 cage/group (4-week study) and 4 mice/cage, 4 cages/group (16-week study) at 22 +/-0.5 degrees Celsius on a 12 hour day/night cycle. Upon arrival, mice were allowed free access to chow food and water for 5 days to allow for stabilization of stress and metabolism in the animals after delivery. Mice were weighed and randomized into diet groups. Semi-purified high-fat diets were prepared and pelleted by Research Diets Incorporated as shown in Table 1. In two separate studies, mice were fed experimental diets of punicic acid for 4 or 16 weeks. Body weights and food intake (fed minus remainder in cage) were measured three times weekly.

| Table 1. Composition of Experimental Diets (g/kg). High Fat Diet: 20 energy% of protein, 45.5 energy% of fat; Free access for 4 or 16 weeks. |
|----------------|----------------|----------------|
| Ingredient      | Control         | PA (1%)        | PA (2%)        |
| Casein          | 233.1           | 233.1          | 233.1          |
| Cornstarch      | 84.8            | 84.8           | 84.8           |
| Dextrose        | 116.5           | 116.5          | 116.5          |
| Sucrose         | 201.4           | 201.4          | 201.4          |
| Cellulose       | 58.3            | 58.3           | 58.3           |
| Lard            | 178.3           | 178.3          | 178.3          |
| Soybean oil     | 57.7            | 43.4           | 29.1           |
| Punicic acid (72% purity) | - | 14.3 | 26.6 |
| c9t11-CLA       | -               | -              | -              |
| t10c12-CLA      | -               | -              | -              |
| Salt Mix        | 52.4            | 52.4           | 52.4           |
| Vitamin Mix     | 11.7            | 11.7           | 11.7           |
| L-cystine       | 3.5             | 3.5            | 3.5            |
| Choline bitartrate | 2.3           | 2.3            | 2.3            |
| **Total**       | **1000.0**      | **1000.0**     | **1000.0**     |
**Insulin Tolerance Test (ITT).** The ITT was conducted at day 23 in the 4-week study in order to measure insulin sensitivity. Mice were fasted overnight for 12 hours and then injected intraperitoneally with 2 U/kg body weight insulin (Humulin® R, Eli Lilly and Co., Indianapolis, IN). Glucose was measured from tail vein blood using a One Touch Ultra® (LifeScan, Inc., Milpitas, CA) glucose meter immediately prior to insulin injection (time 0) and 15, 30, 45, 60, 90, and 120 minutes following the injection. Incremental area under the glucose curve (AUC) was calculated as the net area contained between individual baselines (set by the glucose value at time 0) and curves (Wolever, Jenkins, Jenkins, & Josse, 1991).

**Necropsy.** Mice were euthanized by overdose of isofluorane and cardiac puncture for exsanguinations. Excision of tissues included adipose depots, quadriceps muscle, and liver. Tissues were weighed and immediately frozen in liquid nitrogen for analysis of fatty acids and mRNA levels. The mass of each tissue was expressed as a percentage of body mass on the day of necropsy.

**Fatty Acid Analysis.** For quantifying fatty acid composition of liver, muscle, and epididymal fat, lipids were extracted by the modification of Folch and lipid extracts separated into neutral and phospholipids using a silica column procedure. Fatty acid methyl esters were prepared by incubating the fractions with tetramethylguanididine at 100°C and analyzed by gas chromatography using a 30m Omegawax 320 capillary column. Helium flow rate was 30 ml/min. Oven temperature was programmed to start at 175°C for 4 minutes then increase to 220°C at a rate of 3°C/min (Belury & Kempa-Steczko, 1997).

**Quantitative Real-time PCR.** Expression of RNA levels was analyzed by quantitative real-time PCR (Prism 7300 Sequence Detection System; Applied Biosystems, Foster City, CA, USA). Epididymal adipose was collected at necropsy and frozen at -80°C. Total RNA from epididymal adipose tissue was isolated using an RNeasy Lipid Tissue kit (Qiagen, Valencia, CA, USA). Total RNA was reversed transcribed with random hexamers using MultiScribe reverse transcriptase (Applied Biosystems). After cDNA synthesis, real-time PCR analysis was performed using predesigned primers and probes supplied by Applied Biosystems (TaqMan Gene Expression Assays, Applied Biosystems). The four primers investigated were: PPARγ, AP2 (FABP4), UCP1, and UCP2. Target gene expression was expressed as 2^{-DDct} using comparative CT method and normalized to the expression of 18S ribosomal RNA and the 0% PA control group (Livak & Schmittgen, 2001).
Statistical Analysis. All data are presented as mean ± standard error (s.e.). Comparisons were analyzed using one way ANOVA (MINITAB). Differences were considered significant at p < 0.05.

RESULTS

Body Weight, Food Intake, and Food Efficiency. Mice were fed diets composed of 0% PA, 1% PA, or 2% PA for 4 weeks or 16 weeks. In each of the two studies, there was evidence for increased body weight (Figures 2 and 3) and food intake (Figure 4) in the mice fed the 2% PA diet as compared to the 0% and 1% PA diet groups. Body weights for mice in the 2% PA feeding study during the 16-week study were significantly increased as compared to the 0% and 1% PA feeding groups. Additionally, food efficiency increased significantly in mice fed the 2% PA diet during the 16-week study (Figure 4). Quadriceps muscle mass decreased significantly in mice fed the 1% PA and 2% PA diets during the 4-week study. The liver and epididymal adipose tissue masses were not significantly different between feeding groups in the 4-week study (Figure 5).

Fatty Acid Composition. The decrease in quadriceps muscle mass was accompanied by a dose-responsive increase in the quantity of punicic acid (c9t11c13-CLNA) and its saturated metabolite, c9t11-CLA in mice fed the 1% PA and 2% PA diets. Tissues measured for fatty acid content were the quadriceps muscle and liver tissues of mice fed the 4-week PA diet (Table 2).

Likewise, in the 16-week feeding study, the epididymal adipose tissue increased in its fatty acid content of punicic acid (c9t11c13-CLNA) and c9t11-CLA in a dose-responsive manner (Table 2). Therefore, punicic acid has the ability to accumulate in the skeletal muscle, liver, and epididymal fat of mice.

Insulin Tolerance Test (ITT). In the ITT of the 4-week PA study, the blood glucose level decreased rapidly with insulin injections. The blood glucose levels after insulin injection differed significantly between the 2% PA and the 0% PA and 1% PA feeding groups (Figure 6). Beginning at 15 minutes after insulin injection, the blood glucose level for the 2% PA feeding group (at 123.7 mg/dl) remained lower than the glucose levels for both the 0% PA and 1% PA feeding groups throughout the 120 minute ITT. At 120 minutes after insulin injection, the glucose levels were as follows: 0% feeding group (135.7 mg/dl), 1% PA feeding group (152.8 mg/dl), and 2% PA feeding group (91.7 mg/dl). These data indicate that the 0% PA and 1% PA feeding groups were more insulin resistant and the 2% PA group was more insulin sensitive.
Figure 2. Body weights of 4-week (N=8) and 16-week (N=16) Punicic Acid Studies.
Figure 3. Change in Body Weight for 4-week (N=6) and 16-week (N=18) Punicic Acid Studies. Values for each measurement that have different letters are significantly different at p<0.05.
Figure 4. Food intake and Food Efficiency for 4-week (N=6) and 16-week (N=16) Puninic Acid Studies. Values for each measurement that have different letters are significantly different at p<0.05.
Figure 5. Liver, Quadriiceps, and Epididymal Tissue Weights for Purinic Acid 4-week Study. Values for each measurement that have different letters are significantly different at p<0.05.
Table 2. Percentage of Punicic Acid in Fatty Acid Analysis of Liver Tissue (4-week study), Quadriceps Muscle Tissue (4-week study), and Epididymal Adipose Tissue (16-week study).

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<td>in Liver</td>
<td>0.000% ± 0.000</td>
<td>0.958% ± 0.091</td>
<td>1.837% ± 0.277</td>
<td>0.010% ± 0.057</td>
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<td>% c9t11-CLA</td>
<td>0.344% ± 0.026</td>
<td>1.469% ± 0.067</td>
<td>2.813% ± 0.517</td>
<td>0.282% ± 0.036</td>
<td>1.385% ± 0.238</td>
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Expression of Genes Involved in Adipocyte Differentiation, Lipid Metabolism, and Energy Consumption. Quantitative Real-time PCR of epididymal adipose tissue from the 16-week PA feeding study was conducted using four genes: PPAR\(\gamma\), AP2, UCP1, and UCP2. PPAR\(\gamma\) is a transcription factor in the PPAR family. It plays a crucial role in adipocyte differentiation (Sharma & Staels, 2007). PPAR\(\gamma\) may up-regulate genes such as adipocyte protein-2 (AP2), otherwise known as fatty acid binding protein-4 (FABP4). AP2 is a fatty acid binding protein which facilitates the transfer of lipophilic molecules from the outer cell membrane to the intracellular PPAR\(\gamma\) receptor. AP2 regulates fatty acid uptake and intracellular transport and indicates the ability of a fatty acid to be incorporated into an adipocyte (Chmurzyńska, 2006). Lastly, UCP1 and UCP2 are uncoupling proteins. The former is found in the mitochondria of brown adipose tissue, the latter is expressed ubiquitously throughout the body, most significantly in skeletal muscle. Both are used to generate heat through the uncoupling of oxidative phosphorylation, thus enhancing energy dissipation (Konishi et al., 2008).

Results of RT-PCR analysis presented no significant differences in the expression of PPAR\(\gamma\), AP2, and UCP1 in epididymal fat between diet groups. In contrast, the expression of UCP2 was significantly increased in the epididymal fat tissue of the 2% PA feeding group (Figure 7).
**Figure 7.** RT-PCR analysis of 16-week PA feeding study epididymal adipose tissue. mRNA expression was measured for PPARγ, AP2, UCP1, and UCP2. Values for each measurement that have different letters are significantly different at p<0.05.
DISCUSSION

The in-vivo data from the 4-week and 16-week feeding studies show that punicic acid exerts physiological effects on mice. Mice fed the greatest concentrations of punicic acid (2%) experienced increased total body weight gain and reduced muscle mass. Food intake also increased for the 2% PA feeding group of both studies. These results were not expected given the ability of punicic acid’s metabolite, c9t11-CLA, to reduce adipose tissue in previous mouse and human studies (Steck et al., 2007). The mice fed 2% PA exhibited significantly increased body weights and food efficiency in the 16-week feeding study. This indicates that the mice fed 2% PA required less food to gain weight when compared to the 0% PA or 1% PA feeding groups. These data highlight the importance of time (16 weeks versus 4 weeks) in punicic acid’s ability to potentiate changes in body weight and food efficiency.

Fatty acid content was altered by higher punicic acid concentrations. Diets containing increased levels of punicic acid resulted in a dose-responsive accumulation of conjugated linolenic acid in skeletal muscle, liver, and epididymal fat of mice. While punicic acid accumulated in these tissues, it did not cause weight loss. In the 4-week study, quadriceps muscle mass decreased in the 1% PA and 2% PA feeding groups while liver and epididymal fat remained relatively constant between feeding groups. While tissue mass only decreased significantly for skeletal muscle, these data indicate a correlation between increased body weight and punicic acid accumulation in liver, skeletal muscle, and adipose tissue.

While punicic acid may not be an effective weight suppressant in mice, it is interesting to consider its potential insulin-sensitizing effect. As displayed in Figure 6, the ITT data from the 4-week PA feeding study show the 2% PA fed mice as the most insulin sensitive group. This presents a conundrum, since significant weight gain (as seen in the 2% PA feeding group) has been shown to lead to greater insulin resistance (Ruan & Pownall, 2009). In the 4-week study, the 2% PA feeding group showed increased insulin sensitivity in comparison to the 0% PA and 1% PA feeding groups. A future study designed to investigate the correlation between punicic acid, adipose tissue, and insulin sensitivity is warranted.

To help characterize the molecular mechanism of punicic acid, four genes involved in fat and energy metabolism were investigated at the RNA level. Because we observed an increase in body weight and insulin sensitivity in mice fed the highest dosage of punicic acid, we first investigated the transcription factor PPARγ. PPARγ is predominately expressed in an adipose-selective manner in both rodents and
humans (Hauner, 2002). As a master regulator of fat cell formation, PPARγ has emerged as a key controller of lipid metabolism and the progression of obesity (Lehrke & Lazar, 2005).

In addition, PPARγ has been shown to increase insulin sensitivity by adipocyte differentiation (Lee, Olson, & Evans, 2003). That is to say, PPARγ increases insulin sensitivity by storing fat where it belongs — in adipose tissue — rather than in non-adipose depots. PPARγ activation results in the up-regulation of genes involved in insulin sensitivity, which ultimately reduces the risk for cardiovascular disease.

As a member of the nuclear receptor family, PPARγ is modulated by direct binding of lipid metabolites. It has been reported that polyunsaturated fatty acids are weak ligands of PPARγ (Hauner, 2002). It remains to be determined if punicic acid is a ligand for PPARγ. Since the 2% PA feeding group increased in both body weight and insulin sensitivity, we were interested to see an increase in RNA levels of PPARγ in epididymal (white adipose) fat tissue. Interestingly, we saw no significant differences in PPARγ expression between diet groups. This indicates that perhaps the adipose cells were not differentiating and merely expanding in size.

To further study the effect of punicic acid on lipid metabolism, the RNA expression of AP2 was investigated. AP2 is a PPARγ responsive gene which regulates fatty acid uptake and intracellular transport (Chmurzyńska, 2006). Similar to PPARγ, it too showed no significant differences in RNA expression between diet groups. While this may lead one to believe that fatty acids were not taken up by adipocytes, it is important to note that AP2 is only one of several PPARγ responsive genes. To fully understand punicic acid’s influence on adipocyte differentiation, future studies should investigate the transcription of other PPARγ-responsive genes involved in glucose and lipid metabolism. These would include lipoprotein lipase, fatty acid transporter protein, fatty acyl-CoA synthase, malic enzyme, glucokinase, and GLUT4 glucose transporter (Hauner, 2002).

Additionally, PPARγ serves as a receptor for thiazolidinediones (TZDs), a class of oral anti-diabetic drugs which work through insulin sensitization. TZDs are the most potent triggers for adipocyte differentiation. These drugs help improve metabolic control in type II diabetics, which ultimately decreases the risk for obesity and cardiovascular disease (Hauner, 2002). TZDs reduce insulin resistance in adipose, muscle, and liver tissue. Interestingly, there is evidence from animal studies and clinical trials that treatment with TZDs also results in moderate weight gain, which could be a consequence of increased fat cell mass.
This phenomenon parallels with treatment of punicic acid in mice. Mice fed concentrations of 2% PA resulted in enhanced weight gain. As a potential weak ligand of PPARγ, punicic acid could exhibit a similar mode of action as that of TZDs. Further research must be done to clarify punicic acid’s binding affinity to PPARγ.

Punicic acid may also play a role in the metabolism of uncoupling protein-2 (UCP2). UCP2 is expressed ubiquitously throughout the body. It is expected to enhance energy dissipation in tissues such as white adipose (epididymal fat) (Kelly et al., 1998). The increase in body weight in the 2% PA fed mice could be attributed to the enhancement of energy dissipation mediated by UCP2 in mature adipocytes.

UCP2 maps to a region on distal mouse chromosome 7 that has been linked to the phenotypes of obesity and type II diabetes. Previous research reported increased UCP2 expression in adipose tissue by high-fat diet feeding (Surwit et al., 1998). Correspondingly, mice in the 4-week and 16-week punicic acid studies were fed a high-fat diet (24% by weight) of punicic acid. Together, these associations indicate that the consumption of a high fat diet in the form of punicic acid may selectively up-regulate UCP2 expression in white adipose tissue. This is important because an increase in UCP2 may correspond to increased oxidative stress. UCP2 has been suggested to affect the production of reactive oxygen species, which can ultimately damage DNA, lipid membranes, and tissues (Schrauwen & Hesselink, 2002). Determining exactly how UCP2 is related to energy metabolism and obesity in rodents and humans requires future study.

The mechanism for punicic acid contributing to obesity remains to be determined. Findings from this senior honors research thesis indicate that punicic acid has the ability to modulate weight gain, food intake, food efficiency, and energy metabolism in mice. Additionally, punicic acid could be an effective insulin-sensitizing agent as a ligand of PPARγ. Lower doses of punicic acid (1%) did not show significant levels of weight gain in mice. The biphasic response of this study indicates that the dosage of punicic acid is important in potentiating changes in metabolism. Future studies should investigate punicic acid’s ability to alter lipid and energy metabolism by changes in gene expression and insulin sensitivity.

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