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A MOUSE MODEL WITH CARNITINE PALMITOYLTRANSFERASE-1A DEFICIENCY REVEALS ITS CRUCIAL ROLES IN GESTATION AND GLUCOSE TOLERANCE

by

LARA RACHEL NYMAN

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2005

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ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree PhD Program Cellular and Molecular Physiology

Name of Candidate Lara Rachel Nyman

Committee Chair Philip A. Wood

Title A Mouse Model With Carnitine Palmitoyltransferase-1a Deficiency Reveals Its Crucial Roles in Gestation and Glucose Tolerance

A mouse model for carnitine palmitoyltransferase 1a (liver isoform, mouse gene = *Cpt1a*, protein = CPT-1a) was used to investigate the physiological function of CPT-1a and its role in human health and the potentially fatal pediatric disease caused by CPT-1a deficiency and investigate CPT-1a deficiency and its role in the development of impaired glucose tolerance. We used a replacement gene targeting strategy in ES cells that resulted in the deletion of exons 11-18, thus producing a null allele.

Homozygous deficient mice (CPT-1a-/-) were not viable. There were no CPT-1-/- pups, embryos, or fetuses detected from day 10 of gestation to term. The inheritance pattern from heterozygous matings was skewed and biased toward CPT-1a+/- mice (>80%). CPT-1a +/- mice had decreased *Cpt-1a* mRNA expression in liver, heart, brain, testis, kidney, and white fat. This resulted in 54.7% CPT-1 activity in liver from CPT-1a+/- males as compared to CPT-1a+/+ controls. Fasting free fatty acid concentrations were significantly elevated, while blood glucose concentrations were significantly lower in 6-week-old CPT-1a+/- mice compared to controls.

The CPT-1a+/- mouse model was used investigate the link between excess fat and the pathogenesis of type 2 diabetes. Male CPT-1a+/- mice were found to be more insulin sensitive when fed a high-carbohydrate diet (HCD) or a high-fat diet (HFD). This was

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associated with increased expression of *Cpt-1b* (muscle isoform) in liver and possibly lower pyruvate dehydrogenase kinase-4 expression in skeletal muscle as compared to CPT-1a+/+ mice. Lower fasting serum insulin levels in CPT-1a+/- mice fed HFD at both 7 months and 12-14 months further supported this result demonstrating higher glucose sensitivity than CPT-1a+/+ mice. CPT-1a+/- mice were more glucose tolerant when fed the HCD but less glucose tolerant when fed the HFD. Furthermore, CPT-1a+/- mice fed HFD or HCD had fewer and smaller pancreatic islets identified by β-cell staining for insulin. Overall, these findings suggest that expression of both CPT-1a and CPT-1b may play a role in the pathophysiology of the development of type 2 diabetes and are influenced further by diet.

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INTRODUCTION

HOMOZOGOUS CARNITINE PALMITOYLTRANSFERASE 1A (LIVER-ISOFORM) DEFICIENCY IS LETHAL IN THE MOUSE

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CARNITINE PALMITOYLTRANSFERASE
1A (CPT-1A) AFFECTS GLUCOSE AND INSULIN SENSITIVITY

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LIST OF ABBRIEVIATIONS

ACADM	medium chain acyl-CoA dehydrogenase gene
ACOX-1	acyl-coenzyme A oxidase-1
ACC	acetyl-CoA carboxylase
BMI	body mass index
BSA	Bovine serum albumin
B6;129	C57BL/6NTac, 129S6/SvEvTac
129	129S6/SvEvTac
CPT	Carnitine palmitoyltransferase
DAG	Diacylglycerols
DM2	type 2 diabetes mellitus
DXA	Dual-energy x-ray absorptiometry
FISH	Fluorescence in situ hybridization
GLUT 4	Glucose transporter 4
GSIS	Glucose stimulated insulin secretion
ES	Embryonic Stem
FAO	Fatty acid oxidation
HCD	High-carbohydrate diet
HE	Hematoxylin and eosin
HFD	High-fat diet
IGT	Impaired glucose tolerance

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IR	Insulin resistance
ITT	Insulin tolerance test
LCCoA	Long-chain acyl-CoA
NEFA	Non-esterified fatty acids
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
PDH	Pyruvate dehydrogenase complex
PDK 2 & 4	Pyruvate dehydrogenase kinase isoforms 2 and 4
PPAR- α	Peroxisomal proliferator activator receptor α
SRD	Standard rodent diet
TG	Triglycerides
VLDL	Very low-density lipoprotein

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INTRODUCTION

Fully functional and properly regulated fatty acid metabolism is essential for health. The function and regulation of this pathway are intimately tied to the genetics of the individual along with his or her environment and activity level. The incidence of abnormal health conditions associated with excess fat including obesity, dyslipidemia, insulin resistance, metabolic syndrome, and type 2 diabetes mellitus (DM2) are increasing at alarming rates. The common theme of this dissertation is the investigation of the genetic influences of a pivotal step of mitochondrial fatty acid β-oxidation, namely carnitine palmitoyltransferase-1a (CPT-1a-liver isoform) and the environmental stresses of high-fat and high-carbohydrate diets.

In order to set up the background scenario of the studies described in this dissertation, I will provide an overview of mitochondrial fatty acid oxidation (FAO), the crucial role that CPT-1 plays in regulating this important pathway, what happens when there is an inherited deficiency of CPT-1, and finally how this step and its downstream pathway are decisive components in normal glucose tolerance and insulin sensitivity, which ultimately are tied with development of DM2.

CPT-1 and fatty acid oxidation

CPT-1 activity is crucial for normal processes of FAO. FAO is an essential metabolic process by which fat (triglyceride) is broken down into free fatty acids and glycerol. The fatty acids are used for fuel. The energy available from fat storage far exceeds that of

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protein, glycogen, or glucose. It not only has a higher energy yield per gram but also has greater storage capacity. Fat is mainly stored as triglyceride in adipose tissue although it is also stored in smaller amounts, at least in the healthy individual, in muscle. Fatty acid oxidation, an intramitochondrial process, is a critical provider of nonglucose sources of energy in the fasted state and is responsible for supplying 80% to 90% of cellular energy requirements during prolonged fasting [1]. Additionally, although most tissues are reliant on FAO to provide cellular energy during the fasted state, long-chain FAO is the primary source of energy for heart and skeletal muscle in both the fed and fasting state [2]. Furthermore, infants, who generally have limited glycogen stores, are heavily reliant on long-chain FAO for energy [3]. Therefore, if there is any impairment in the fatty acid oxidation process, it may lead to hypoglycemia as it does in children who are deficient in FAO enzymes vital in this pathway.

Steps of FAO

FAO is a metabolic process consisting of three major components: 1) cellular uptake and activation of fatty acids, 2) carnitine-acylcarnitine cycle across the mitochondrial membrane, and 3) β-oxidation pathway spiral. Upon fasting, long-chain fatty acids are mobilized from adipose tissue and transported into circulation, mostly bound to albumin. Fatty acids are taken up by the liver and other tissues and activated to form acyl-CoAs in the cytosol of the cell. This step allows the fatty acid to become an active intermediate and thus permits its continuance toward β-oxidation.

Acyl-CoA is then processed through the carnitine cycle. First, CPT-1 catalyzes the transfer of an acyl group to a carnitine, forming the acyl-carnitine substrate. More-

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over, the generation of acyl-carnitine sanctions the transport across the mitochondrial membrane via carnitine-acylcarnitine translocase. Carnitine palmitoyltransferase-2 then catalyzes the conversion of acyl-carnitine back to the acyl-CoA form, allowing it to proceed with the succeeding steps of the mitochondrial β-oxidation spiral pathway (Fig. 1).

The β-oxidation pathway spiral consists of a series of dehydrogenation and other (hydratase and thiolase) steps which are depicted in Fig. 2. The dehydrogenation steps serve as a series of reactions that result in shorter chain lengths of very long chain acyl-CoAs, long-chain acyl-CoAs, medium-chain acyl-CoAs, and short-chain acyl-CoAs. Finally, 3-ketoacyl-CoA thiolase catalyzes the formation of a shorter form of acyl-CoA and acetyl CoA through this thiolysis reaction. Thus, the products of a completed cycle of β-oxidation include acetyl-CoA and a two carbon shorter form of acyl-CoA. These end products are further utilized to provide energy required to maintain metabolic homeostasis. Acetyl-CoA can be advanced into the tricarboxylic acid cycle for the generation of more ATP or it can be used for the production of ketones, which is of grave importance to organs such as brain and heart, notably in times of fasting. An inherited enzyme deficiency within the FAO pathway can lead to fasting intolerance, diarrhea, vomiting, and Reye-syndrome-like disease (a disease primarily characterized by vomiting, diarrhea, hypoglycemia, and hyperammonemia) [4].

CPT-1 expression

CPT-1 is expressed as different isoforms in virtually all tissues in the body. In mice, rats, and humans, CPT-1a is known as the liver isoform because it is the predominant isoform expressed in liver, kidney, islet, lung, spleen, brain, intestine, and ovary

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[5,6]. CPT-1b is known as the muscle isoform because it is the primary isoform expressed in skeletal muscle, heart, testis, and both brown and white adipose tissue, except in mice where CPT-1a predominates in white adipose tissue [5,6]. CPT-1c, a third isoform recently described, has been found to be expressed predominantly in brain [7].

Dysfunction of carnitine palmitoyltransferase 1 activity may be pivotal in the development of multiple disease processes

CPT-1 is a vital enzyme needed for proper metabolic function. In children with inherited CPT-1a deficiency, there is a high risk for Reye syndrome-like disease and potential death [8]. Patients with CPT-1b deficiency have never been identified, implicating it as an enzyme crucial for life. CPT-1a deficiency may also play a crucial role in the development of impaired glucose tolerance which is thought to be the earliest identifiable metabolic abnormality in the pathogenesis of DM2 [9]. The focus of the first manuscript will include studies using the mouse model with a null allele of CPT-1a in order to understand the physiological function of CPT-1a and its role in the potentially fatal pediatric disease caused by CPT-1a deficiency. The second manuscript will include the role of CPT-1a deficiency in the development of impaired glucose tolerance (IGT) and DM2. Finally, in the conclusions section, I summarize the collective information from these studies and discuss the implications of this work and speculate about future studies.

Lipogenesis and CPT-1 inhibition by malonyl-CoA

Malonyl-CoA, the product of acetyl-CoA carboxylase (ACC), and also a substrate of the opposing pathway of *de novo* fatty acid synthesis, potently inhibits both CPT-1a and b. Thus, in the liver, during a fed state, when ACC activity is high, malonyl-CoA

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production increases, and CPT-1a is inhibited. Consequently, this promotes fatty acid synthesis, or lipogenesis [10].

Lipogenesis occurs in the cytosol. *De novo* synthesis of fatty acids occurs in liver, mammary gland, brain, lung, kidney, and adipose tissue. This process includes the carboxylation of acetyl-CoA to form malonyl-CoA which takes place in the presence of ATP and ACC [11]. The reaction includes both the carboxylation of the biotin component with ATP and the transfer of the carboxyl group to acetyl-CoA to form malonyl-CoA [11]. The end product of this process is free palmitate, a long-chain fatty acid. Both citrate and insulin regulate this process by activating ACC by dephosphorylation. Thus, the formation of malonyl-CoA, both a CPT-1 inhibitor and a building block for fatty acid synthesis, is increased by insulin [11].

Regulation, kinetics, and sensitivity to inhibitor

Beyond transcriptional regulation, CPT-1 activity is regulated by at least four mechanisms: 1) changes in malonyl-CoA levels, 2) changes in malonyl CoA sensitivity, 3) variations in enzyme activity assumed to be due to enzyme synthesis or breakdown [12], and 4) sensitivity of the enzyme to the fluidity of its inherent membrane environment [13]. CPT-1a is thought to be regulated both by changes in malonyl-CoA levels and by changes in phosphorylation by protein kinase CKII [14] which are thought to lead to changes in sensitivity to malonyl-CoA inhibition [14]. Alternatively, CPT-1b is thought to be regulated solely by changes in malonyl-CoA levels [14].

In most mammals, CPT-1a has been found to be less sensitive to the inhibitory effects of malonyl-CoA and have a greater affinity for carnitine than CPT-1b [15]. Fur-

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thermore, the production and clearance of malonyl-CoA may differ depending on the tissue in which it is produced. Moreover, it is important to note that ACC, the producer of malonyl-CoA, is also expressed in two different isoforms [16]. Similar to CPT-1, there are different tissues in which each isoform (ACC-1 or ACC-2) is the primary isoform expressed. This is important to note because ACC-2 is the predominant isoform expressed in primarily the same tissues (oxidative tissues) in which CPT-1b is expressed predominantly. The same is true for CPT-1a and ACC-1, that is, they are both the primary isoforms expressed in lipogenic tissues [17]. Therefore, although CPT-1b has greater sensitivity to malonyl-CoA inhibition, ACC-1, the producer of malonyl-CoA, has lower sensitivity to insulin and therefore is less responsive to insulin than ACC-2. In brief, the location and milieu of CPT-1 may also be contributing factors in its regulation either directly or indirectly.

Inherited CPT-1a deficiency

Inborn errors of fatty acid oxidation cause severe metabolic disturbances predominantly in young children. CPT-1a (gene = *CPT-1a*, protein = CPT-1a) deficiency has been found in more than 30 patients [18]. Disease occurs most commonly in children 0-2 years [19]. CPT-1a deficient patients generally have CPT-1a activity ranging from less than 1% to 34% of controls. CPT-1a deficiency in liver is often characterized by fasting hypoglycemia, hypoketonemia, fatty liver, and Reye syndrome-like disease, as well as sudden death in infants 8-18 months old [20]. For the most part, acute effects in young patients are reported to recede with aging. Pathogenesis is unclear, although unquestionably, metabolic deficiencies involving fat metabolism exist in the disease.

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Abberant fatty acid metabolism is pivotal in the developmental aspects of type 2 diabetes mellitus

In parallel to the pediatric disease of CPT-1a deficiency, the disease process of DM2 is intimately linked to aberrant fatty acid metabolism. This is demonstrated by a disproportionate amount of pre-diabetic individuals presenting with elevated free fatty acids and triglycerides, together with excessive deposition of fat in nonlipogenic tissues such as skeletal muscle. Impaired CPT-1a activity may be a critical player linking aberrant fatty acid metabolism to disease. It has been proposed that altered lipid metabolism may in fact be pivotal in the development of DM2 [21].

Type 2 diabetes affects 2-5% of the world's population [22], and yet its etiology still remains unclear. Although the primary causes are thought to be polygenic [23] and heterogeneous [24], DM2 patients commonly present with similar disease. Impaired glucose tolerance as a result of reduced glucose and insulin sensitivity, excessively high rates of hepatic glucose production [25], and abnormal insulin secretory patterns [26] are trademarks of the early stages of disease. Deranged fatty acid metabolism has been linked to the disturbance in glucose uptake, abnormal insulin secretory patterns, and excessive glucose production.

Insulin sensitivity is a measure of responsiveness to the insulin secreted by the β-cells

Insulin sensitivity reflects the potency of insulin action which includes glucose uptake, inhibition of lypolysis, and inhibition of hepatic glucose output to name a few. Both insulin resistance and β-cell dysfunction contribute to the development of DM2.

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Glucose sensitivity is a measure of β-cell responsiveness to glucose

Glucose sensitivity reflects the responsiveness of the β-cells to an increase in blood glucose. Indices of β-cell responsiveness include the magnitude of insulin release, the degree of sensitivity to the stimulus, the amplification range of the response, and the pulsatory rate of oscillations of the secretion of insulin [28]. Interpretations of glucose tolerance tests vary; however, many logically attribute at least the initial 30 min of any change in glucose levels after administration to glucose sensitivity or β-cell responsiveness.

Impaired glucose tolerance is the earliest identifiable defect seen in the development of DM2 [6]

Glucose tolerance is thought to be the functional combination of both glucose and insulin sensitivity. Although, there are some who believe that excursions from the glucose curve after glucose administration are best explained by β-cell responsiveness to increases in blood glucose [27].

Insulin resistance in DM2

Insulin resistance has been defined as a reduced capacity of insulin to elicit insulin action. Insulin resistance is thought to be pivotal in the development of DM2. It is also thought to be associated with obesity, dyslipidemia, and several other disease states all having in common an accumulation of excess fat in tissue. Although the latter fact seems agreed upon without controversy, the temporal sequence remains unclear. Several studies have attempted to elucidate the role of aberrant fatty acid metabolism in the development of insulin resistance (IR).

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Elevated glucose levels in DM2

Blood glucose levels in patients with overt DM2 are typically elevated. Factors that may contribute to the elevations in blood glucose beyond those accounted for by secretory dysfunction of insulin and impaired glucose uptake may include a higher rate of hepatic glucose production (also thought to be due to insulin resistance) or impaired glucose clearance rates in diabetic patients compared with non-diabetic patients. Recent evidence suggested that the excess rates of glucose production may be accounted for by a 60% increase in hepatic gluconeogenesis [29]. Thus, the excessive production of glucose may partially induce or exacerbate the situation in which formerly appropriate levels of insulin secretion are no longer able to meet the demands needed for blood glucose regulation.

β-cell dysfunction in and before DM2

β-cell failure is commonly thought of as a state in which the amount of insulin secretion is not sufficient to meet the demands required to maintain normal blood glucose levels. However, it is also important to note that the beginning stage of DM2, also known as the "pre-diabetic stage", commonly includes hyperregulated or dysregulated secretion of insulin despite normal blood glucose levels [21]. Some studies suggest that IR, including both reduced peripheral uptake as well as a compromised ability to moderate hepatic glucose output, may be the cause of β-cell failure. Alternatively, some believe that there is a considerable body of evidence indicating that abnormal insulin, that is high or low insulin secretion, depending on stage of disease, is the primary defect [30]. In further support of this view are numerous studies finding that individuals with impaired glucose

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tolerance (commonly thought to be the "pre-diabetic state") already have impaired insulin secretion [30]. Regardless of whether one comes before the other, there is strong evidence supporting fatty acid involvement in both IR and β-cell dysfunction.

Fatty acid metabolites in insulin resistance and abnormal insulin secretion

Evidence suggests that impaired fatty acid metabolism may play a role in the malfunctioning insulin secretory process even in the premature stages of the disease [31]. The mechanism proposed is similar to that proposed for the mechanism by which insulin resistance in muscle has been shown to occur. In skeletal muscle, a fatty acyl-CoA induced reduction in GLUT 4 (glucose transporter) activity or recruitment has been implicated in the development of skeletal muscle insulin resistance. Lipids are also involved in β-cell signaling and the stimulation of insulin release. The intracellular concentration of these lipids, derived from the blood, glucose metabolism, or endogenous lipids [32], is critical for understanding both hypersecretion and hyposecretion and the pulsatile nature in which insulin is released. However, studies have recently suggested that excessive intracellular lipid content alone may not account for the abnormalities, while it may act as a marker for the presence of other possibly disparaging fatty acid metabolites [33]. These metabolites may provide a mechanistic link between increased intracellular lipid availability and deranged insulin secretion as well as impaired glucose utilization in tissues.

It is well accepted that long-chain acyl-CoAs (LCCoA) and diacylglycerols (DAG) play regulatory roles in several steps within cellular and molecular processes involved in the disease pathogenesis of DM2. Predictably, a defect in CPT-1 may first lead to an accumulation of LCCoAs and DAGs and possibly ceramides in the cytosol of both

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the β-cell and skeletal muscle cell. This may result in interference with several steps in β-cell metabolism, trafficking, and pro-insulin synthesis via stimulatory and inhibitory changes in protein kinase expression or action. As is seen in the literature repeatedly, functions of protein kinases are crucial for the maintenance of proper uptake of glucose into cells and the insulin secretion process [22].

Drug therapy indicates a pivotal role for aberrant fatty acid oxidation in type 2 diabetes

Therapeutic effects of pharmacological agents have enabled insight into disease processes leading to a particular system's failure. Metformin is widely used as an insulin sensitizer in the treatment of DM2 [22]. Effects of metformin include a reduction in blood glucose, inhibition of adipose tissue lipolysis, a decrease in free fatty acids, and a reduction in very low-density lipoprotein (VLDL) production [22]. Some of the known actions of metformin include an increase in insulin sensitivity in muscle and liver, decreased hepatic glucose production (gluconeogenesis), and an increase in peripheral glucose uptake. Another major effect of metformin is a reduction in malonyl-CoA, which is a potent CPT-1 inhibitor, resulting in less inhibition of FAO or more CPT-1 activity. Since metformin does not promote weight gain or hypoglycemia while acting independent of insulin secretion, its mechanisms of action may be extremely useful for elucidating the role for CPT-1 in insulin sensitivity.

C75, an inhibitor of fatty acid synthase and a newly discovered CPT-1 agonist, has recently been suggested to have profound effect on weight loss, diminished adipose mass, and resolution of fatty liver. Although excess body fat is not directly linked to DM2 in that not everyone who has DM2 is obese, weight loss has a profound effect on

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insulin sensitivity. C75 is thought to act by way of increasing CPT-1 activity, fatty acid oxidation, and energy production resulting in a reduction of adipose tissue and liver fat and weight loss. Furthermore, *in vivo* inhibition of CPT-1 by etomoxir has been shown to reverse the C75 induced increase in energy expenditure [22]. Evidence suggests that CPT-1 is an ideal target for investigating a role of obesity in DM2. The results from studies investigating the mechanism of C75 further confirm a role for CPT-1 in the development of DM2 and, more importantly, the need to look further at the mechanisms involved.

CPT-1 inhibitors and insulin resistance

Dobbins et al. showed that prolonged inhibition of both CPT-1a and CPT-1b promoted lipid accumulation and insulin resistance in rats [34]. The significance of the study was based on previous findings suggesting that insulin resistance correlated most strongly with intramuscular triglyceride levels rather than body mass index (BMI), percent body fat, waist to hip ratio, or age. Further confirming the need for their study was work showing that prolonged exposure to diets promoting for fat storage was central in the development of insulin resistance, caused by a build-up of fatty acyl-CoAs in skeletal muscle. McGarry's group used both diet and pharmacological methods to promote the accumulation of lipids. Etomoxir, a CPT-1 inhibitor (both a and b isoforms), was used to block the entry of fatty acids into the mitochondria. The study compared four groups: the first group consisted of rats on a low-fat diet for four weeks, and the second group of rats were on a high-fat diet. The third group had rats on a low-fat diet with etomoxir and the fourth included rats on a high-fat diet with etomoxir. They found that etomoxir signifi-

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cantly impaired insulin sensitivity (measured by hyperinsulinemic-euglycemic clamp). They also found that etomoxir alone significantly reduced insulin-mediated glucose uptake in skeletal muscle by comparing changes in the diet alone groups to those with diet and etomoxir. These results support the hypothesis that CPT-1 and aberrant FAO play a significant role in metabolic derangements found in the development of DM2.

CPT-1 inhibitors and insulin secretion

CPT-1 may also modulate insulin secretion [35]. Although glucose is the most potent stimulus for insulin secretion, transient elevations in fatty acids have been shown to enhance glucose stimulated insulin secretion, although prolonged elevations have been shown to decrease the β-cell's sensitivity to glucose [36]. Results with CPT-1a inhibitors [24] reported an increase in insulin secretion. Moreover, an increase in glucose-stimulated insulin secretion has been demonstrated to be accompanied by an increase in malonyl-CoA levels and an increase in the cytosolic concentration of LCCoA esters. Prentki et al. [37] suggested that the accumulation of cytosolic LCCoAs might be explained by a decrease in β-oxidation. Etomoxir has been used to inhibit CPT-1 and has also been found to potentiate glucose stimulated insulin secretion (GSIS) in the pancreas while inhibiting FAO in islets. These results further support evidence suggesting a pivotal role for CPT-1 in GSIS of the normal β-cell, along with attendant elevation of cytosolic LCCoA concentration [38]. Moreover, studies using CPT-1 inhibitors to suppress endogenous fatty acid oxidation are also powerful stimulators of insulin secretion [39]. In summary, excess fatty acids that are not being oxidized, most likely due to a decreased

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flux through β-oxidation brought about by a decrease in CPT-1 activity, may have varying effects on insulin secretion depending on the duration of exposure.

Rationale for role of CPT-1 in DM2 study

In order to provide sufficient evidence that the following study is indeed necessary and worthy, evidence revealing some of the limitations of previous studies and how these gaps will be filled are provided below:

1) Chemical inhibitors of FAO may not be specific to CPT-1a and instead may include inhibition of CPT-1b and CPT-2, making it difficult to dissect which is responsible for the effect. Additionally, insulin secretion studies demonstrating increases in GSIS using CPT-1 inhibitors have been criticized. The criticism has been that compounds such as etomoxir and 2-bromopalmitate are modified fatty acids and may act as endogenous lipids initiating the increase in GSIS independent of CPT-1 inhibition [40].

2) High-fat diets are often not isocaloric to the control low-fat diets (ex. low-fat diet = 3.5 kcal/g and high-fat diet = 4.4 kcal/g).

3) Durations of the different studies looking at some of these issues may not have been sufficient to elucidate the role of deranged fatty acid metabolism in the development of DM2.

4) Further limitations include studies of β-cell dysfunction. Researchers have predicted that a build-up of fatty acyl-CoAs within the muscle cell may be responsible for the insulin signaling dysfunction. Various studies investigating β-cells *in vitro* have suggested that proper function is dependent on the fatty acid or fat acid metabolite content

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within. To our knowledge, no group so far has looked at the role of β-cell acyl-CoA content *in vivo* and the pathogenesis of β-cell dysfunction.

Summary

In summary, the background information presented here confirms inherited CPT-1a deficiency is a pediatric disease that results in severe metabolic alterations. Moreover, these metabolic alterations share similarities with common biochemical markers of insulin resistance. We postulated that aberrant FAO is pivotal in the development of IR and that CPT-1 is a key player. However, the information reviewed here also demonstrates the need to further examine excessive fatty acyl-CoA build-up and whether CPT-1a or CPT-1b activity is critical to its prevention. Results produced by metformin, C75, diet, exercise, and etomoxir in rats and β cells contribute to forming the hypothesis that a mouse with a genetic deficiency in CPT-1a may be an excellent model for elucidating some of the mechanistic alterations that induce disease states such as insulin resistance and β-cell dysfunction. If an increase in CPT-1 activity (metformin induced) helps alleviate factors involved in the development of DM2 and inhibition of CPT-1 (etomoxir induced) promotes IR and impaired glucose tolerance, we would predict that a genetic (CPT-1a) enzyme deficiency would induce or exacerbate disease.

β-cell dysfunction and peripheral IR in the early stages of obesity-related DM2 coexist [22] and may eventually lead to loss of glucose-stimulated insulin secretion and frank diabetes. Because excess fat or, more precisely, a build-up of intracellular fatty acid metabolites has been implicated in each one of these derangements, we proposed that all three abnormalities may be secondary to a single primary defect [41]. In order to eluci-

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date the mechanisms underlying the link between IR and intracellular fatty acyl-CoA build-up, we have developed a heterozygous gene knockout mouse model for CPT-1a, which is a key enzyme in the regulation of intracellular long-chain fatty acid oxidation.

I hypothesized that CPT-1a, the isoform expressed in both liver and β-cell, may play a critical role in the development of both insulin resistance and β-cell dysfunction. I expected a genetically deficient CPT-1a mouse model to help elucidate some of the mechanisms by which diabetic patients develop this progressive disease, specifically, the mechanism by which a build-up of fatty acid intermediates in the cytosol may interfere with normal insulin secretion and glucose uptake. I predicted that pathogenesis occurring in the β-cell and in those cells involved in peripheral glucose uptake coexist, leading to abnormal secretory patterns and peripheral insulin resistance and eventually hyperglycemia, indicating frank DM2. We proposed using this excellent mouse model to get at the mechanistic link between CPT-1, increased lipid availability, and the pathogenesis of DM2.

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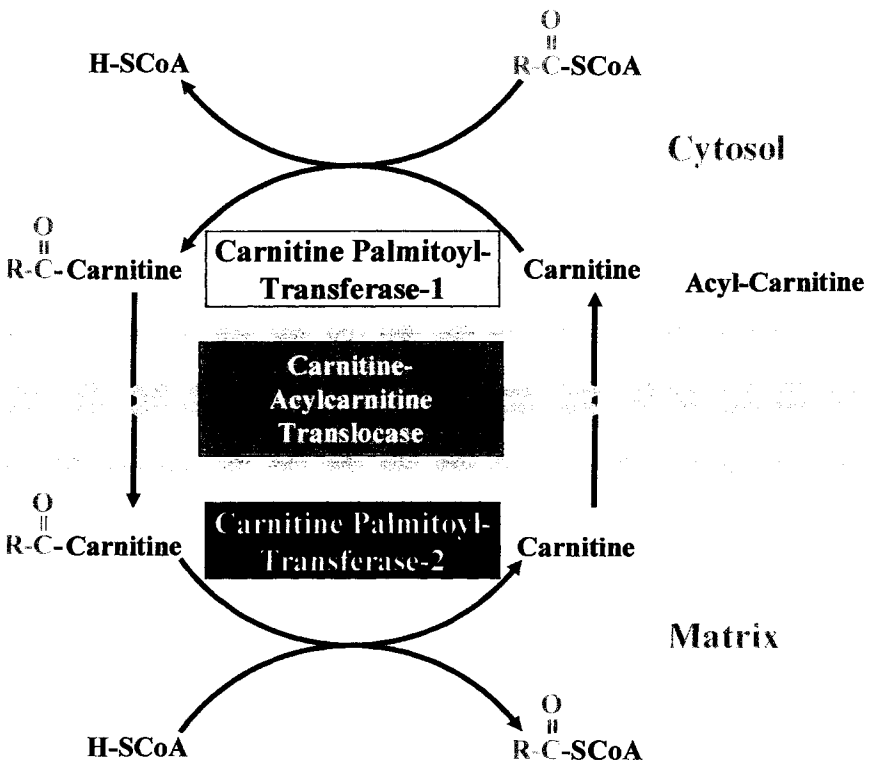

Fig. 1. CPT-1a catalyzes the conversion of acyl-CoA to acylcarnitine, a necessary step needed to cross the mitochondrial membranes and enter into β-oxidation.

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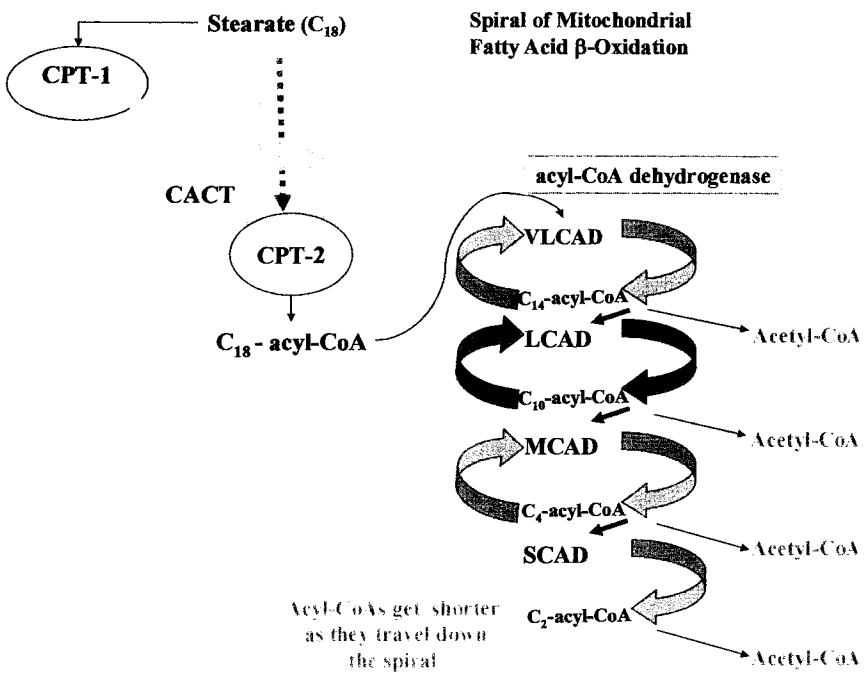

Fig. 2. Following conversion of acylcarnitine back to acyl-CoA by CPT-2, the acyl-CoA passes into the β-oxidation pathway spiral where it is oxidized to produce acetyl-CoA.

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HOMOZYGOUS CARNITINE PALMITOYLTRANSFERASE 1A (LIVER ISOFORM) DEFICIENCY IS LETHAL IN THE MOUSE

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Abstract

To better understand carnitine palmitoyltransferase 1a (liver-isoform, gene = *Cpt-1a*, protein = CPT-1a) deficiency in human disease, we developed a gene knockout mouse model. We used a replacement gene targeting strategy in ES cells that resulted in the deletion of exons 11-18, thus producing a null allele. Homozygous deficient mice (CPT-1a -/-) were not viable. There were no CPT-1 -/- pups, embryos or fetuses detected from day 10 of gestation to term. FISH analysis demonstrated targeting vector recombination at the expected single locus on chromosome 19. The inheritance pattern from heterozygous matings was skewed in both C57BL/6NTac, 129S6/SvEvTac (B6;129 - mixed) and 129S6/SvEvTac (129-coisogenic) genetic backgrounds biased toward CPT-1a +/- mice (>80%). There was no sex preference with regard to germ-line transmission of the mutant allele. CPT-1a +/- mice had decreased *Cpt-1a* mRNA expression in liver, heart, brain, testis, kidney, and white fat. This resulted in 54.7% CPT-1 activity in liver from CPT-1a +/- males but no significant difference in females as compared to CPT-1a +/+ controls. CPT-1a +/- mice showed no fatty change in liver and were cold tolerant. Fasting free fatty acid concentrations were significantly elevated, while blood glucose concentrations were significantly lower in 6-week-old CPT-1a +/- mice compared to controls. Although the homozygous mutants were not viable, we did find some aspects of haploinsufficiency in the CPT-1a +/- mutants, which will make them an important mouse model for studying the role of CPT-1a in human disease.

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Introduction

Carnitine palmitoyltransferase 1 (CPT-1) deficiency has been identified as a potentially fatal human inborn error of metabolism. CPT-1 exists in at least two isoforms, CPT-1a, often called the liver isoform, and CPT-1b, also known as the muscle isoform. These two isoforms appear to have different expression levels depending on tissue, developmental stage, hormonal regulation, and species. In adult mice, rats, and humans, CPT-1a is the predominate isoform expressed in liver, kidney, lung, spleen, brain, intestine, pancreatic islets, and ovary [1,2]. Alternatively, CPT-1b is the primary isoform expressed in heart, skeletal muscle, testis, and both brown and white adipose tissue, except in mice where CPT-1a predominates in white adipose tissue [1,2]. CPT-1a and CPT-1b have conventionally been considered the only two isoforms of CPT-1; however recently, a third isoform, CPT-1c, has been described and is expressed predominantly in brain [3].

Unlike medium- and short-chain fatty acids, long-chain fatty acids cannot enter mitochondria via simple diffusion. Both isoforms of CPT-1 (CPT-1a and CPT-1b) catalyze the transfer of the acyl group of long-chain acyl-CoAs to a carnitine, forming an acylcarnitine product. The resulting acylcarnitine is translocated across the mitochondrial membrane via carnitine-acylcarnitine translocase. Finally, carnitine palmitoyltransferase 2 catalyzes the conversion of acylcarnitine back to acyl-CoA, allowing it to proceed with the succeeding steps of the mitochondrial β-oxidation pathway. CPT-1, but not CPT-2, is sensitive to inhibition by malonyl-CoA. Thus, CPT-1 has a pivotal regulatory role in the inward mitochondrial flux of fatty acid substrates, as well as serving as a key point for regulation of mitochondrial β-oxidation [4].

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β-oxidation of fatty acids is a vital cellular process responsible for supplying 80-90% of cellular energy requirements during prolonged fasting [5]. Although most tissues are reliant on fatty acid oxidation to provide cellular energy during the fasted state, long-chain fatty acid oxidation is the primary source of energy for heart and skeletal muscle in the fed and fasting state [6]. Furthermore, infants, who generally have limited glycogen stores, are heavily reliant on long-chain fatty acid oxidation for energy [7].

CPT-1a deficiency has been found in more than 30 children [8]. Adults and children with inborn errors of fatty acid oxidation exhibit severe metabolic disturbances including hypoketotic-hypoglycemia, and fatty liver [9]. Disease occurs most commonly in children from birth to 2 years of age, although adult patients have presented as well. CPT-1a deficient patients may have residual CPT-1a activity, measured in fibroblasts, with values ranging from 0 to 65% of controls [8,10]. Although overall pathogenesis is complex, impaired β-oxidation of fatty acids is an essential part of the disease. As a consequence of its pivotal role in fatty acid oxidation, CPT-1a deficiency can be fatal. The acute effects of CPT-1a deficiency occur primarily in young patients as a result of catabolic stress and are reported to recede with aging. In order to better understand mechanisms in acute disease, we developed a gene knockout mouse model for CPT-1a deficiency.

Materials and methods

Construction of targeting vector and gene targeting in ES cells

The *CPT-1a* targeting vector was constructed from genomic DNA fragments derived from a mouse 129X1/SvJ genomic P1 clone, PV1. The P1 clone was identified by

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screening a mouse 129X1/SvJ strain genomic library by PCR (Genome Systems, St. Louis, MO) (Fig. 1). Exons 11-18 were deleted by a replacement gene targeting strategy (Fig. 1) by gene transfer into ES cells. The targeted ES cells were used to generate mice with a null allele (*Cpt-1a*^{tm1Uab}). ES cells (TC-1) were originally derived from 129S6/SvEv mice. Screening for recombinant ES cell clones was done by G418 selection (350 μg/ml) for 7 days. Surviving colonies were picked and expanded for Southern blot analysis.

Mice

Chimeric mice were produced by microinjection of gene targeted ES cells into C57BL/6NTac (B6) embryos. The chimeric founders were bred to 129S6/SvEvTac (129) or B6 for perpetuation of mice used in these studies. We attempted to produce all three genotypes (wild-type, heterozygous mutants and homozygous mutants) on both B6;129 and 129 backgrounds for these studies.

Mice were fasted for ~24 h and euthanized with a lethal dose of avertin before collecting blood for biochemical markers and collecting tissue for activity assays. The mice were also fasted for ~24 h prior to cold tolerance testing. Alternatively, the mice used to measure mRNA expression were fasted for ~24 h and euthanized with CO_2 inhalation. Blood was collected from the retro-orbital venous plexus with the exception of the mice used to measure mRNA expression where blood was collected by cardiac puncture. Blood samples were allowed to coagulate at room temperature, and then placed on ice and centrifuged for serum collection. All animal protocols were approved by the Institu-

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tional Animal Care and Use Committee of the University of Alabama at Birmingham. Mice were fed a standard rodent diet (Teklad LM-485).

Genotyping

Mice were genotyped by standard southern blot analysis of tail DNA. Genomic DNA was digested with *Eco*RI (Promega). Standard southern blot procedures were carried out as described in [11] for the purpose of genotyping. A genomic probe located 3' to exon 19 was generated by PCR using primers (F-5'ATGTACTTGACCAAGGAGGAGCACACAGT3') and (R-5'ATGAAGAAACTCAGTGTTGCTCTGGAGAGA3'). The targeted allele (3.8 kb) was distinguished from the wild-type allele (11.8 kb) (Fig. 2) by restriction fragment analysis using this probe.

Fluorescence in situ hybridization

Metaphase chromosome spreads were prepared from cultured bone marrow obtained from CPT-1a +/- mice. In brief, cultured cells were arrested in metaphase with Colcemid (Gibco), treated with hypotonic solution (buffered 0.075 M KCl) and fixed in 3:1 methanol/acetic acid [12]. The cell suspension was subsequently dropped on glass slides and examined for metaphase spreads.

To determine the chromosomal localization of *Cpt-1a* targeting vector in the mutant mice studied, fluorescence in situ hybridization (FISH) was performed using a modification of previously published techniques [13,14]. The *Cpt-1a* targeting vector (Fig. 1) was direct-labeled with Spectrum Green by nick translation (Vysis, Inc.) and hybridized

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to metaphase spreads prepared from mouse bone marrow cultures. After overnight hybridization, the slides were washed in 0.4X SSC/0.3% NP-40 at 73 °C for 2 min, followed by a 1 min wash in 2X SSC/0.1% NP-40 at ambient temperature. The slides were counterstained with DAPI and analyzed using a Leitz microscope. Color images of metaphase spreads showing hybridization with the *Cpt-1a* probe were obtained with Cytovision (Applied Imaging).

Gestation studies

After finding no live-born CPT-1a -/- mice, timed matings were used to investigate the suspected gestational lethality of that genotype. Female CPT-1a +/- breeders were checked for plugs for several days to determine the time of conception. The day on which the female was found plugged was considered day 0, and embryos and fetuses were collected from day 10 until term. The mice were euthanized with CO_2, fetuses were excised, and DNA was isolated followed by Southern blot analysis.

Skewed inheritance pattern investigation

In order to investigate a parental sex preference of transmission of the mutant allele, breeders were set up with either the male or the female transmitting the mutant allele and paired with wild-type mates. Live born pups of both crosses were genotyped by Southern blot.

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mRNA analysis

Northern blots were used to evaluate *Cpt-1a* and *Cpt-1b* tissue expression in both wild-type and mutant mice. Liver, brown fat, brain, heart, skeletal muscle, testis, ovary, and white adipose tissue were collected aseptically for RNA analysis. A 100 mg sample of tissue was placed in 1 ml of Triazol reagent (Invitrogen), homogenized, and processed for RNA isolation. Gel electrophoresis, blotting, probe synthesis (Strip EZ DNA Ambion), and hybridization were carried out as described previously [15].

Evaluation of carnitine palmitoyltransferase 1 activity

Frozen liver specimens were homogenized to yield a 10% (w/v) homogenate in 10 mM potassium phosphate/150 mM NaCl, pH 7.4, supplemented with protease inhibitor cocktail and protein phosphatase inhibitor cocktail I and II (Sigma) at a 1/100 dilution. CPT-1 activity was determined in duplicate using the modified radiochemical forward assay by measuring the formation of ^{14}C-labeled palmitoylcarnitine from [^{14}C]carnitine and palmitoyl-CoA at 37 °C and is defined as the activity in nmoles/min/g wet weight inhibited by 200 μM malonyl-CoA [16,17]. Briefly, 10 μl aliquots of the 10% (w/v) liver homogenate were preincubated for 2 min with 50 μM palmitoyl-CoA at fixed palmitoyl-CoA/BSA ratio (0.85) alone or plus 200 μM malonyl-CoA in 250 μl reaction mixture containing 50 mM MOPS, 80 mM KCl, 1.0 mM EGTA, and 2.0 mM KCN. The reaction was initiated with [^{14}C]carnitine (2.0 mM final concentratioin), specific radioactivity 1823 dpm/nmole, and stopped after one and a half minute with 1.0 ml 1N HCl. Following extraction of [^{14}C]palmitoylcarnitine with water-saturated butanol and reextraction of the

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butanol phase with butanol-saturated water an aliquot of the organic layer was used for scintillation counting.

Serum metabolite assays

Glucose concentration was measured in 10 µl sera using an Ektachem DT II system (Johnson and Johnson Clinical Diagnostics.) Total non-esterified fatty acids (NEFA) were measured by an enzymatic, colorimetric method ("NEFA-C" reagents, Wako Diagnostics, Richmond, Virginia). The assay was modified to accommodate a reduced sample size (10 µl), and use of a microplate reader for measurement of optical density at 550 nm. Triglyceride concentrations were measured in 10 µl sera with the Ektachem DT II system (Johnson and Johnson Clinical Diagnostics.)

Cold intolerance testing

Cold tolerance of male and female CPT-1a +/- and CPT-1a +/+ mice was assessed by exposure to 4 °C temperatures for a 3 h interval as we described previously [18]. Rectal temperatures were measured prior to cold exposure and repeated hourly using Barnant Thermocouple thermometer. Following our standard protocol, cold challenge experiments were terminated when rectal temperatures dropped to 25 °C or after a total of 3 h of exposure. If the rectal temperature did not drop more than 3 °C after 3 h the mice were deemed cold tolerant.

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Histologic characterization

Two female and two male, CPT-1a +/- and +/+ mice, age 3-4 weeks, were examined. At necropsy, the mice were examined for internal and external abnormalities. Lungs, heart, liver, kidneys, adrenals, spleen, lymph nodes, brown fat, white fat, skeletal muscle, male and female internal reproductive organs, and brain were fixed by immersion in alcoholic formalin (75 parts ethanol:10 parts 40% formalin:15 parts distilled water). Fixed tissues were processed routinely, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (HE).

Statistical Analysis

Results were reported as mean values with standard deviations. Student's *t* test and x^2 analyses were used to test for significant differences. Results were considered significantly different with a $p<0.05$.

Results

Generation of Cpt-1a mutant mice

The targeted mutagenesis strategy for mouse *Cpt-1a* included the deletion of exons 11-18 by replacement with a neo-expression cassette. Southern blot screening identified clone N10 as having a targeted *Cpt-1a* allele. ES cells from clone N10 were injected into blastocysts which yielded several chimeric mice. Agouti pups were produced from two chimeric males indicating germ-line transmission from the N10 ES cells. Germ-line transmission of the mutant allele was further confirmed by the identification of both a heterozygous male and female which were used as a breeding pair.

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Lethality

After Southern blot analysis of over 1000 offspring from heterozygous matings, no homozygous mutants were found. Therefore, we concluded that homozygous CPT-1a deficiency was lethal in the mouse mutant. To ascertain whether the trait was due to an additional, unrecognized integration of the targeting vector, we performed FISH analysis using the targeting vector as the probe.

Fluorescence in situ hybridization

A representative result showing hybridization of the direct-labeled (Spectrum Green) *Cpt-1a* targeting vector probe to the centromeric region of chromosome 19 is shown in Fig. 3. In the metaphase spreads with hybridization signal detected on both copies of chromosome 19, signal intensity varied between homologs perhaps indicating that signal intensity was allele specific. There were no other hybridization signals detected indicating the expected single location of the targeting vector on chromosome 19.

Developmental stage of lethality

After confirming the gene targeting replacement vector chromosomal location, we postulated that the CPT-1a -/- genotype produced a lethal trait that was gestational. In order to establish the timing of the suspected loss, embryos/fetuses were collected from 129 mice and genotyped by Southern blot from day 10 to term. Over 76 embryos and fetuses were genotyped, and still no homozygous mutants were found (Fig. 4). These results indicated that this was an early lethal condition occurring prior to day 10. Moreover, the embryo/fetal genotypes did not follow an expected Mendelian pattern of trans-

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mission of the mutant allele. There was an unexpectedly high number of heterozygous embryos and fetuses. This experiment was repeated using mice with a 129/B6 mixed background and the results were indistinguishable (data not shown).

Skewed inheritance patterns

Genotyping results of live-born pups from heterozygous matings also revealed an unexpectedly high number of heterozygous as compared to wild-type pups. The over representation of heterozygous offspring was found in both 129 and B6;129 mixed background strains (Fig. 5), although there was no difference in average litter size. From these results, we assumed that CPT-1a -/- offspring were not viable. Therefore, we would expect 67% of offspring to be CPT-1a +/- genotype; however, we found >80%. There was no significant difference in transmission of the mutant allele from either the male or female parent of origin (Table 1). Female CPT-1a +/- and male +/+ breeding pairs had ~49% heterozygous pups and 51% wild-type pups, while male CPT-1a +/- and female CPT-1a +/+ breeding pairs produced 62% CPT-1a +/- and 38% wild-type offspring.

Table 1
Evaluation of sex transmission bias of mutant allele

Matings 129 strain background	Offspring CPT-1a +/- (%)	Offspring CPT-1a +/+ (%)
CPT-1a +/- (F) x CPT-1a +/+ (M) 36 litters, 155 total pups	50	50
CPT-1a +/- (M) x CPT-1a +/+ (F) 22 litters, 111 total pups	62	38

There were no significant differences found.

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Evaluation of mRNA expression in various tissues

In wild-type mice we found the highest levels of expression of *Cpt-1a* in liver, kidney, heart, brain, testis or ovary, and white adipose tissue (male). The most marked reductions in mRNA expression of male and female CPT-1a +/- mutants were found in kidney, liver, heart, white fat (most obvious in males), brain, and testis. However, in tissues that we found to have low levels of *Cpt-1a* expression such as brown fat, skeletal muscle, and white fat (in females) the differences were negligible (Fig. 6).

CPT-1 activity

CPT-1 activity was measured in mice euthanized after a 24 h fast to confirm the presence of a null allele. This modified radiochemical forward assay is optimized to measure malonyl-CoA sensitive CPT activity i.e., CPT-1 activity. CPT-1a +/- 6- to 8-week-old male mice showed 54.7% enzyme activity in liver as compared to wild-type controls (Fig. 7). This decrease in CPT-1 activity in livers from heterozygote male mice is highly significant ($P<0.001$). However, in female CPT-1a +/- mice, enzyme activity did not appear to be significantly different compared to wild-type controls (Fig. 7) ($p=0.436$).

Serum metabolite phenotypes of CPT-1a +/- mice

Serum glucose, free fatty acids, and triglycerides were measured in 5 male, 6-week-old CPT-1a +/+ and 6 male CPT-1a +/- mice (Fig. 8). Serum glucose concentrations after a 24 h fast were significantly lower in male 6-week-old CPT-1a +/- mice. Additionally, serum free fatty acids after a 24 h fast were found to be significantly elevated as compared to controls in CPT-1a +/- mice. Conversely, fasting serum triglyceride

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levels were not significantly different in the mutants although the CPT-1a +/- mutants showed a trend toward hypertriglyceridemia (Fig. 8).

Cold tolerance testing

Both male and female CPT-1a +/- and +/+ mice were cold challenged after a 24 h fast and CPT-1a +/- mice were found to be completely cold tolerant. This finding was demonstrated by indistinguishable rectal temperature responses found between CPT-1a +/- and CPT-1a +/+ controls (data not shown) throughout the cold challenge experiments.

Histologic phenotype

Phenotyping by histology concluded that all organs and tissues were normal for mice of this strain and age.

Discussion

The targeting strategy was based on a replacement vector design, which included the replacement of exons 11-18 with a neo cassette, which would induce a null mutation. This provided for readily distinguishable genotype detection between the wild-type and mutant alleles.

As we have shown, there were no homozygous mutants found. Using FISH, we confirmed that the lethality was specifically due to the *Cpt-1a* mutation rather than a possible integration at another locus. We recognized an apparent preconception or gestational lethality of CPT-1a -/- mice after we found no live-born CPT-1a -/- offspring, as well as no homozygous embryos/fetuses found from day 10 to term of gestation. This re-

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sult implicates that CPT-1a must play a critical role in metabolic function of the embryo. Furthermore, the studies of parental transmission of the mutant allele indicated no gametogenesis problem, as CPT-1a +/- pups were produced from gametes of both male and female parents.

This lethal trait was unexpected given that there are human patients with homozygous deficiency of CPT-1a [8]. Background strain did not appear to be a factor as we found no difference in survival of homozygous pups on either 129 or a B6;129 mixed background. Previously, we described gestational lethality as part of the phenotype in long-chain acyl-CoA dehydrogenase deficient mice, which appears to occur early in gestation, prior to formation of the blastocyst [19]. Thus, perhaps this finding could be expected considering that the energy provided from fatty acid oxidation appears critical for embryonic development [20-22].

Furthermore, previous studies investigating expression patterns have found that both CPT-1a and CPT-1b are expressed in a range of tissues depending on species and stage of development [1,2,23-27]. In some instances the predominant expression of either isoform has been found to be transient throughout stages of development. Alternatively, some findings have indicated that CPT-1a or b is the sole isoform expressed at both immature and mature stages of development in a particular tissue like the finding that CPT-1a is the sole isoform expressed in pre- and post-differentiated white adipocytes as well as in immature testis [1,2].

Considering that there are differences in regulation, kinetics, and sensitivity to malonyl-CoA inhibition, it would seem that CPT-1a and b have unique functions [16,28,29]. We further speculate that the lethality may be due to the fact that there is a

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stage of development where CPT-1a is the sole isoform expressed, and therefore its absence prevented critical functions necessary for further development particular to mouse embryonic development. Moreover, CPT-1a may act uniquely to CPT-1b in certain tissues and stages of development, and therefore the absence of CPT-1a expression may cause complete loss of function in certain tissues. Evidence for unique function includes differences found in kinetics and sensitivity to malonyl-CoA inhibition as well as results finding the transition of the dominance of one isoform to the other, as is demonstrated in the immature testis and the mature testis [2].

Our finding of skewed inheritance patterns biased toward heterozygous offspring differed from that of the expected Mendelian pattern 1(+/+):2(+/-):1(-/-). However this altered genetic distribution is not the only one of its kind as there are several different examples of allelic frequencies not following the one-two-one ratio. One hypothesis may be that the altered inheritance pattern is a consequence of unequal meiotic segregation in a heterozygous parent. Previous findings of altered inheritance patterns found a female mouse strain in the wild that carries an aberrant form of chromosome 1 [30]. They found that during meiosis the aberrant chromosome is preferentially transmitted (85%) to the secondary egg rather than either polar body, resulting in ratio distortion of the female's offspring [30]. Alternatively, there have been findings of non-Mendelian inheritance patterns that have been explained as a consequence of the *t*-haplotype system where various inversions on a region of mouse chromosome 17 suppress recombination leading to dominant transmission of the *t*-haplotype. This occurs after meiosis and before fertilization [31,32]. Thus, the *Cpt-1a* mutation on chromosome 19 [33] also appears to be preferentially transmitted; however there was no indication of a chromosome abnormality.

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Presently, we have been unable to determine the mechanism. The skewed inheritance pattern found in the offspring of CPT-1a +/- mice may be another of the numerous examples of non-Mendelian inheritance found in the literature without explanation. In adult mice, rats, and humans, CPT-1a was found to be the predominate isoform expressed in liver, kidney, lung, spleen, brain, intestine, pancreatic islets, and ovary [1,2,25]. Alternatively, CPT-1b was found to be the predominate isoform expressed in rat and human mature white fat whereas, in mice CPT-1a was previously found to be the predominant isoform expressed in mature white fat. In the present study, we found expression of both *Cpt-1a* and *Cpt-1b* in white fat, although male mice appeared to have *Cpt-1a* expressed as the predominant isoform in most cases, while female mice appeared to have a greater expression of *Cpt-1b* in white fat. Furthermore, previous studies published on rat tissues found that CPT-1a was highest in ovary, liver, and kidney [1,2]. Similarly, from our studies we conclude that mice have the highest *Cpt-1a* expression in kidney, liver, male white fat, and ovary. These results extend previous findings of rat and mouse *CPT-1a* tissue expression patterns. The *Cpt-1a* mutation led to an approximate 50% decrease in expression of *Cpt-1a* in tissues where *Cpt-1a* was the predominant isoform expressed. Our findings support previous findings in rat [1,23-27] and extend them with the finding of a sex difference in mature mouse white fat expression pattern.

Enzyme activity matched our expression patterns indicating that there was 54.7% CPT-1 activity in the male CPT1a +/- mice as compared to wild-type. In contrast, female CPT1a +/- mice were found to have no significant difference in enzyme activity as compared to female wild-type mice. The mechanism remains to be determined.

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In conclusion, we demonstrated that homozygous CPT-1a deficiency is a lethal trait, apparently occurring very early in gestation at least before day 10. FISH analysis demonstrated the expected targeted locus and an otherwise normal karyotype. Thus, the gestational lethality cannot be explained by random integration of the targeting vector into an additional critical locus for development. Heterozygous matings produce an unexpectedly high percentage of CPT-1a +/- offspring on both an inbred 129 or mixed B6;129 genetic background. We also demonstrated the expected reduction of liver CPT-1 activity in males; however surprisingly we found normal liver CPT-1 activity in females. There were modest changes in glucose and free fatty acids during fasting conditions of young CPT-1a +/- mice, and all show normal cold tolerance.

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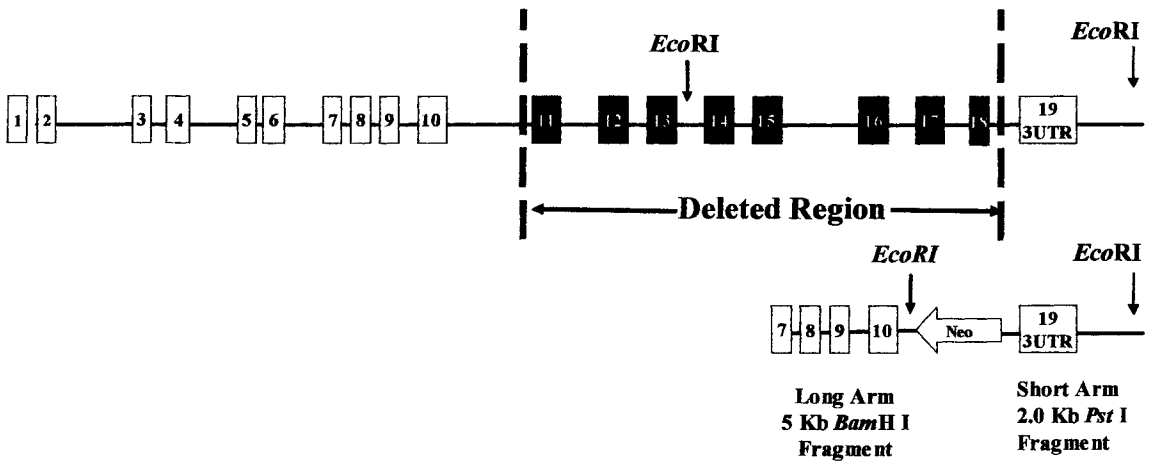

Fig. 1. Gene targeting strategy consisted of replacement of exons 11-18 with a 1.8 kb Neo insertion cassette containing a phosphoglycerate kinase promoter and a bovine poly(A) signal derived from pNTK vector [34]. A genomic probe located 3' to exon 19 was used to detect both restriction fragments as indicated. *Note*: Not drawn to scale.

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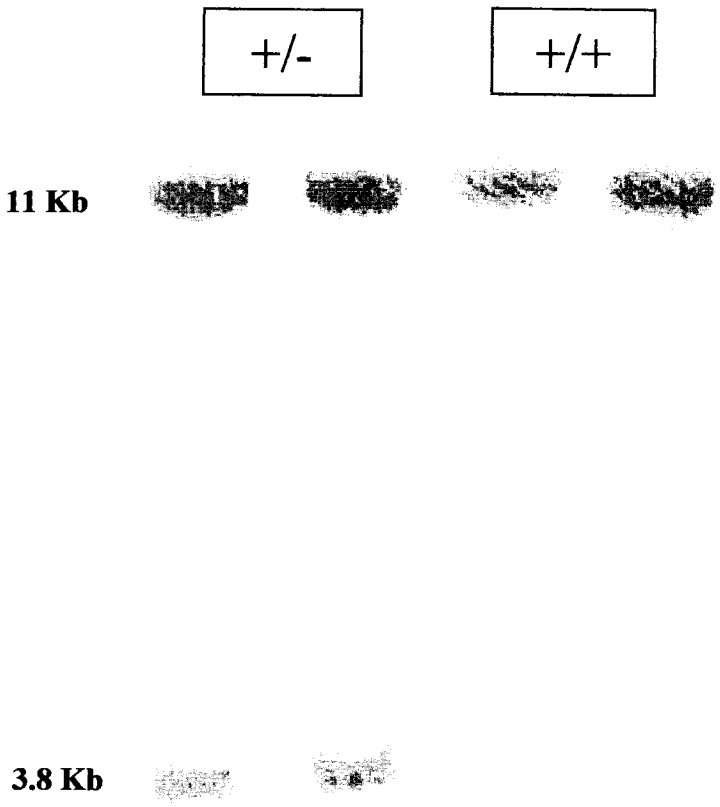

Fig. 2. Genotype characterization included 11.8 kb band representing wild-type allele with an *Eco*RI digest or the combination of the 11.8 kb band with a 3.8 kb band, representing the mutant allele. The presence of both bands signified a heterozygous mutant (CPT-1a +/-). CPT-1a +/+ mice were identified by the presence of just the larger band. No homozygous mutants were found.

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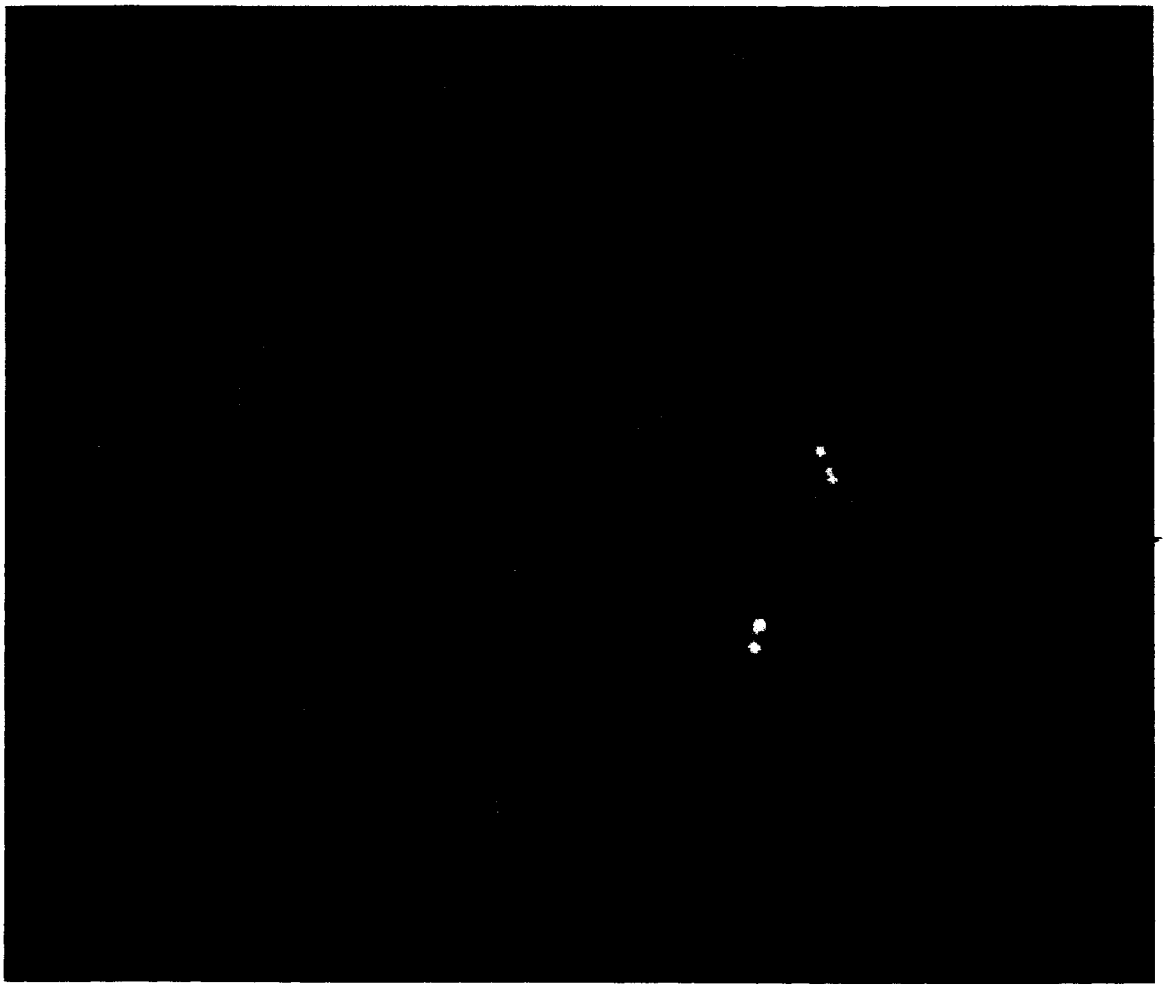

Fig. 3. Localization of *Cpt-1a* targeted locus to chromosome 19. FISH with a direct-labeled *Cpt-1a* targeting vector probe (Spectrum Green) hybridized to metaphase spreads prepared from CPT-1a +/- mice localized to the centromeric region of chromosome 19.

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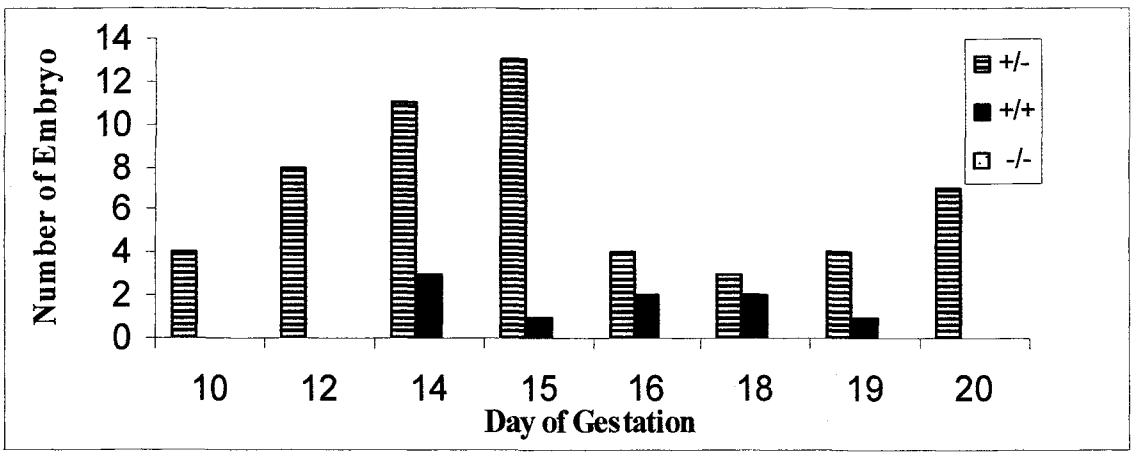

Fig. 4. Gestational genotyping. There were no CPT-1a -/- embryos (129 strain) found from heterozygous matings from day 10 of gestation to term (day 20). Similar results were found on B6;129 mixed background (data not shown). There was an overrepresentation by CPT-1a +/- embryos and fetuses than would be expected by Mendelian inheritance.

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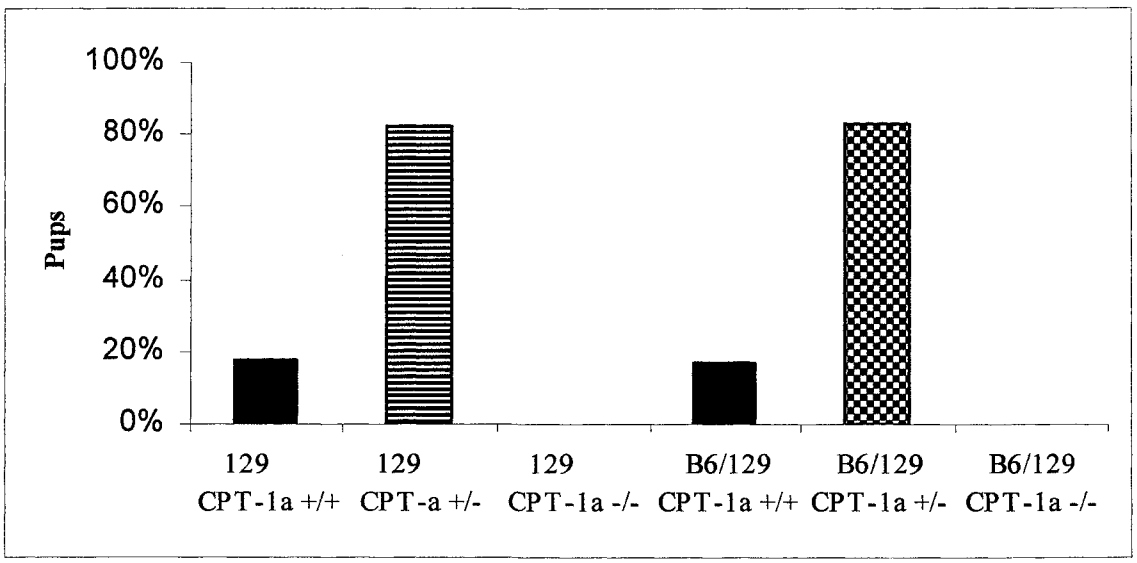

Fig. 5. Skewed inheritance pattern. Both 129 (n = 136) and B6;129 (n = 107) CPT-1a +/- matings produced a larger percentage of CPT-1a +/- pups than would be predicted by Mendelian inheritance (*$p < 0.005$).

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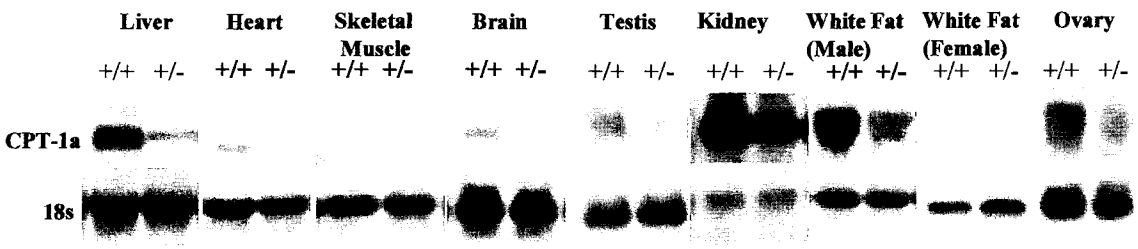

Fig. 6. Expression patterns. *Cpt-1a* mRNA expression in male and female liver, heart, skeletal muscle, testis, kidney, white fat, and ovary from both CPT-1a +/- and CPT-1-a +/+ mice.

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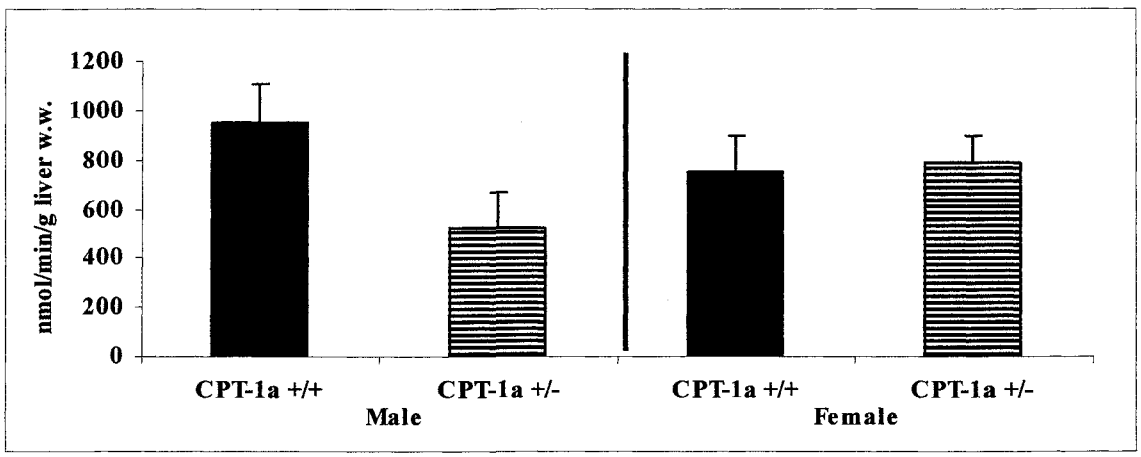

Fig. 7. Liver CPT-1 activity. Male CPT-1a +/- mice (n = 10) had 54.7% CPT-1 activity in liver as compared to the CPT-1a +/+ controls (n = 11) ($p < 0.001$). Female CPT-1a +/- mice (n = 5) showed no difference in CPT-1 activity in liver as compared to the CPT-1a +/+ controls (n = 6).

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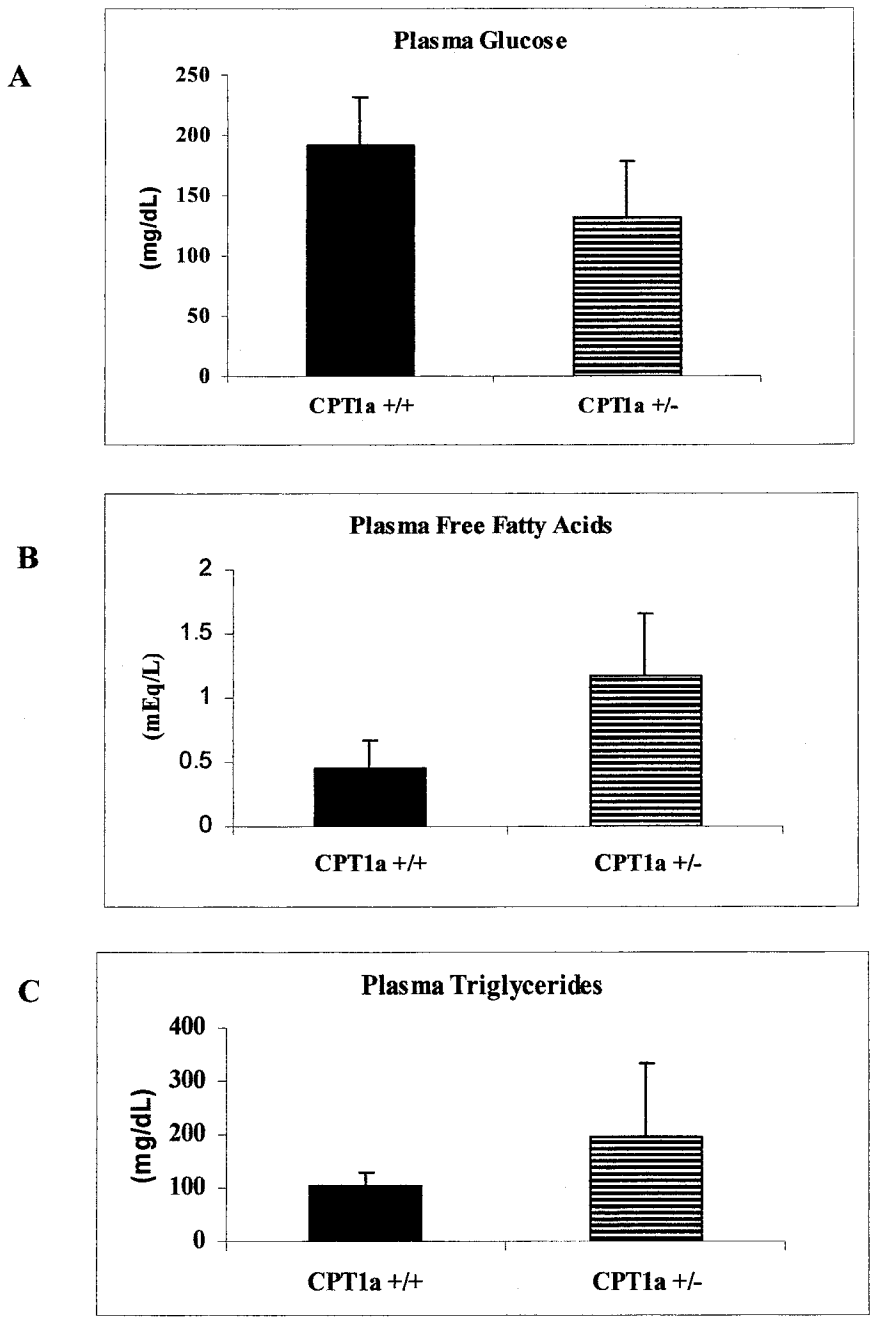

Fig. 8. Serum metabolites. CPT-1a +/- mice ($n = 6$) (6 weeks old) displayed significantly lower blood glucose concentrations ($p < 0.05$), and significantly elevated blood free fatty acid concentrations ($p = 0.01$) compared to CPT-1a +/+.

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CARNITINE PALMITOYLTRANSFERASE 1A (CPT-1A) AFFECTS GLUCOSE AND INSULIN SENSITIVITY

by

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In Preparation for *Diabetes*

Format adapted for dissertation

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Abstract

Carnitine palmitoyltransferase-1a (CPT-1a, liver isoform) is a crucial regulatory step in mitochondrial fatty acid oxidation (FAO) and likely important in the mechanisms of insulin resistance and β-cell dysfunction. A heterozygous gene knockout mouse model for CPT-1a deficiency (CPT-1a +/-) was used to investigate the role of CPT-1 in the development of impaired glucose tolerance. The major results include the following. Body fat was significantly greater in CPT-1a +/- male mice fed a high-carbohydrate diet (HCD) as compared to wild-type mice, whereas CPT-1a +/- mice fed the high-fat diet (HFD) had lower body fat than wild-type controls. Body weight was not affected regardless of diet. Male CPT-1a +/- mice were found to be more insulin sensitive than wild-type controls when fed either an HCD or an HFD. The higher insulin sensitivity was associated with an increased expression of *Cpt-1b* (muscle isoform) in liver compared to CPT-1a +/+ mice and lower pyruvate dehydrogenase kinase-4 (*Pdk*-4) expression in skeletal muscle which was significantly different in HFD but not HCD fed CPT-1a +/- mice as compared to wild-type controls. Lower fasting serum insulin levels in CPT-1a +/- mice fed HFD at both 7 months and 12-14 months further supported this result demonstrating higher glucose sensitivity than CPT-1a +/+ mice. CPT-1a +/- mice were more glucose tolerant than wild-types when fed the HCD but less glucose tolerant when fed the HFD. Furthermore, CPT-1a +/- mice fed HFD or HCD had fewer and smaller islets, identified by β-cell staining for insulin, than wild-type mice. Overall, these findings suggest that expression or activity of both *Cpt-1a* and *Cpt-1b* may play a role in the pathophysiology of the development of type 2 diabetes and are influenced further by diet.

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Introduction

Impaired glucose tolerance is thought to be the earliest identifiable metabolic abnormality in the pathogenesis of type 2 diabetes [1]. Because of the upsurge in the number of patients being diagnosed with type 2 diabetes, further understanding of its pathophysiology is critical. Although the temporal sequence is still under debate, there are some metabolic components that are clearly tied to the progression from normal glucose tolerance to impaired glucose tolerance and then type 2 diabetes. Glucose tolerance is thought to be a function of both β-cell responsiveness to increases in blood glucose and peripheral tissue responsiveness to the insulin that is secreted. It follows that both insulin resistance and β-cell dysfunction are independently or collectively involved in the progression to frank diabetes.

Aberrant fatty acid oxidation may play a pivotal role in both the pathogenesis of insulin resistance and β-cell dysfunction. A build-up of fatty acid metabolites, including long-chain fatty acyl-CoAs (LCCoAs) and diacylglycerides (DAGs) in the cytosol of skeletal muscle and other tissues, has been implicated in the mechanism proposed for fatty acid-induced insulin resistance [2]. Moreover, studies in rodents have shown chronic exposure to fatty acids alters β-cell function [3,4]. Because inhibition of carnitine palmitoyltransferase 1 may lead to a buildup of these fatty acid metabolites, we postulated that the activity of CPT-1 may be a crucial link between elevated fatty acids and the development of type 2 diabetes.

CPT-1 functions as the rate-limiting step of FAO. This enzyme is responsible for converting LCCoAs into long-chain acylcarnitines, a necessary process allowing these substrates to be translocated across the mitochondrial membrane into the mitochondrial

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matrix where β-oxidation occurs [5]. In mice, there have been three different CPT-1 isoforms discovered [6-8]. Although CPT-1a and 1b are thought to be expressed in most tissues, depending on the stage of development, usually either CPT-1a or b is dominant. CPT-1a is highly expressed in liver. CPT-1b is also expressed in liver; however, depending on the stage of development, it is expressed at much lower levels. Alternatively, CPT-1b is the dominant isoform expressed in skeletal muscle. Further, CPT-1c is thought to be the dominant isoform in brain and testis.

Treatment with CPT-1 inhibitors has provided further evidence for the involvement of decreased CPT-1 activity in the development of type 2 diabetes. McGarry and colleagues found that prolonged inhibition of CPT-1 by etomoxir promoted lipid accumulation and insulin resistance in rats [9]. The study included two separate groups of rats: Group 1 included rats fed an HFD challenge or a low-fat diet challenge. Group 2 included rats fed the high-fat diet with etomoxir or rats fed low-fat diet with etomoxir. They found that both the HFD challenge and the etomoxir treatment led to significantly impaired insulin sensitivity as measured by hyperinsulinemic-euglycemic clamp.

Additionally, potentiating effects leading to excessive or dysregulated secretion of insulin from pancreatic β-cells have been found in studies using both CPT-1 inhibitors and exposure to excess fatty acids [10-14]. These increased or dysregulated oscillatory pulses of insulin, found as a result of CPT-1 inhibition or excess fatty acid exposure, may play a role in the progression to β-cell failure that is seen at the onset of frank diabetes. In brief, previous findings using CPT-1 inhibitors have had adverse effects on both β-cell function and insulin sensitivity.

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To pursue this hypothesis and extend the observations found using CPT-1 inhibitors with their inherent limitations, we have used a mouse model with heterozygous CPT-1a deficiency (CPT-1a +/-) to investigate what effect this deficiency has on glucose homeostasis. Our experimental objective was to show that the activity of CPT-1a plays a pivotal role in the development of impaired glucose tolerance, insulin resistance, and β-cell dysfunction.

Our aim was to replicate some of the factors thought to be involved in the evolution of the epidemic of type 2 diabetes and obesity in the human population by challenging both CPT-1a +/+ or CPT-1a +/- mice with a high-carbohydrate diet (HCD) or an HFD diet. The rationale behind the HCD challenge was that it was to induce conditions that would promote for high levels of malonyl-CoA, a potent CPT-1 inhibitor. Furthermore, reasoning behind the use of the HFD was to challenge this impaired CPT-1 system with excess substrate to promote for build-up of fatty acids. We hypothesized that the HCD-induced increase in the CPT-1a inhibitor and the HFD-induced substrate overload would unmask a phenotype in a mouse model with an already compromised state of FAO. Moreover, we expected to demonstrate that the combination of the environmental stresses of diet and decreased CPT-1a activity as a genetically induced limitation on FAO would be causal at the cellular level for one or all of the following: glucose intolerance, insulin resistance, and β-cell dysfunction.

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Methods

Animals

Mice with a heterozygous deficiency for carnitine palmitoyltransferase 1a (*Cpt1*$^{+/-}$ mice) (129/S6SvEvTacBR background) were previously described [15]. Homozygous CPT-1a deficient mice are not viable [15]. These studies were divided into three different experiments. In study 1 the goal was to evaluate serum glucose, free fatty acids, and triglycerides in the fed and fasting states of CPT-1a +/- and CPT-1a +/+ mice fed a standard rodent diet (SRD). The goal of study 2 was to assess body weights, body fat, fasting serum glucose and insulin levels of CPT-1a +/- and CPT-1a +/+ mice after either an HFD or HCD challenge of 46 weeks. Finally, the goal in study 3 was to further investigate the findings in study 2 using a physiologic approach measuring glucose and insulin tolerance. Collectively, we wanted to follow temporal events in both CPT-1 genotypes of mice using biochemical markers of insulin resistance or diabetes such as postprandial glucose and insulin levels, and more physiologic, functional measures to evaluate glucose and insulin sensitivity when challenged by these extreme diets and also make comparisons to baseline data from the same genotypes fed an SRD.

In study 1, CPT-1a +/- and CPT-1a +/+, male 6- to 8-month-old mice fed an SRD were used to evaluate fed and fasting serum glucose, free fatty acids , and triglycerides (TG).

In study 2, male and female (CPT-1a +/+ and CPT-1a +/-) mice were weaned and maintained on either an HFD or HCD for ~12 months. Body weight was measured weekly for 46 weeks, and body fat was measured 1 week prior to termination. This study

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also included steady-state RNA analysis of *Cpt-1a* and *b* and measurements of fasting serum glucose, triglycerides, and free fatty acids.

For study 3, two separate age-matched male (CPT-1a +/+ and CPT-1a +/-) groups were weaned onto or maintained on either HFD or HCD for either 7 month or 12-14 month diet durations and were evaluated for glucose tolerance by oral glucose tolerance tests (OGTT), for insulin sensitivity by insulin tolerance tests (ITT), for islet morphology by β-cell immunohistochemistry for serum insulin concentrations, for hepatic steatosis by histological analysis, and for serum leptin concentration.

All mice were housed in a pathogen-free barrier facility (12-h light/12-h dark cycle). Mice were negative for mouse pathogens based on serological assays for 10 different viruses, aerobic bacterial cultures of nasopharynx and cecum, examinations for endo- and ectoparasites, and histopathology of all major organs. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Diet and body weights

Mice were fed either an SRD (10% fat by calories-Teklad 7012) or custom diets formulated by Harlan-Teklad, Inc. (Madison, WI). The custom diets consisted of an HFD (Teklad diet-TD 97268) which contained 43% fat (calorie %) by the addition of beef tallow as the major fat source and an HCD (Teklad diet-TD 97267) that was 14% fat (calorie %) with starch making up the bulk of calories. Both custom diets were isocaloric and contained equal amounts of sucrose. The HFD was relatively high in saturated fat and cholesterol when compared to an SRD. In all studies, mice 17-19 days of age were

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weaned onto their respective diets and fed *ad libitum* for different diet durations according to study design.

Measurement of body composition (DXA)

In vivo body composition (total body fat, soft-lean tissue, and bone mineral content) of mice was determined using a PIXImus densitometer (GE-Lunar PIXImus, Madison, WI) as we have previously validated [16]. Briefly, mice were anesthetized using isoflurane and placed in a prostrate position on the imaging plate. Scans were analyzed and the data corrected to the carcass composition standard using our previously validated equations [16]. All DXA measures were conducted in the early afternoon (1300 h). Five hours prior to the DXA scan, any food remaining in the animal's food hopper (or spilled food in the cage) was removed. This period of fasting helped to minimize the potential effect of gut fill on the DXA measures. Removal of food for 5 h during the light period (a time when mice feed relatively little) was not overly stressful for the mice.

Serum metabolite assays

In studies 1 and 2, mice were fasted for 20 to 22 h before collection of serum with the exception of the fed group in study 1. Terminal glucose concentrations were measured in 10 μl sera using an Ektachem DT II system (Johnson and Johnson Clinical Diagnostics). Total non-esterified fatty acids (NEFA) were measured by an enzymatic, colorimetric method ("NEFA-C" reagents, Wako Diagnostics, Richmond, VA). The assay was modified to accommodate a reduced sample size (10 μl), and use of a microplate reader for measurement of optical density at 550 nm. Triglyceride concentrations were

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measured in 10 µl sera with the Ektachem DT II system (Johnson and Johnson Clinical Diagnostics).

Glucose tolerance tests

The OGTT was used to assess the ability of the mouse to respond to and dispose of an oral bolus of glucose. Outcomes of interest included basal (fasted) serum glucose concentration and glucose and insulin concentrations at individual time points post-glucose ingestion. From these data, the rate of decline of blood glucose was determined. At 20 h prior to OGTT, animals were food deprived with water overnight. After the mice were weighed, a baseline blood sample was obtained by tail bleed, and additional blood samples were obtained at 15, 30, 60, and 120 min following glucose administration. Glucose was administered orally at a dosage of 2.0 mg/g body weight. Insulinogenic index calculations were made in order to assess or estimate differences in first phase insulin secretion. The formula used is as follows: [(insulin 30 min-insulin 0 min)/(glucose 30 min-glucose 0 min)] [17]. This represents the change in insulin level 30 min after glucose administration divided by the change in glucose level 30 min after the oral dose of glucose.

Insulin tolerance tests

The ITT was used to measure the degree of insulin sensitivity. Mice were food deprived for 4 h, baseline blood samples were collected, and recombinant human insulin (Humulin R, 100 U/ml stock) was injected IP (0.1 ml per 10 g body weight). Prior to injection, insulin was diluted in saline to a concentration of 0.075 U/ml (1:1330). Addi-

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tional blood samples were obtained at 10, 20, and 30 min following insulin injection. Outcomes of interest included the final (30 min) glucose concentration and the rate of decline of glucose which was calculated from the slope of the descending points after insulin injection. The rate of decline of blood glucose was determined to be a reflection of the degree of insulin sensitivity.

OGTT and ITT serum glucose and insulin

For all OGTT and ITT samples, blood glucose levels were measured with a blood glucose meter (Accu-chek; Roche Diagnostics, Indianapolis, IN), and serum insulin levels were measured with an immunoassay kit (LINCO Research, Inc., St. Charles, MO) as per manufacturer's instructions.

mRNA analysis by Northern blots

Northern blots were used to evaluate *Cpt-1a* and *Cpt-1b,* medium chain acyl-CoA dehydrogenase (*Acadm*) and acyl-coenzyme A oxidase-1 (*Acox-1*) tissue expression in both wild-type and mutant mice. Liver, heart, and skeletal muscle tissues were collected aseptically for RNA collection. A 100 mg sample of tissue was placed in 1 ml of Triazol reagent (Invitrogen), homogenized, and processed for RNA isolation. Gel electrophoresis, blotting, probe synthesis (Strip EZ DNA Ambion), and hybridization were carried out as described [18].

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RNA extraction and real time quantitative RT-PCR analysis

Total RNA was extracted from heart, liver and skeletal muscle (soleus and gastrocnemius) using the TRIzol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA) according to the manufacturer's instructions. First strand cDNA was generated from 5 μg of RNA in a 20 μl volume reaction containing both oligo-(dT) and random hexamers (Invitrogen First Strand Synthesis Kit) according to the manufacturer's instructions. Real-time quantitative RT-PCR was carried out in a 20 μl reaction volume containing 10 μl supermix UDG (Invitrogen Life Technologies, Inc.), 2 μl cDNA, 0.2 μM LUX Cpt-1a and 1b, pyruvate dehydrogenase kinase isoforms 2 or 4 (*Pdk2* or *Pdk4*) primer forward, 0.2 μM LUX *Cpt-1a,1b, Pdk2 or Pdk4* primer reverse (FAM labeled Invitrogen, Life Technologies), 0.1 μM 18S RNA primer forward, and 0.1 μM 18S RNA primer reverse (JOE labeled Invitrogen, Life Technologies). Cycling conditions included incubation at 50 °C for 2 min, a 2 min 95 °C denaturing step, followed by 44 cycles of 95 °C denaturation, 45 sec -58 °C annealing time, and a 30 sec -72 °C extension step. A melting curve analysis was completed following amplification from 55-95 °C to assure product identification and homogeneity.

Each sample was normalized to the housekeeping gene 18S RNA to compensate for any differences in starting quantity of total RNA. Starting RNA was run on a gel to assure quality prior to use, and degraded samples were eliminated. The fold change in *Cpt-1b*, *Cpt-1a*, *Pdk2*, or *Pdk4* mRNA of CPT-1a+/- samples compared to CPT-1a +/+ samples was calculated using the formula, fold change = 2 exp (ΔCt), where Ct is the cycle number when threshold fluorescence is significantly above background, and ΔCt was obtained by first subtracting gene of interest related Ct from 18S related Ct for each sam-

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ple and each CPT-1a +/+ sample. The average CPT-1a +/+ sample difference was subtracted from each sample and used to calculate final numbers.

Histology and morphometric analyses of liver and pancreas

Liver and pancreas were collected from 24 h fasted mice fed an HCD or HFD for 12-14 months. Specimens were fixed in 10% buffered formalin until processing. Comprehensive histologic (or light microscopic) examination of liver and pancreas tissues was done using standard methods of fixation, paraffin embedding, and staining with hematoxylin and eosin (HE). Tissues were examined for any changes or differences between CPT-1a +/- and +/+ mice liver and pancreas. Diet and genotype effects were evaluated by subjective scoring of changes in affected tissues. Slides were coded to conceal the identity of the treatment group from the examiner. After subjective evaluation for abnormalities, the tissues were evaluated by histomorphometry using a Leica DMR research microscope (Leica Microsystems Wetzlar GmbH, Wetzlar, Germany), SPOT RT Slider digital camera (Diagnostic Instruments, Sterling Heights, MI), and Image Pro Plus v4 image analysis software (Media Cybernetics, Silver Spring, MD). Color digital images were made at an objective magnification of 20X. Hepatocellular fatty change was assessed by subjective scoring of HE sections. The evaluator examined the slides without knowledge of the genotype and diet classification of the mice. Microvesicular and macrovesicular fatty changes were scored separately. Each was scored 0 for normal/absent, 1 for mild, 2 for moderate, and 3 for severe, using increments of 0.5 for intermediate degrees, e.g., 0.5 for slight or 1.5 for mild to moderate. Estimated distribution of each type of change was scored 1 for up to 1/4 of the section, 2 for 1/4 to 1/2, 3 for 1/2

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to 3/4, or 4 for 3/4 to all of the section. The severity score for microvesicular and macrovesicular fatty change was determined by multiplying the scores severity and distribution. Microvesicular and macrovesicular severity scores were summed for a total severity score.

Immunohistochemistry

Pancreas was fixed in 10% neutral buffered formalin and then processed by standard paraffin embedding methods, and sections were cut and mounted on slides. Endogenous peroxides were blocked with 0.3% hydrogen peroxide in phosphate buffered saline (PBS) for 30 min. Background staining was decreased by blocking with 1% BSA in PBS for 1 h. Anti-pig insulin (Sigma-Aldrich, Co., St. Louis, MO) developed in guinea pig was used for the primary antibody. This lot worked best at 1:9000 dilution incubated for 2 hours in a humidity chamber at room temperature. Anti-guinea pig IgG (whole molecule) peroxidase conjugate (Sigma-Aldrich, Co., St. Louis, MO) at 1:1000 dilution was used for the secondary antibody and incubated for 30 min at room temperature in a humidity chamber. True blue peroxidase substrate (Kierkegaard and Perry Laboratories, Inc., Gaithersburg, MD) was applied for 10 min and washed in water for 3 min. Nuclear Fast Red counterstain (Vector Labs, Burlingame, CA) was used for counterstaining for 10 min and followed with a 3 min wash in water. Slides were allowed to air dry thoroughly and dipped in xylene, and cover slips were applied with Permount (Fisher Chemicals, Fairlawn, NJ).

Size and numbers of islets in immunohistochemically stained sections of pancreas were determined by histomorphometry. Images of each section were made at 1X with a

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Nikon Super CoolScan 5000ED scanner at 4,000 pixels/inch. For each section, the total area of pancreas and the area of each immunostained islet were determined with Image-Pro Plus 5.1 image analysis software (Media Cybernetics, Silver Spring, MD). The resulting data were exported to a Microsoft Excel spreadsheet for calculation of average islet area in mm^2, number of islets/mm^2 pancreas, and the ratio of total islet area to pancreas area.

Leptin assays

Leptin levels were determined by ELISA (Crystal Chem., Inc., Downers Grove, IL). Aliquots of each sample were diluted 1:10, and 5 µl of each was used for the assay. Samples were incubated on the microplate supplied overnight at 4 °C, and the reaction was completed the following day as per protocol and read at 450 nm and subtracting OD was read at 630 nm. A standard curve was established, and sample data were determined from the slope.

Statistics

All data were expressed as mean values with standard deviations. Student's *t*-tests, and two-way ANOVA were used to analyze significant differences between genotype and diet. Liver macrovesicular and microvesicular fat scores were analyzed using the Kruskal-Wallis test, the rank sum test with Tukey's probability correction for multiple comparisons, and analysis of variance of ranks. *P* values less than 0.05 were considered significantly different.

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Results

Study 1

In study 1, we evaluated both fed and fasting serum free fatty acids and triglycerides. Because insulin resistant individuals often have elevated free fatty acids even in the fed state, we investigated this possible characteristic in both CPT-1a +/- and +/+ male mice fed SRD.

Fasting and fed serum free fatty acids and triglycerides

CPT-1a +/- mice fed SRD for 6-8 months demonstrated significantly lower serum free fatty acids ($p = 0.018$) in the fed state but no significant differences in the fasted state (Table 1.). Serum triglycerides in the fed state of these CPT-1a +/- mice were also significantly lower ($p = 0.01$); however, there was no significant difference found in the fasted state as compared to CPT-1a +/+ mice (Table 1).

Study 2

In study 2, we evaluated clinical markers of obesity, insulin resistance and type 2 diabetes such as body weight, body fat, and liver fatty change in CPT-1a +/- males and females as compared to the CPT-1a +/+ mice on HCDs or HFDs.

Body fat and body weight

Using DXA analysis, body fat was significantly greater in male CPT-1a +/- mice on HCD for 46 weeks ($p = 0.04$) than for CPT-1a +/+ mice (Fig. 1a). CPT-1a +/- mice showed no significant differences in body weight (Fig. 2a) as compared to CPT-1a +/+

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mice. Neither body fat (data not shown) nor body weight (Fig. 2b) was significantly different in female CPT-1a +/- versus CPT-1a +/+ mice fed an HCD for 46 weeks. In contrast to the findings on the HCD, body fat was significantly greater in male CPT-1a +/+ mice ($p = .004$) on HFD diet for 46 weeks compared to CPT-1a +/- mutants (Fig. 1b). There were no significant differences in body weights of male CPT-1a +/+ and CPT-1a +/- mice (Fig. 2a). Female CPT-1a +/- mice showed no significant difference in either body fat (data not shown) or body weight (Fig. 2b) after 46 weeks on HFD as compared to CPT-1a +/+ females.

Hepatic steatosis

CPT-1a +/- male mice fed either of the challenge diets demonstrated significantly higher total (macro + microvesicular combined) fatty change ($p = 0.01$) as compared to CPT-1a +/+ controls (Fig. 3). Microvesicular fat, in mice fed either diet, was significantly higher than macrovesicular fat, independent of genotype ($p = 0.003$) (Fig. 3). Microvesicular fat was significantly higher in CPT-1a +/- mice when fed either diet as compared to CPT-1a +/+ mice ($p = 0.03$) (Fig. 3).

Overall, in study 2, we found male CPT-1a +/- mice to be more negatively affected by the HCD with increased body fat. In contrast, they had less body fat than male CPT-1a +/+ on the HFD. Microvesicular liver steatosis is expected for β-oxidation disorders.

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Study 3

In study 3, we evaluated the time course of functional changes when male mice of both genotypes were fed the HCD and HFD. Beyond clinical markers, we pursued direct physiological evaluations that further examined for the development of impaired glucose tolerance and the development of type 2 diabetes. Further evaluation included morphological characterization of islets (β-cells) and liver for fatty change and steady state mRNA for CPT-1a and b expression.

Glucose tolerance

In both the 7-month HCD and the 12- to 14-month HCD groups, CPT-1a +/- mice were more glucose tolerant than CPT-1a +/+ mice as measured by OGTT (7-month HCD, $p < 0.001$; 12- to 14-month HCD, $p < 0.015$) (Fig. 4a and 6a). Insulin levels, measured throughout OGTTs, were not significantly different between genotypes (Fig. 4b and 6b). However, in both diet duration groups, CPT-1a +/- mice were found to have significantly higher average first phase insulin responses to a glucose load, based on insulinogenic index values (Fig. 4c and 6c), indicating greater glucose sensitivity (7-month HCD, $p = 0.014$; 12- to 14-month HCD, $p = 0.047$).

In the 7-month HFD group, there was no significant difference in glucose tolerance between CPT-1a +/- and CPT-1a +/+ mice (Fig. 7a). In contrast, the CPT-1a +/- mice fed the HFD diet for 12-14 months were significantly less glucose tolerant ($p < 0.023$) (Fig. 7a) than CPT-1a +/+ mice as measured by OGTT. CPT-1a +/- mice demonstrated significantly lower insulin levels (7-month HFD, $p < 0.002$; 12- to 14-month HFD, $p < 0.001$) (Fig. 7b and 8b) throughout the OGTT as compared to the insulin levels

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of CPT-1a +/+ controls. However, first phase insulin secretion calculated by insulinogenic index showed no significant difference between CPT-1a +/- and CPT-1a +/+ mice for either diet duration group (Fig. 7c and 8c).

Insulin tolerance

HCD male CPT-1a +/- mice were significantly more insulin sensitive (7 month-HCD $p < 0.002$, 12-14 month-HCD $p < 0.003$) (Fig. 8a and 8c) than were CPT-1a +/+ controls as was found by their steeper rate of decline of blood glucose levels in response to the IP injection of insulin. In fact, the standard protocol had to be modified due to increased sensitivity found in the mutants because of the induction of severe hypoglycemia. Additionally, HFD-fed CPT-1a +/- mice were significantly more insulin sensitive (7 month-HFD, $p = 0.04$; 12- to 14-month HFD, $p = 0.026$) (Fig. 8b and 8d) than CPT-1a +/+ controls as was found by their steeper rate of decline of blood glucose levels in response to an IP injection of insulin.

Basal insulin levels

Fasting insulin levels were not significantly different among both CPT-1a +/- and CPT-1a +/+ mice fed HCD for either duration (data not shown). However, HFD CPT-1a +/- mouse fasting insulin levels were significantly lower (7 month-HFD, $p < 0.01$; 12-14 month HFD, $p = 0.027$) (Table 1) than CPT-1a +/+ control mice also indicating a state of increased insulin sensitivity.

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Morphometric analysis of β-cells within islets

Immunostaining of pancreas sections for insulin identified β-cells and islets. This analysis showed that 12- to 14-month-fed HCD CPT-1a +/- mice had lower percent islet area/pancreas area ($p < 0.001$), lower total islet area (mm^2) ($p = 0.001$), and lower average islet area (mm^2) ($p = 0.001$) (Fig. 9a, b, and c). Similarly, 12- to 14-month-fed HFD CPT-1a +/- mice had lower percent islet area/pancreas area ($p = 0.006$), lower total islet area (mm^2) ($p = 0.005$), lower total islet number ($p = 0.001$), and a lower average number of islets per mm2 pancreas ($p = 0.014$) (Fig. 10a,b,c,and d).

Analysis of steady state mRNA levels by Northern blot

Northern blot results showed a trend toward increased *Cpt-1b* mRNA expression in liver of CPT-1a +/- mice as compared to CPT-1a +/+ mice fed an HCD (data not shown). There was also a trend toward increased *Cpt-1b* expression in skeletal muscle, although neither result by this analysis was significantly different (data not shown). *Cpt-1a* expression was lower in CPT-1a +/- as was expected as a result of the heterozygous deletion (data not shown).

Peroxisomal fatty acid oxidation requires *Acox-1* expression, and complete mitochondrial fatty acid oxidation requires medium-chain acyl-CoA dehydrogenase (*Acadm*) expression. Furthermore, both genes are downstream targets of peroxisomal proliferator activator receptor α (PPAR- α), a master regulatory transcription factor of mitochondrial and peroxisomal fatty acid oxidation, which includes *Cpt-1b*. There were no significant differences in liver expression of *Acox-1* or *Acadm* in CPT-1a +/- mice fed either HFD or

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HCD (data not shown). However both *Acox-1* and *Acadm* expression in CPT-1a +/- mice appeared higher as compared to wild-type controls (Fig. 11).

To further evaluate *Cpt-1a* and *b* mRNA expression, we used the more quantitative and sensitive method of Q-RT-PCR.

Quantitative real time PCR analysis of steady state mRNA levels

HCD CPT-1a +/- mice showed significantly higher *Cpt-1b* expression in liver as measured by Q-RT-PCR than CPT-1a +/+ controls ($p = 0.007$) (Fig. 12a). HFD-fed CPT-1a +/- mice showed a trend toward higher *Cpt-1b* expression in liver as measured by Q-RT-PCR than CPT-1a +/+ controls, although the differences were not significant (Fig. 12b).

Pyruvate dehydrogenase kinase is a critical regulator of glucose oxidation. Decreased PDK-4 activity has been associated with increased insulin sensitivity [19] Thus, increased PDK activity would inactivate pyruvate dehydrogenase and decrease glucose oxidation. *Pdk4* expression in liver of HCD-fed CPT-1a +/- mice was significantly higher ($p < 0.05$) (Fig. 13a), although *Pdk2* expression in liver of HCD CPT-1a +/- mice was not significantly different from CPT-1a +/+ mice (Fig. 13b). *Pdk 4 and 2* expression in liver of HFD CPT-1a +/- mice was higher although neither result was significantly different (Fig. 15a and b).

In skeletal muscle of HCD CPT-1a +/- mice, *Pdk 2* and *4* expression was not significantly different (Fig. 14a and b). In contrast, HFD-fed CPT-1a +/- mice demonstrated significantly lower *Pdk 4* expression than CPT-1a +/+ mice ($p = 0.03$) (Fig. 16a). *Pdk2* expression was lower as well in HFD CPT-1a +/- mice, but the result was not significant

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(Fig. 16b). There were no significant differences in heart *Pdk 4* and *2* expression in HFD CPT-1a +/- mice as compared to CPT-1a +/+ mice (Fig. 17).

Serum leptin measurements

Previous studies in rats had shown that chemical inhibition of CPT-1 activity had marked effects on leptin levels and body fat [20]. Thus, in this study leptin levels were measured in all four diet groups. Neither genotype nor diet appear to affect leptin levels because no significant differences in leptin levels were found between any of the groups (Fig. 18).

Discussion

We hypothesized that the long-term effects of a high calorie diet concurrent with a deficiency in mitochondrial fatty acid oxidation (CPT-1a deficiency) would be causal at the cellular levels for development of both insulin resistance and β-cell dysfunction, which are pivotal characteristics involved in the development of type 2 diabetes. Our hypothesis was based on previous studies using CPT-1 inhibitors [9] and the extensive body of literature associating excess fatty acids with both the development of insulin resistance and β-cell dysfunction. Understanding the mechanism behind this link between excess fatty acids and the development of type 2 diabetes is crucial for prevention and treatment.

The most striking finding of these studies was that the CPT-1a +/- male mice were found to be more insulin sensitive than CPT-1a +/+ male mice when fed either an HCD or HFD. Additionally, they were more glucose tolerant when fed the HCD but less glucose tolerant when fed the HFD. Although these results initially seemed contradictory

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to our initial hypothesis, they may be explained by the significant finding of increased *Cpt-1b* expression in liver in CPT-1a +/- mice as compared to CPT-1a +/+ mice.

In study 1, we expected to find that CPT-1a +/- male mice had elevated free fatty acids and TG in the fed state because we initially predicted this mouse model to have characteristics indicating the development of type 2 diabetes with or without the stress of an HFD or HCD. In the fasting state, free fatty acids are generally elevated in a normal animal. This is a result of fat mobilization from adipose tissue which is regulated by insulin. Free fatty acids elevated in the non-fasting states have been correlated with insulin resistance. Insulin inhibits lipolysis, and, because insulin is usually secreted in a more robust, pulsatile manner in the post-prandial state, lipolysis is generally inhibited in the post-prandial state. In a diseased or insulin resistant state, the actions of insulin, such as inhibition of lipolysis, may not be as potent, and, therefore, it may result in increased free fatty acids and TG in the fed state.

Thus, we investigated free fatty acids and TG in the fasting and fed states and found no significant difference in fasting levels. In contrast, serum free fatty acids and TGs were significantly lower in CPT-1a +/- mice as compared to CPT-1a +/+ mice in the fed state. This result is consistent with our finding of increased insulin sensitivity in CPT-1a +/- male mice as compared to CPT-1a +/+ mice.

The major findings demonstrated in study 2 were that CPT-1a +/- mice had significantly higher body fat after approximately 1 year on the HCD but significantly lower body fat after ~1 year on the HFD. Furthermore, microvesicular fatty change was significantly greater in CPT-1a +/- livers as compared to diet-induced liver steatosis in CPT-1a +/+ mice. The fact that CPT-1a +/- mice demonstrated greater amounts of microvesicular

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liver fat fed both challenge diets than wild-types may not be contradictory to them being more insulin sensitive. Microvesicular fat accumulation has been associated with ATP depletion and fatty acid toxicity with risk for eventual liver inflammation followed by cirrhosis and liver failure [21]. Therefore, the appearance of microvesicular fat could be a consequence of the FAO deficiency [22,23] that is unrelated to their heightened insulin sensitivity [24,25]. This is further supported by a recent study using CPT-1 inhibitor treatment in mice showing severe microvesicular fatty liver in the absence of insulin resistance [25]. Additionally microvesicular fat deposition has also been demonstrated to be a result of chronic lipid peroxidation [22], indicating that an increase in peroxisomal FAO may partially explain the mechanism for the development of microvesicular fat [22]. Our results demonstrating increased *Acox-1* expression in CPT-1a +/- skeletal muscle are in accord with this finding.

Moreover, although salicytic acid has been associated with inhibition of mitochondrial FAO [26] and induction of microvesicular fat [24], it also has been shown to be preventative against fat-induced insulin resistance [27]. Because of the inherent contradictions of the collective findings mentioned above, we feel that the finding of greater hepatic microvesicular fat in the CPT-1a mice is not contradictory to the results demonstrating that CPT-1a mice are more insulin sensitive than CPT-1a +/+ mice. In summary these results along with the biochemical markers warranted further study which is why we completed study 3.

Evidence supporting our initial hypothesis included a study by McGarry and colleagues finding rats fed a high-fat or low-fat diet with etomoxir (CPT-1 inhibitor) were significantly less insulin sensitive than untreated rats. They demonstrated that both eto-

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moxir and diet significantly reduced the glucose infusion rate under hyperinsulinemic-euglycemic clamp conditions, indicating that both diet and inhibition of CPT-1 resulted in insulin resistant states in the rat. We surmised that the reason for the differences in a genetically induced limitation on FAO versus the published study's drug induced limitation on FAO is that etomoxir treatment was administered at a high dosage in order to inhibit both CPT-1a and b isoforms, because CPT-1b is less sensitive to etomoxir [28]. In our study, the heterozygous deletion was targeted only toward the CPT-1a isoform. Our data suggest that CPT-1b compensation in liver may be advantageous in terms of prevention of insulin resistance.

Moreover, in study 3, CPT-1a +/- male mice fed the HCD (7 months and 12- to 14-months) were more glucose tolerant as evidenced by lower glucose levels throughout the OGTT. Because glucose tolerance is a result of both β-cell sensitivity to glucose and peripheral insulin sensitivity, we investigated the possibility that CPT-1a mice had higher insulin secretion and were more sensitive to insulin. We estimated β-cell responsiveness in part by measuring first phase insulin secretion. Our findings suggested that CPT-1a +/- male mice were more glucose sensitive as was demonstrated by their higher first phase insulin secretion during the OGTT.

Insulin tolerance tests were then used to investigate insulin sensitivity in CPT-1a +/- male mice as compared to CPT-1a +/+ male mice fed the HCD. The results demonstrated that they were not only more glucose sensitive but also more insulin sensitive, thus explaining CPT-1a +/- male mice being more glucose tolerant as compared to CPT-1a +/+ on HCD.

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Alternatively, HFD-fed CPT-1a +/- mice were less glucose tolerant than CPT-1a +/+ mice. Once again we investigated the mechanism by which this may have been occurring by first looking at glucose sensitivity by insulinogenic index. In contrast to the HCD-fed CPT-1a +/- mice, CPT-1a +/+ mice fed the HFD showed no significant differences between the genotypes. In fact, in contrast to the HCD-fed mutants, the insulinogenic indexes of HFD-fed CPT-1a +/-mice included a trend toward lower insulinogenic indexes. CPT-1a +/- mice fed the HFD perhaps suffered deleterious effects on β-cell function from the combination of excess substrate delivery from the HFD and the inherited FAO deficiency. In support of this deduction, those with low insulinogenic index are at particularly high risk to develop diabetes, and arteriosclerotic complications are fairly frequent in this category [17]. These data suggest that CPT-1b compensation may not have occurred in the β-cell or was not able to compensate enough in the HFD-fed CPT-1a +/- mouse β-cell. Thus, we speculated that their phenotype may have actually been worse had the CPT-1a +/- mice fed HFD not been more insulin sensitive than CPT-1a +/+ mice fed the HFD.

Insulin tolerance tests revealed that, although CPT-1a +/- mice may have been less glucose tolerant and less glucose sensitive when fed HFD diet, they were still more insulin sensitive. Thus, we speculate that they may have been less glucose tolerant as a result of β-cell dysfunction induced by the combination of HFD and CPT-1a deficiency.

Free fatty acids are known to be insulinotropic as a protection against hypoinsulinemia in the fasting state when glucose is very low. Fatty acids promote for minimal insulin secretion so that there is still some inhibition of lipolysis from adipose tissue and not an unregulated, excessive release of fatty acids from adipose tissue in the fasting state

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[29]. However, when pancreatic β-cells are exposed to excessive amounts or extended duration of fatty acids this is known to be deleterious. There is evidence that conditions of excessive fatty acids lead to apoptosis [30,31], reduced pre-proinsulin biosynthesis, reduced post-translational proinsulin processing, and inadequate responses to glucose stimulation which may be responsible for β-cell dysfunction and impaired glucose tolerance [32].

We measured β-cell (islet) insulin content by immunostaining for insulin. We expected to see either hyperplasia or hypertrophy as a result of excessive secretion from a predicted fatty acid build-up or necrosis as a result of long-term excess fatty acid exposure. Surprisingly, we found that, when fed either challenge diet, CPT-1a +/- mice had lower percent islet to pancreas area indicating that they did not have as many insulin containing β-cells as did the CPT-1a +/+ mice. One interpretation of this finding is that increased insulin sensitivity in the CPT-1a +/- mice prevented them from diet-induced hyperplasia or hypertrophy. In comparison, on the HFD, we could interpret this finding as a -1 in that the lower number of β-cells may have been a result of apoptosis or reduction in the production of insulin granules within the β-cells [32,33] The fatty acid-induced decrease in β-cell insulin content would explain the decrease in glucose sensitivity found in HFD-fed CPT-1a +/- mice. In support of these conclusions, a recent study demonstrating that over-expression of SREBP-1c in β-cells, which resulted in β-cell triglyceride build-up, caused the onset of impaired glucose tolerance which they partially attributed to the reduction in number and size of islets [34].

The mechanism for increased insulin sensitivity in liver of CPT-1a +/- as compared to the CPT-1a +/+ mice may be as follows: post-transcriptionally, CPT-1b is regu-

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lated solely by concentrations of malonyl-CoA, whereas CPT-1a is regulated by concentrations of and changes in sensitivity to malonyl-CoA which is dependent on CPT-1a phosphorylation [35]. Thus, CPT-1a has been found to be unique in responding to different physiological states such as starvation, insulin deficiency, and hypo- and hyperthyroidism by changing its sensitivity to inhibitor several fold [35]. These changes amplify the effects of fluctuations in the cytosolic concentration of malonyl-CoA occurring under these conditions [35].

As stated above, we chose the HFD challenge with the intention of overloading an already comprised metabolic system with substrate. Alternatively, we chose the HCD challenge to increase production of malonyl-CoA to promote for excess inhibition in addition to an already genetically imposed limitation on FAO. So we expect that, if the CPT-1b expression in liver is advantageous to CPT-1a +/- mice fed the HCD, it may be because excess malonyl-CoA may not be as potent an inhibitor for CPT-1b up-regulated in liver. In this scenario, CPT-1b compensation in liver may actually be better than an equivalent CPT-1a expression normally found to predominate in liver. That is, there may be compartmentalization or other factors such as membrane fluidity involved that may limit CPT-1b inhibition in this unusual hepatic environment thus facilitating increased FAO [36].

We searched exhaustively for a mechanism to explain this surprising result. We used mRNA expression studies of marker genes of peroxisomal and mitochondrial FAO, which are also downstream targets of PPAR-α. We found that *Cpt-1b* expression in liver was increased, possibly explaining the advantageous effects of the mutation. We speculated that leptin might be involved because of recent studies using CPT-1 inhibitor treat-

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ment in rats [20]. These investigators demonstrated higher leptin levels after treatment of normal rats with the CPT-1 inhibitor TGDA. They found that the reduction in FAO increased leptin levels implicating a reduction in FAO leads to a build-up of fatty acids which then may initiate feedback causing leptin levels to increase. In contrast, we found a lower ratio of leptin to body weight in CPT-1a +/- males fed the diets than CPT-1a +/+ mice fed the challenge diets indicating that they had lower serum leptin levels once they were normalized for body weight.

In liver both *Pdk2* and *4* are expressed and involved in the regulation of glucose oxidation via inhibition of the pyruvate dehydrogenase complex (PDH). PDK 2 and 4 accomplish this inhibition by way of phosphorylation of this complex [36]. In the fed state, activation of PDH promotes glucose oxidation, the formation of malonyl-CoA, and fatty acid synthesis from pyruvate [36]. In the fasted state, both *Pdk2* and *Pdk4* expression are thought to be increased, and thus PDH inhibited and glucose oxidation minimized facilitating the transfer of available pyruvate to gluconeogenesis or non-glucose substrate oxidation. A reduction in PDK 4 has been associated with increased insulin sensitivity [19]. Our findings that *Pdk4* expression is higher in the liver of CPT-1a +/- mice on HCD compared to CPT-1a +/+ suggests that hepatic glucose oxidation may be limited by PDH inhibition. Alternatively, *Pdk 4* expression in skeletal muscle of HFD CPT-1a +/- mice is lower than CPT-1a +/+, suggesting that the CPT-1a deficiency is promoting for glucose oxidation in skeletal muscle and may be contributing to the increase in insulin sensitivity.

In conclusion, we found CPT-1a +/- mice to have lower free fatty acids and lower TG in the fed state which was contradictory to our initial hypothesis; however, these findings support increased insulin sensitivity and, in the case of HCD fed CPT-1a +/- mice,

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increased glucose tolerance as compared to wild-type mice. We found that CPT-1a +/- mice had higher hepatic microvesicular fat when fed either diet than did CPT-1a +/+ mice. This may or may not be inconsistent with the OGTT and ITT findings in that recent studies demonstrating greater microvesicular fat in liver as a result of CPT-1 inhibition found no difference in insulin sensitivity [25]. Furthermore, CPT-1a +/- male mice fed the HFD had higher body fat although no differences were found in body weight. In contrast, CPT-1a +/- male mice fed the HFD had lower body fat, and, again, no difference was found between body weights of CPT-1a +/- andCPT-1a +/+ mice. Finally, female CPT-1a +/- mice showed no significant differences between body fat or body weight when fed either HFD or HCD.

CPT-1a +/- mice were found to be more insulin sensitive when fed either an HCD or an HFD, which we speculate may be a result of increased CPT-1b activity as compared to male CPT-1a +/+ mice, and possibly lower *Pdk* 4 expression in skeletal muscle which was significant in HFD, but not HCD fed CPT-1a +/- mice. Findings of lower fasting insulin levels in CPT-1a +/- mice fed HFD for either 7 months or 12-14 months further supported this finding. CPT-1a +/- mice were more glucose tolerant when fed the HCD but less glucose tolerant when fed the HFD. We speculated that this difference was due to an impaired insulin response to glucose as a result of both the HFD and the FAO deficiency. This was further supported by the results indicating that CPT-1a +/- mice fed HFD had lower percent islet/pancreas area (mm^2), lower total islet area, lower number of islets, and lower average number of islets per mm^2 of pancreas. In contrast the CPT-1a +/- mice fed HCD also were found to have lower percent islet/pancreas area, lower total

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islet area, and lower average number of islets per mm^2 of pancreas; however they were still more glucose tolerant, and this may be attributed to their greater insulin sensitivity.

Although it was beyond the scope of this study, we feel that future studies examining both β-cell function and β-cell gene expression would complement our understanding thus far of the link between excess fatty acids and the development of type 2 diabetes. Overall we conclude that the study is extremely pertinent to both prevention and treatment of both β-cell dysfunction and insulin resistance.

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Table 1. Phenotype Evaluation Chart

			Fasted	**Fed**			**Fasted**	**Fed**	
Serum Glucose (mg/dl)			**+/-CPT-1a**	**+/-CPT-1a**			**+/+CPT-1a**	**+/+CPT-1a**	
HFD 6-8 Months		n=8	66±9.8*			n=10	54±11*		p=0.03
HFD 12-14 Months		n=9	59±24			n=9	56±15		
HCD 6-8 Months		n=9	58±16			n=10	69±49		
HCD 12-14 Months		n=9	51±12			n=10	50±11		
Serum Insulin (ng/ml)									
HFD 6-8 Months		n=13	0.21±.06*			n=11	0.37±.19*		p=0.01
HFD 12-14 Months		n=9	0.19±.08*			n=8	0.37±.21*		p=0.03
HCD 6-8 Months		n=10	0.16±.08			n=10	0.44±.44		
HCD 12-14 Months		n=9	0.16±.1			n=9	0.21±.09		

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Table 1 (Continued)

Serum Triglycerides (mg/dl)								
SRD 6-8 Months		n=7	141±65	105±30*		n=7	138±36	168±49*	p=0.01
		Fasted	**Fed**			**Fasted**	**Fed**		
HFD 12-14 Months		n=10	146±28			n=13	140±38		
HCD 12-14 Months		n=12	156±56			n=11	144±22		
Serum Free Fatty Acids (mEq/L)								
SRD 6-8 Months		n=7	1.6±.38	.9±.2*		n=7	1.6±.3	1.3±.2*	p=0.018
HFD 12-14 Months		n=10	0.82±.23			n=13	0.98±.24		
HCD 12-14 Months		n=12	1.19±.4			n=11	0.91±.24		

Table 1. (HFD=High-fat diet, HCD=High-carbohydrate diet, SRD=Standard rodent diet) Fasting glucose and insulin for male CPT-1a +/- and CPT-1a +/+ on HFD or HCD for 7 months or 12-14 months. Fasting and fed (SRD only) free fatty acids and triglycerides for male mice on SRD for 6-8 months and HFD and HCD for 7 months or 12-14 months.

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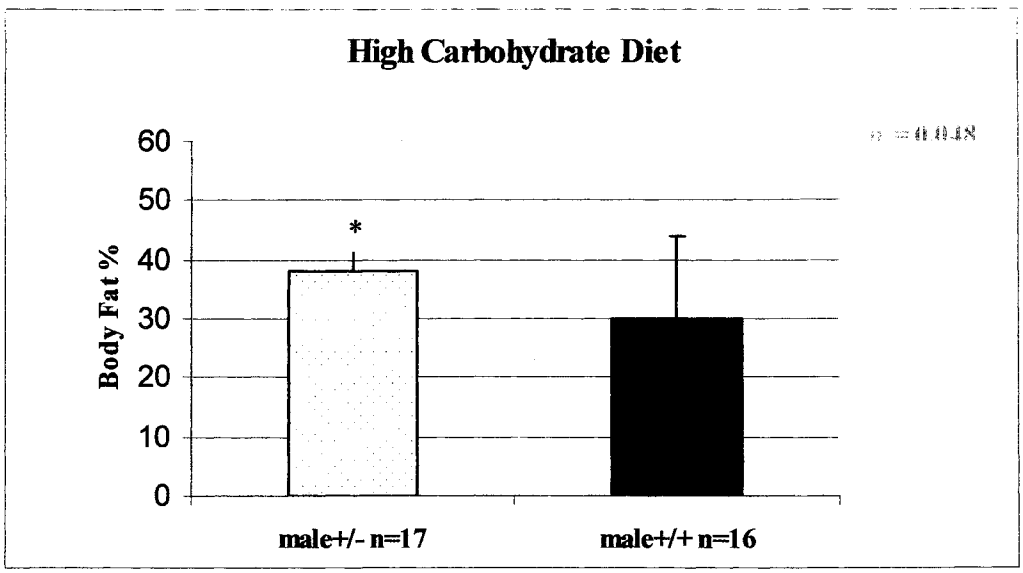

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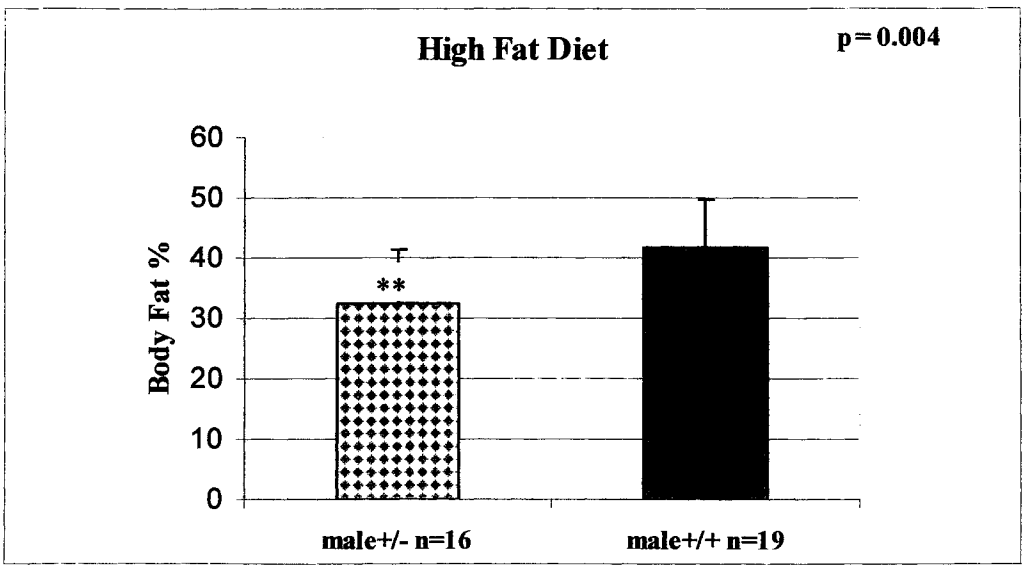

Fig. 1. Effect of HCD and HFD and CPT-1a deficiency on body fat in male mice fed diet for 42 weeks. Body-fat was determined by DXA analysis 1 week prior to sacrifice. Significant differences as compared to CPT-1a +/+ male mice are indicated (*HCD p = 0.048, **HFD p = 0.004).

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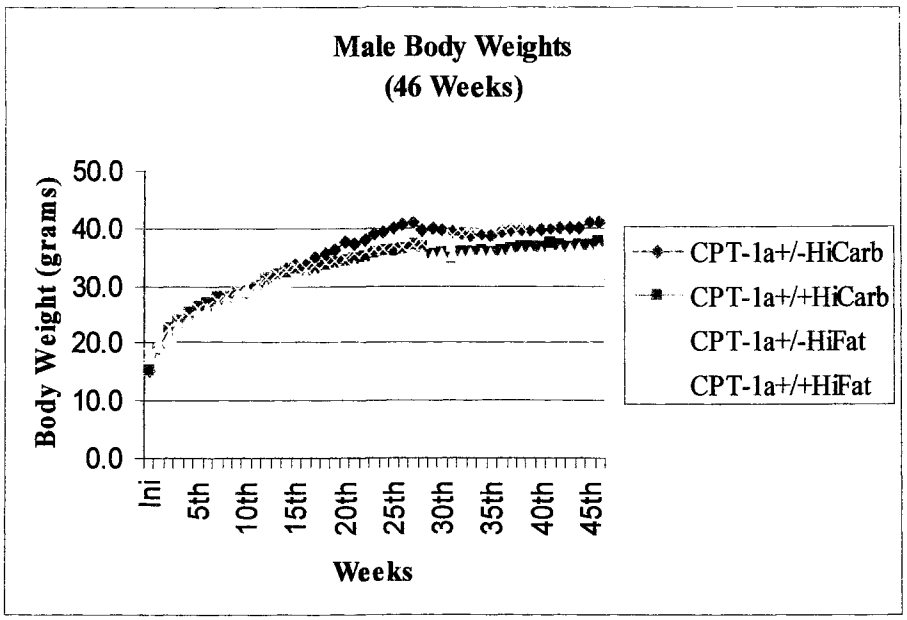

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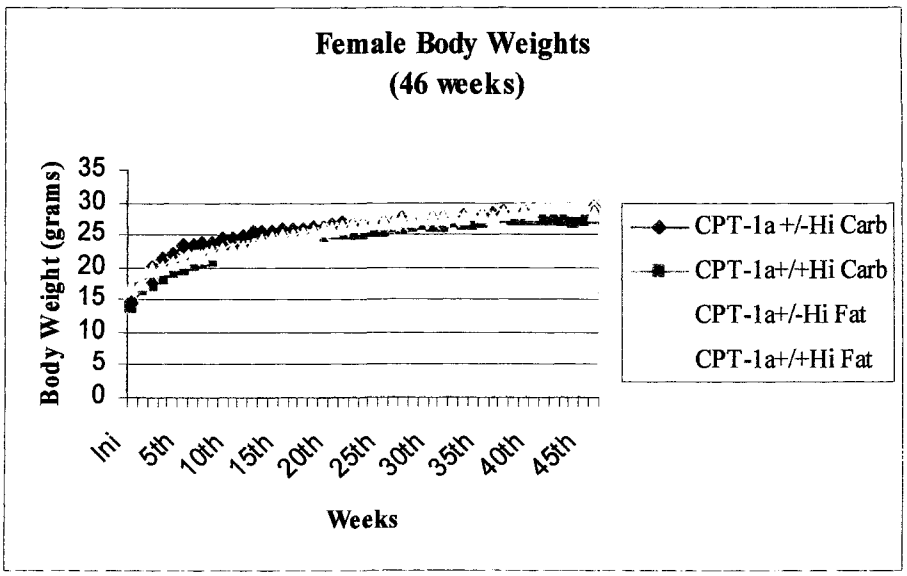

Fig. 2. Body weights of male and female CPT-1a +/- mice as compared to CPT-1a +/+ mice fed HCD or HFD for ~12 months. There were no significant differences in body weight (measured weekly for the first 46 weeks) between CPT-1a +/- and CPT-1a +/+ mice.

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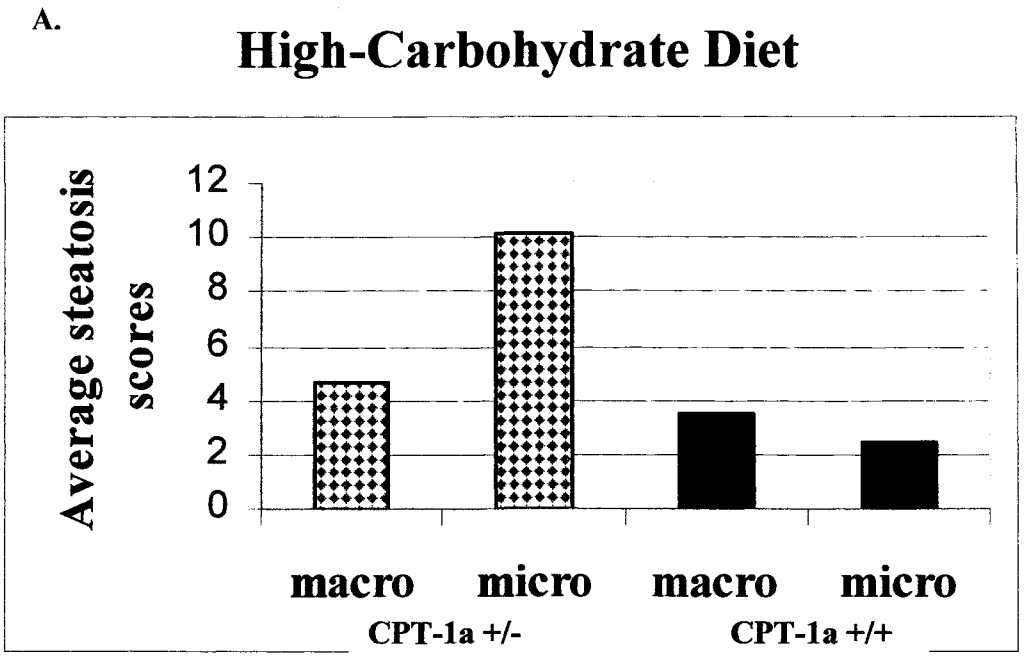

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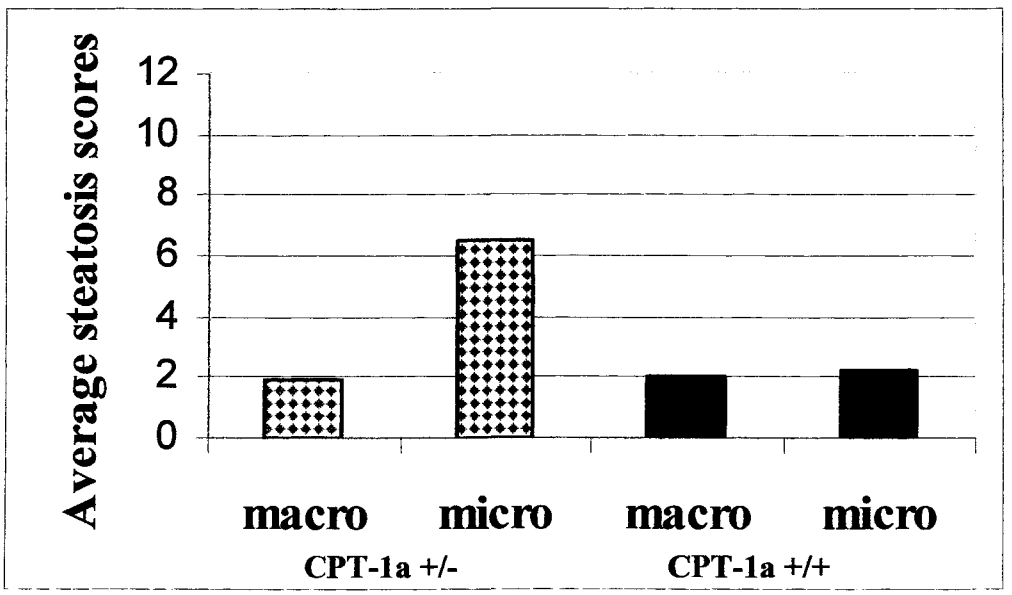

Fig. 3. Liver steatosis in CPT-1a +/- mice as compared to CPT-1a +/+ mice fed high-carbohydrate diet for ~12 months or high-fat diet for ~12 months. Microvesicular fat was significantly greater than macrovesicular fat in mice fed the HCD independent of genotype ($p = 0.003$) CPT-1a +/- livers demonstrated significantly higher microvesicular fat on either diet ($p = 0.03$).

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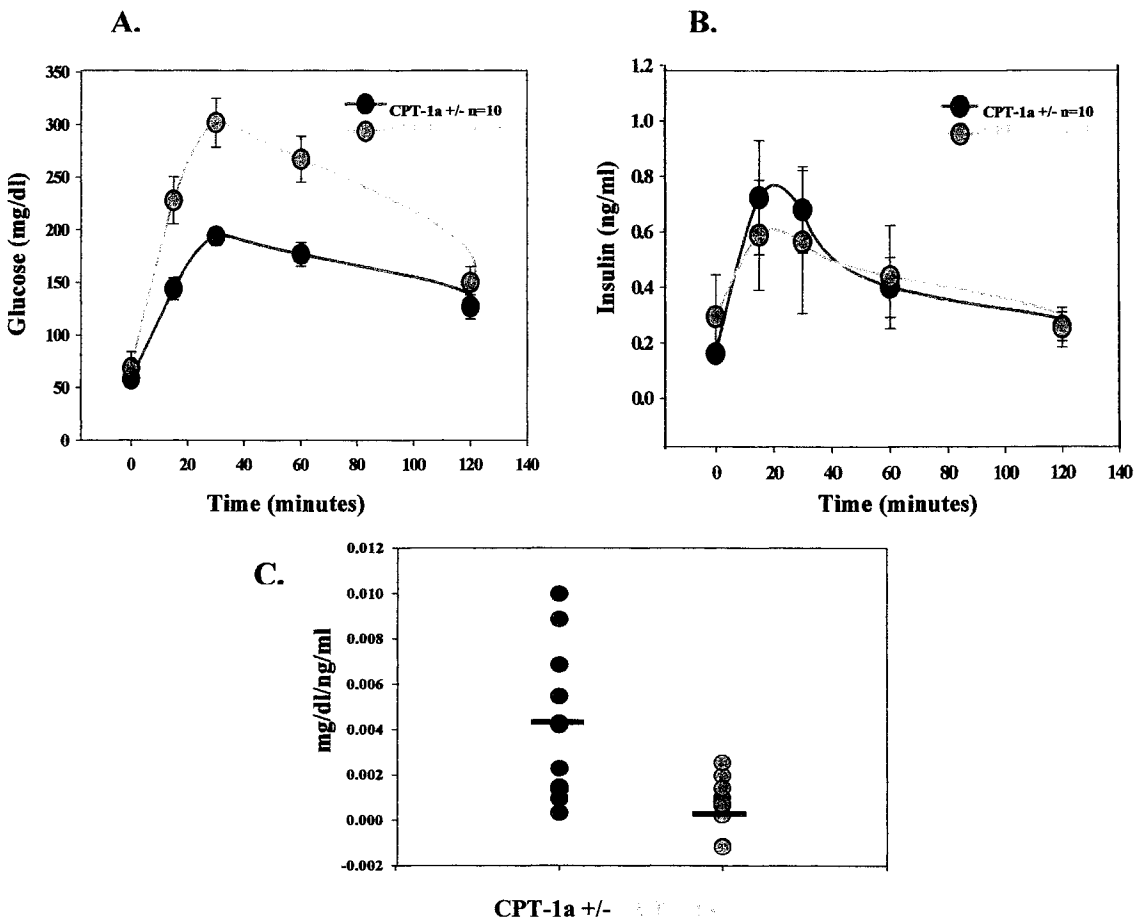

Fig. 4. Glucose tolerance test–HCD 7 months. (A) Serum glucose ($p = 0.001$) and (B) insulin levels for CPT-1a +/- CPT-1a +/+ mice on high-carbohydrate diet for 7 months. (C) Insulinogenic index (Δ insulin / Δ glucose from 0-30 min after glucose administration) ($p = 0.014$).

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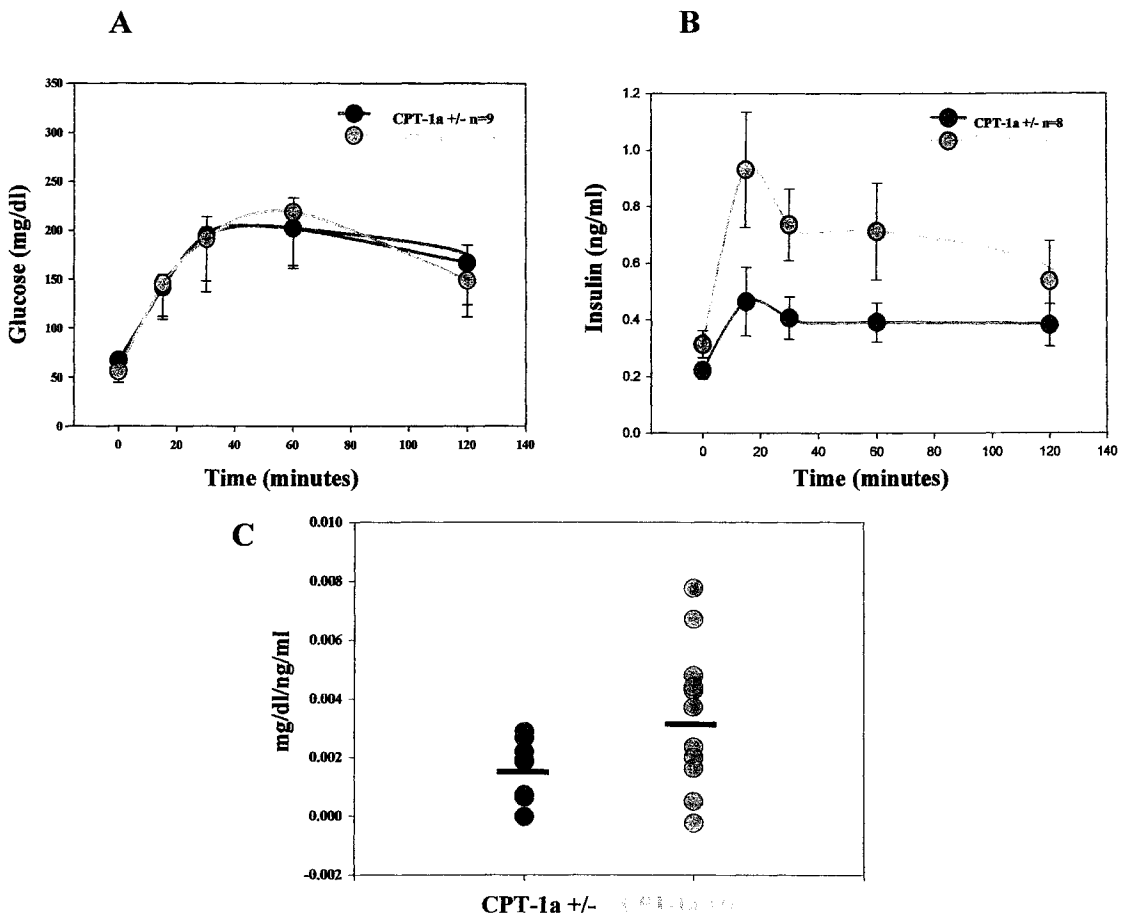

Fig. 5. Glucose tolerance test–HFD 7 months. (A) Serum glucose ($p < 0.002$) and (B) insuline levels for CPT-1a +/- CPT-1a +/+ mice on high-fat diet for 7 months. (C) Insulinogenic index (Δ insulin / Δ glucose from 0-30 min after glucose administration).

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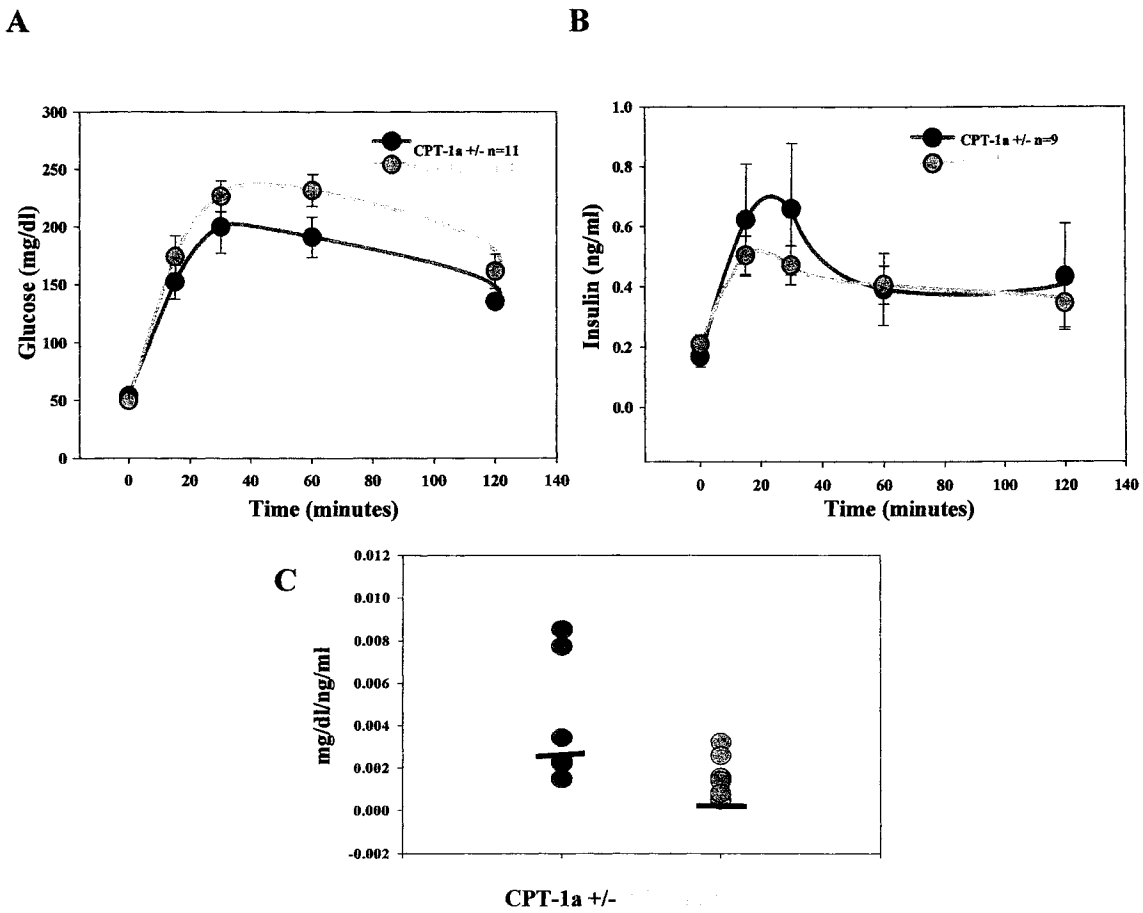

Fig. 6. Glucose tolerance test–HCD 12-14 months. (A) Serum glucose ($p < 0.015$) and (B) insulin levels for CPT-1a +/- and CPT-1a +/+ mice on high-carbohydrate diet for 12-14 months. (C) Insulinogenic index (Δ insulin / Δ glucose from 0-30 min after glucose administration) ($p = 0.047$).

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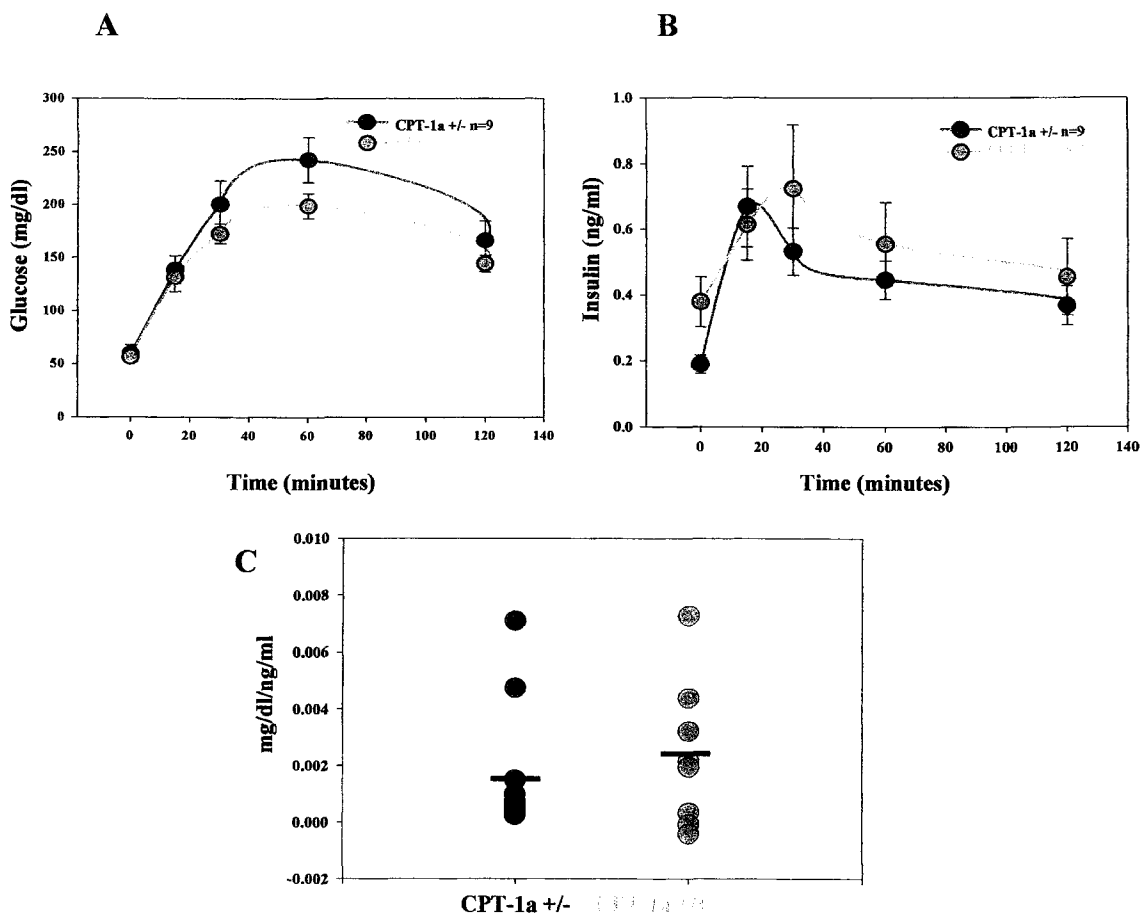

Fig. 7. Glucose tolerance test on HFD for 12-14 months. (A) Serum glucose ($p < 0.001$) and (B) levels for CPT-1a +/- CPT-1a +/+ mice on high-fat diet for 12-14 months. (C) Insulinogenic index (Δ insulin / Δ glucose from 0-30 min after glucose administration).

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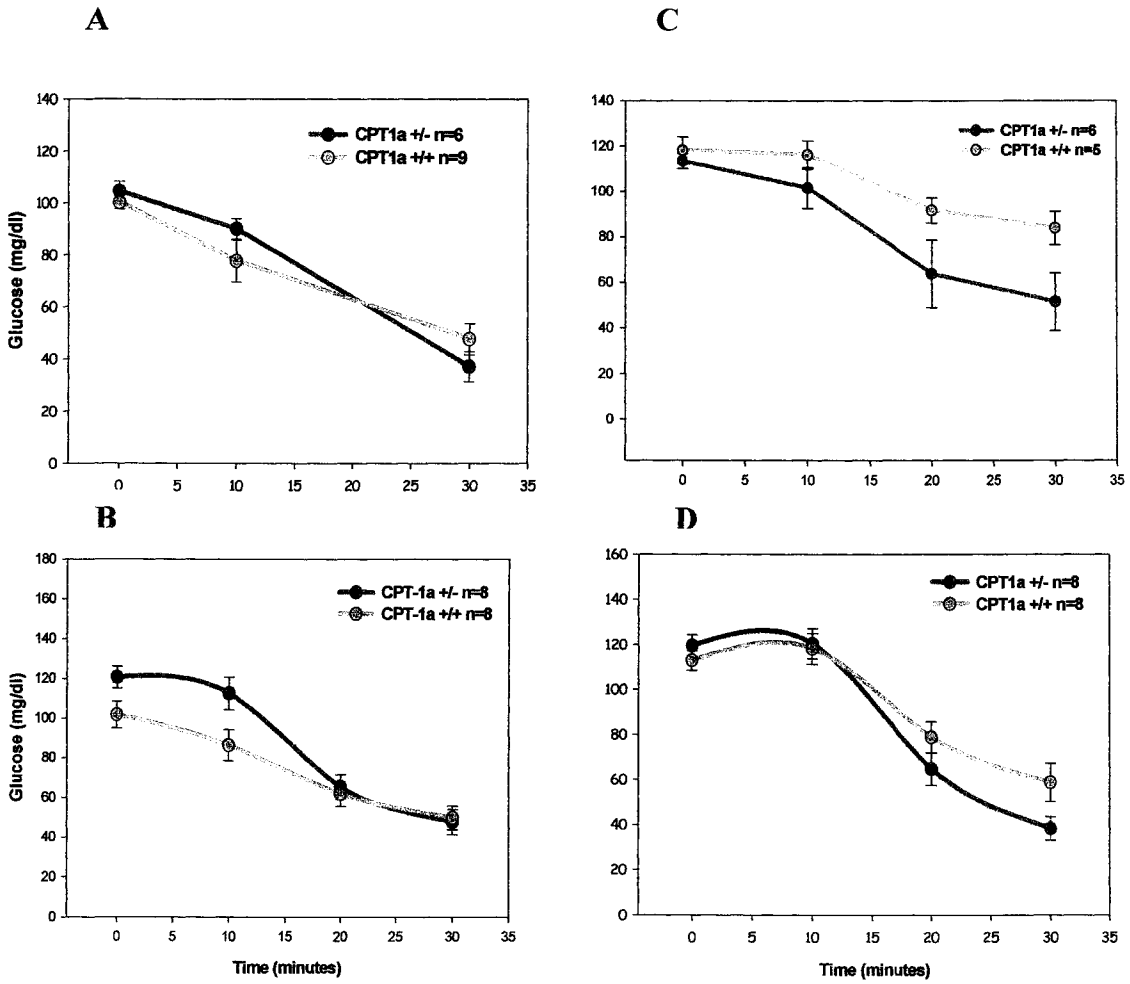

Fig. 8. Insulin tolerance tests. (A) Serum glucose for CPT-1a +/- and CPT-1a +/+ mice on high carbohydrate diet for 7 months ($p < 0.002$), (B) high-fat diet for 7 months ($p = 0.04$), (C) high-carbohydrate diet for 12-14 months ($p<0.003$), and (D) high fat diet for 12-14 months ($p = 0.026$) for 30 min after insulin injection.

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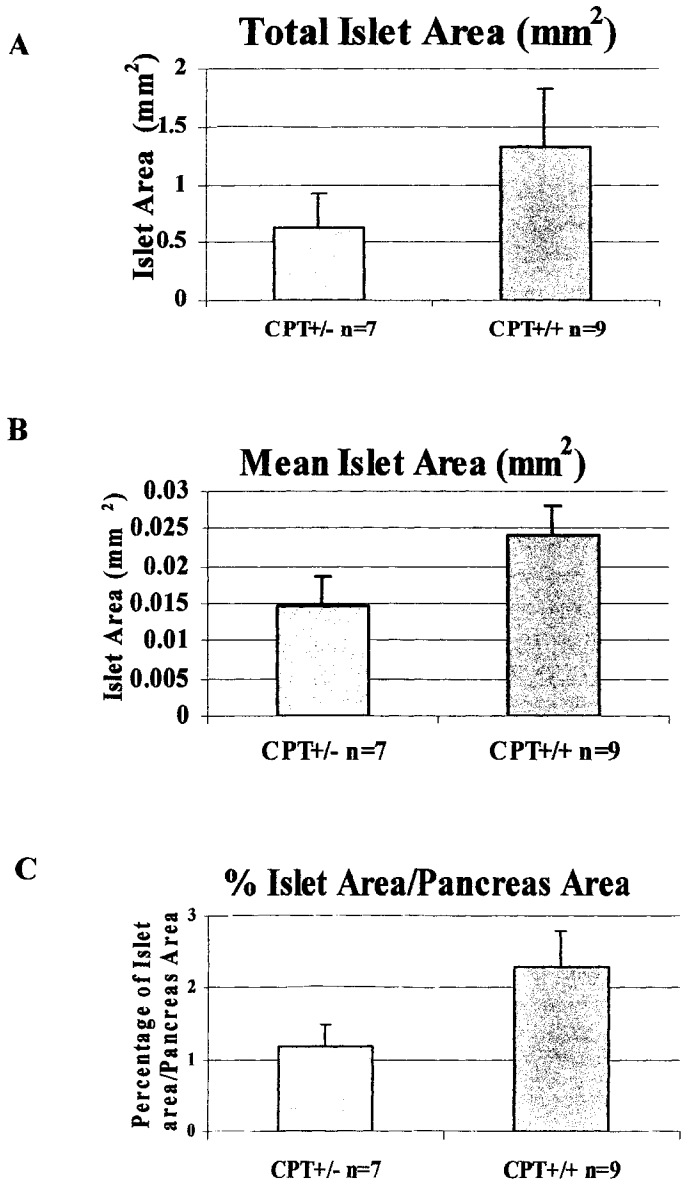

Fig. 9. Immunohistochemistry for islets from mice fed HCD. (A) Differences in total islet area ($p = 0.001$), (B) % islet to pancreas area ($p < 0.001$), and (C) mean islet area ($p = 0.001$) in CPT-1a +/- vs. CPT-1a +/+ mice on HCD.

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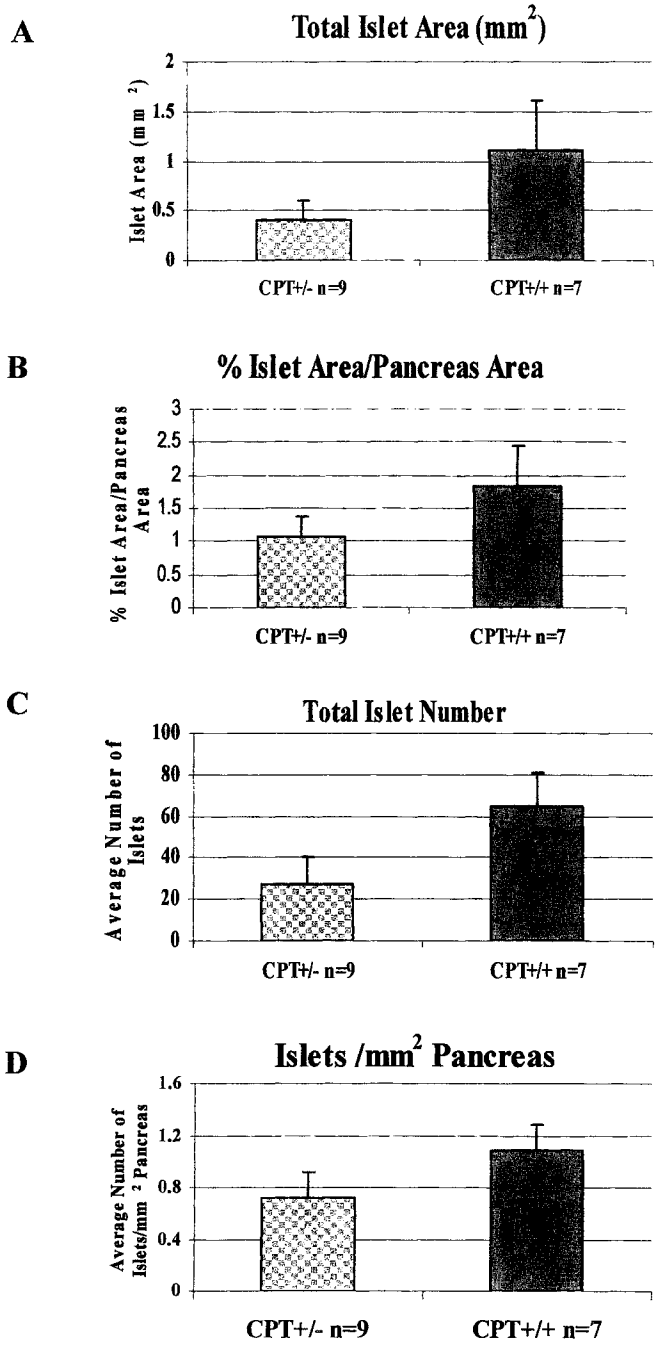

Fig. 10. Immunohistochemistry for islets from mice fed HFD. (A) Differences in islet area ($p = 0.005$), (B) % islet to pancreas area ($p = 0.006$), (C) islet number ($p = 0.001$), and (D) islets per mm2 of pancreas ($p = 0.014$) in CPT-1a +/- vs. CPT-1a +/+ mice on high-fat diet.

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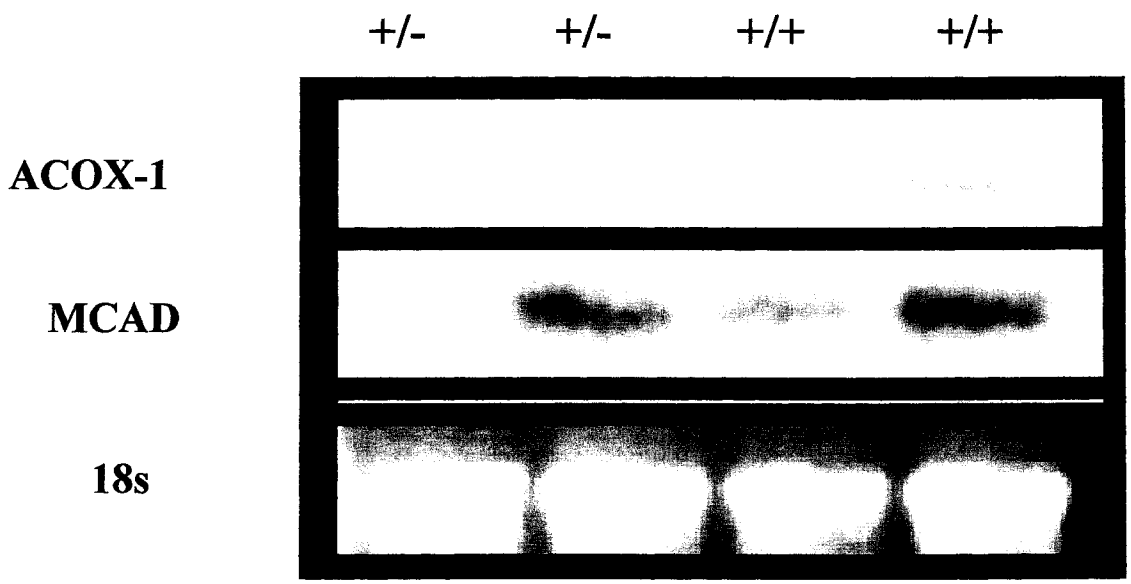

Fig. 11. Skeletal muscle ACOX-1 or (*Acox-1*) and MCAD or (*Acadm*) expression appeared to be higher in CPT-1a +/- mice as compared to CPT-1a +/+ mice.

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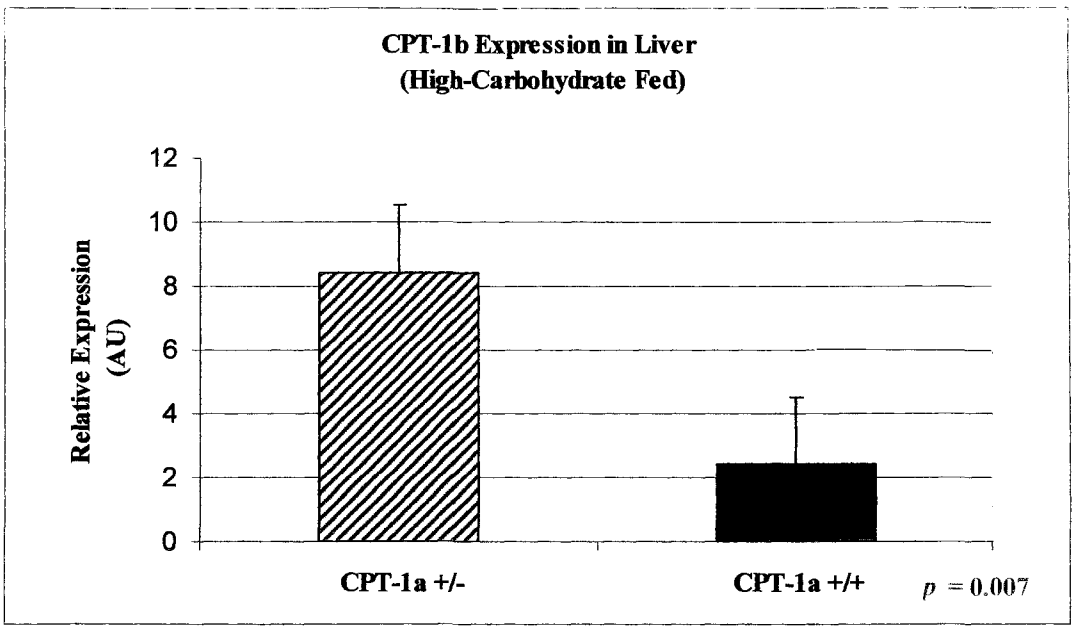

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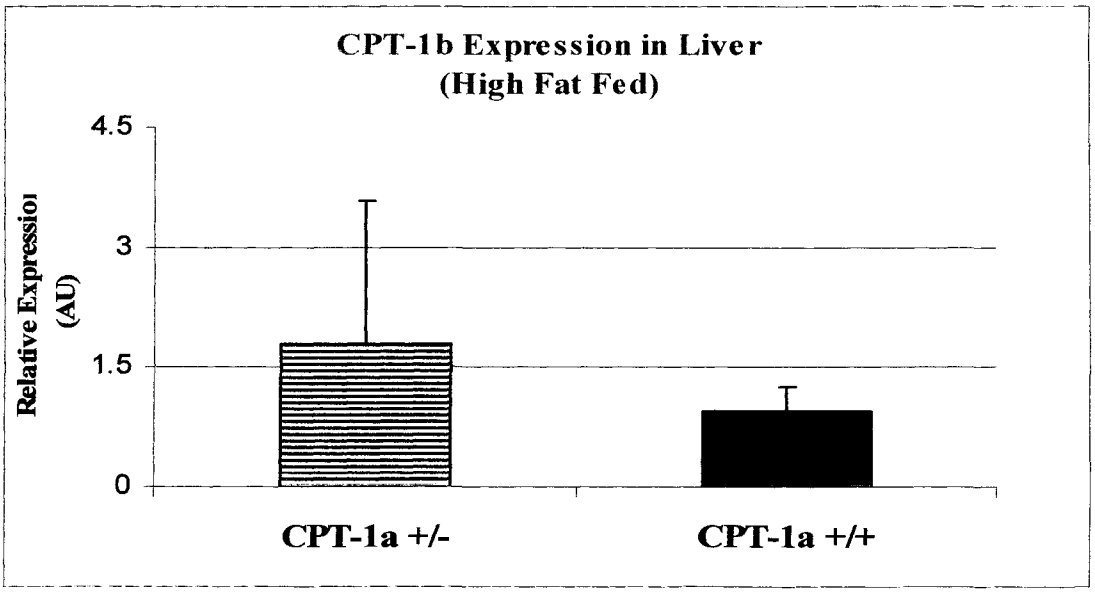

Fig. 12. Quantitative real time PCR analysis of steady state Cpt-1b mRNA levels. (A) Cpt-1b expression in liver as measured by quantitative RT-PCR in HCD fed CPT-1a +/- mice as compared to controls ($p = 0.007$) and (B) HFD fed CPT-1a +/- as compared to controls (NS).

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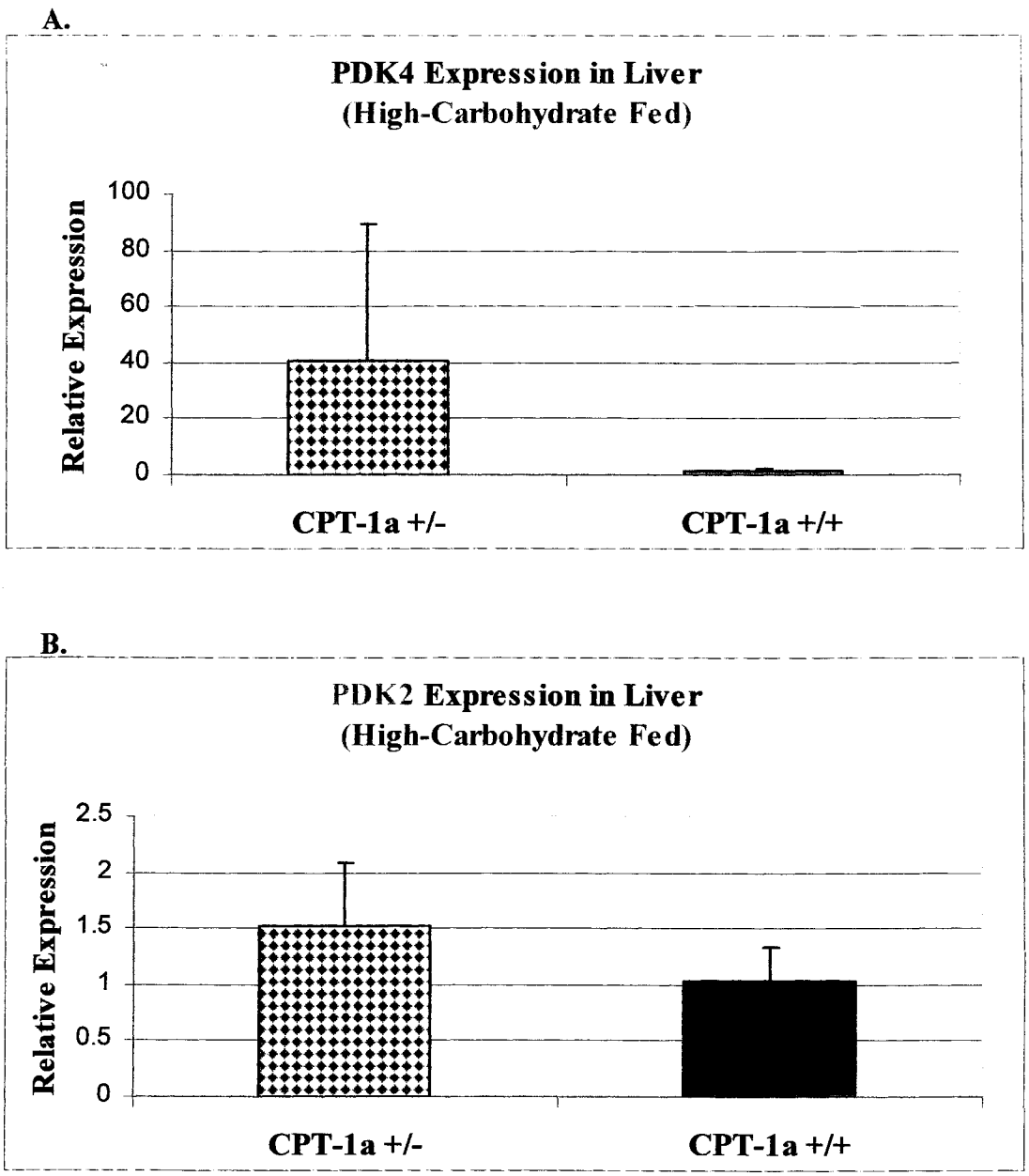

Fig. 13. Quantitative real time PCR analysis of steady state *Pdk* 4 and 2 mRNA levels in liver from mice fed HCD. (A) *Pdk* 4 expression in liver as measured by quantitative RT-PCR in HCD-fed CPT-1a +/- mice as compared to controls ($p < 0.05$) and (B) *Pdk* 2 expression in HCD-fed CPT-1a +/- mice as compared to controls (NS).

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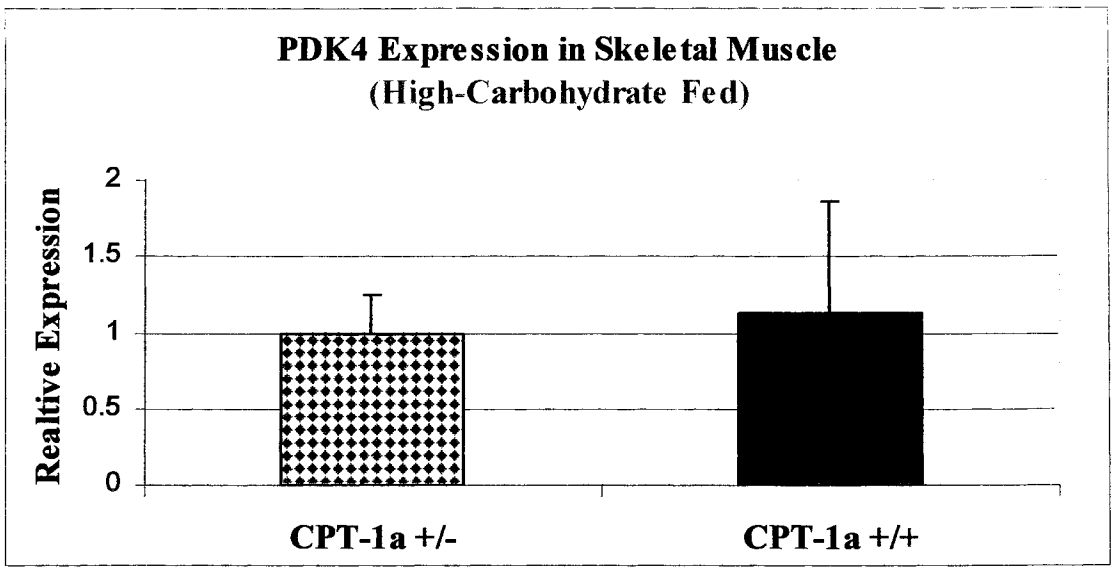

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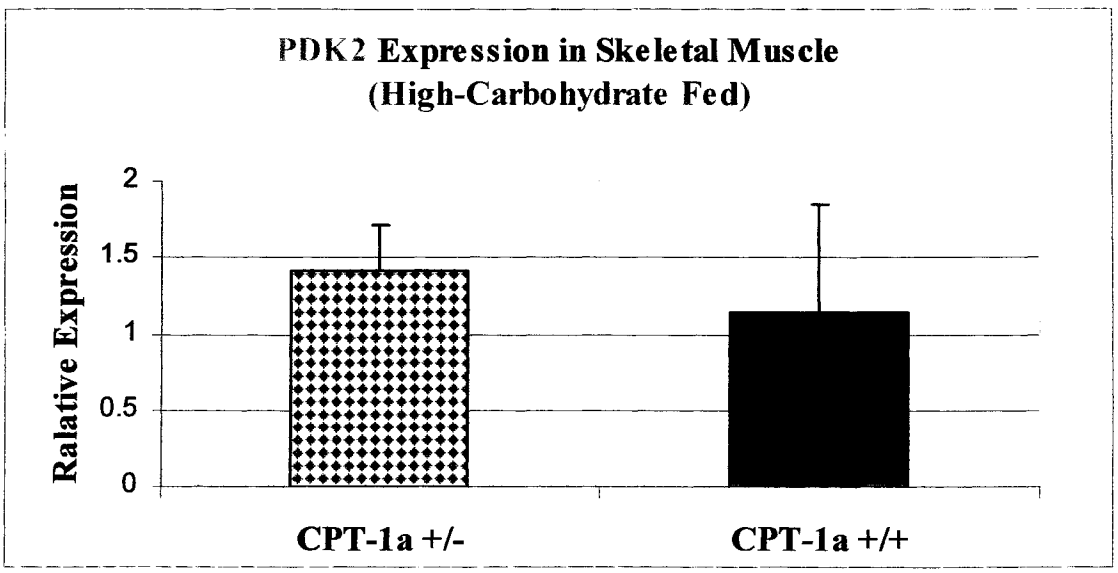

Fig. 14. Quantitative real time PCR analysis of steady state *Pdk* 4 and 2 mRNA levels in skeletal muscle of mice fed HCD. (A) *Pdk* 4 expression in skeletal muscle as measured by quantitative RT-PCR in HCD-fed CPT-1a +/- mice as compared to controls (NS) and (B) *Pdk* 2 expression in HCD fed CPT-1a +/- as compared to controls (NS).

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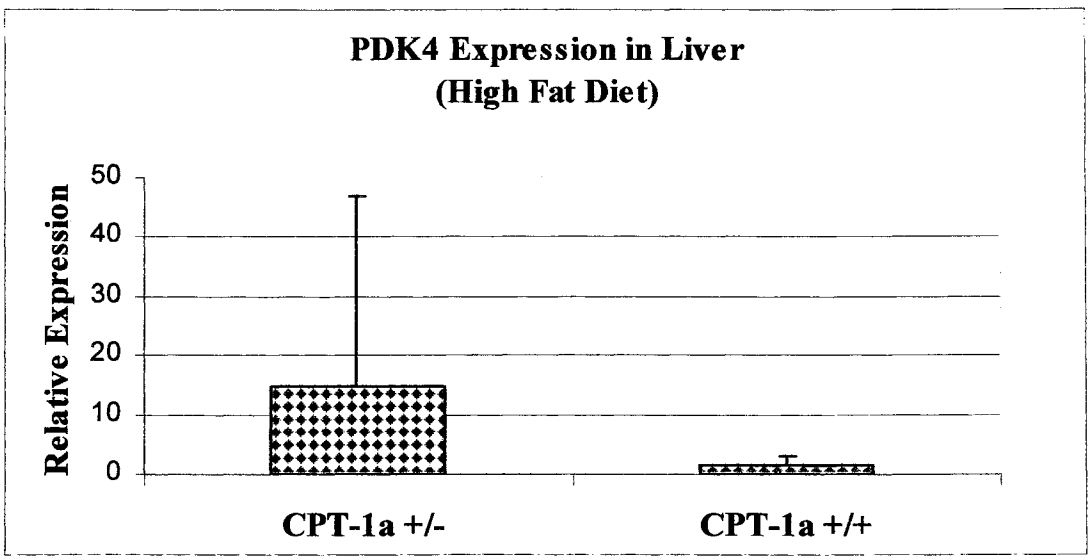

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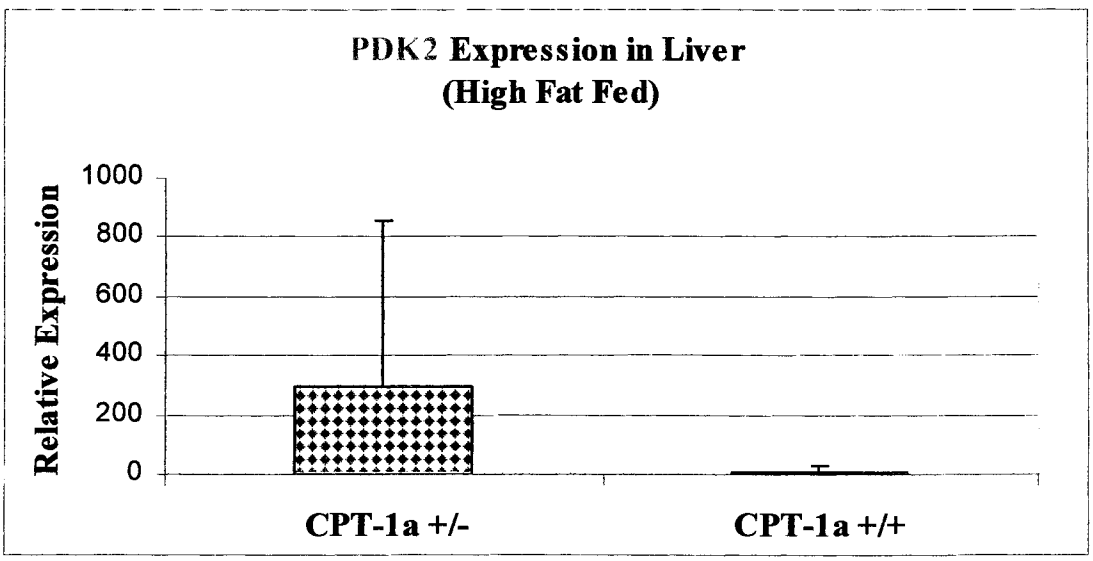

Fig. 15. Quantitative real time PCR analysis of steady state *Pdk* 4 and 2 mRNA levels in liver of mice fed HFD. (A) *Pdk* 4 expression in liver as measured by quantitative RT-PCR in HFD-fed CPT-1a +/- mice as compared to controls (NS)and (B) *Pdk* 2 expression in HFD-fed CPT-1a +/- as compared to controls (NS).

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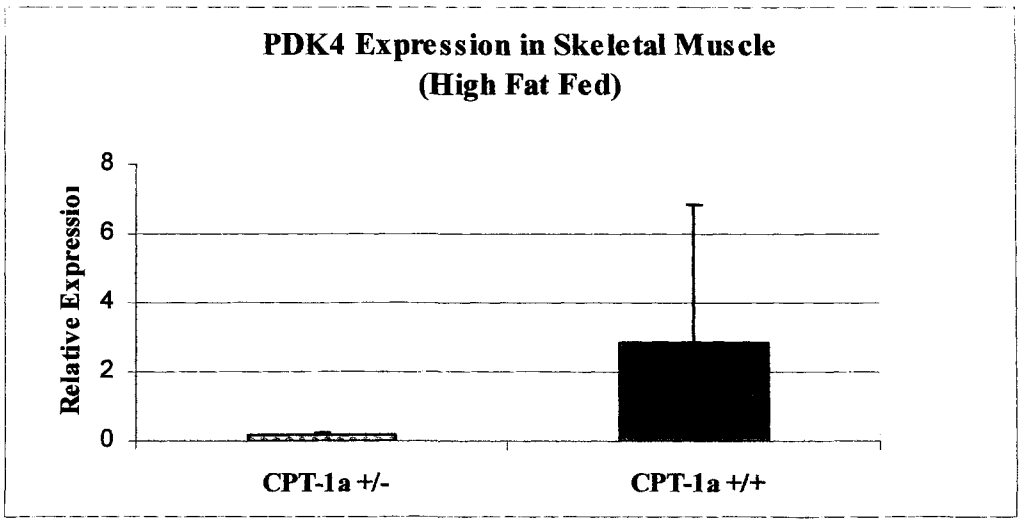

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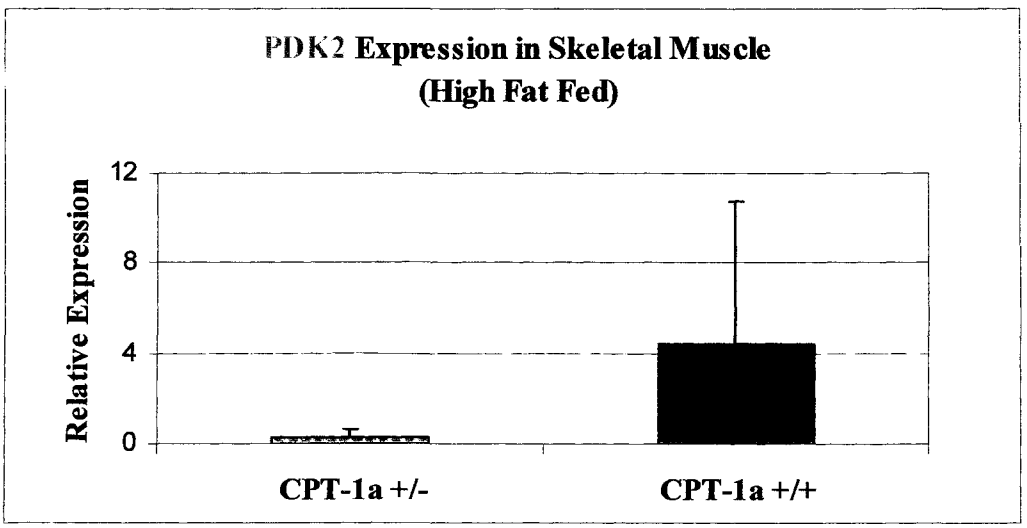

Fig. 16. Quantitative real time PCR analysis of steady state *Pdk* 4 and 2 mRNA levels in skeletal muscle of mice fed HFD. (A) *Pdk* 4 expression in skeletal muscle as measured by quantitative RT-PCR in HFD-fed CPT-1a +/- mice as compared to controls ($p = 0.03$) and (B) *Pdk* 2 expression in HFD-fed CPT-1a +/- as compared to controls (NS).

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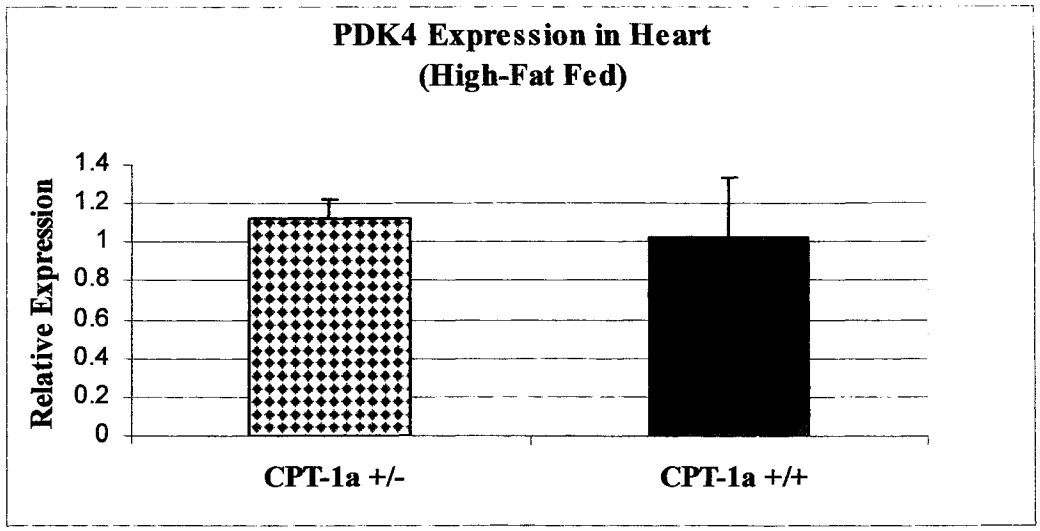

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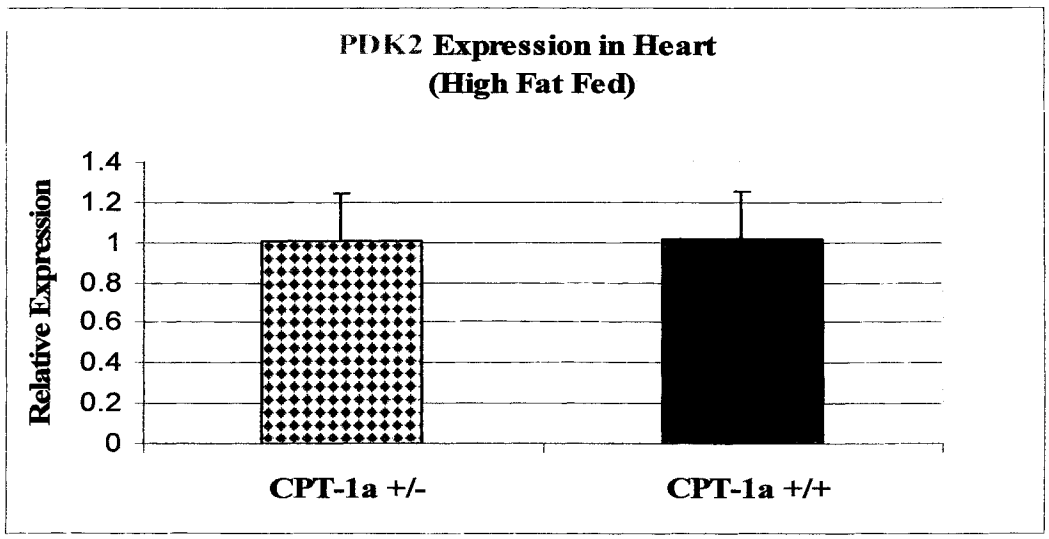

Fig. 17. Quantitative real time PCR analysis of steady state *Pdk* 4 and 2 mRNA levels in heart from mice fed HFD. (A) *Pdk* 4 expression in heart as measured by quantitative RT-PCR in HFD-fed CPT-1a +/- mice as compared to controls (NS) and (B) *Pdk* 2 expression in HFD-fed CPT-1a +/- as compared to controls (NS).

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A. B.

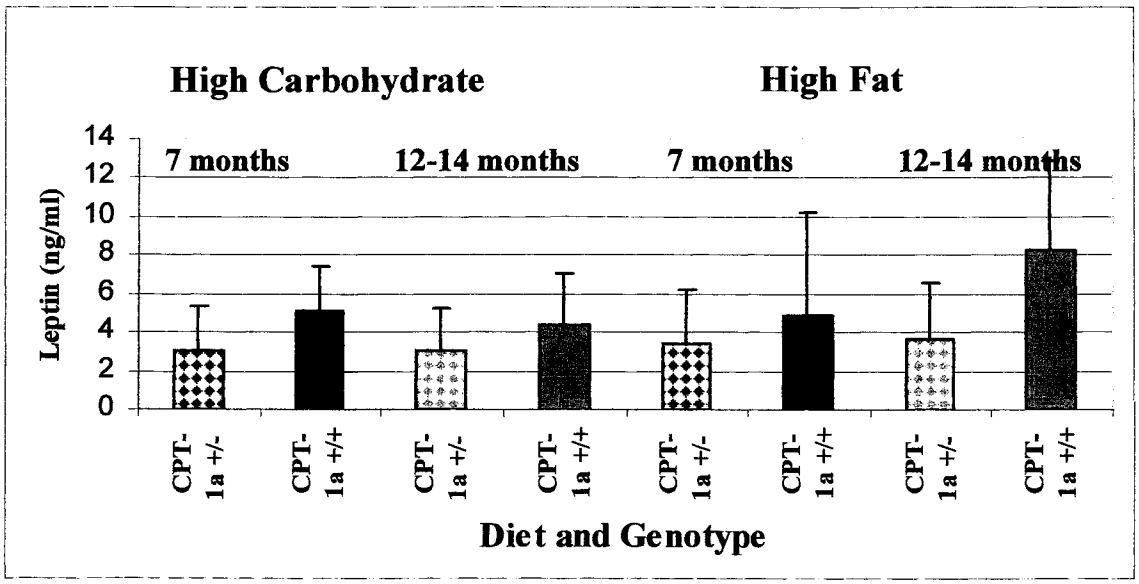

Fig. 18. CPT-1a +/- serum leptin levels show no significant differences as compared to wild-type mice when fed either diet for either the 7-month or 12- to 14-month duration.

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CONCLUSIONS

CPT-1a catalizes the conversion of fatty acyl-CoA into a fatty acyl-carnitine thus allowing entrance into the mitochondrial matrix where β-oxidation occurs. This step functions as the rate limiting step in mitochondrial fatty acid oxidation, a vital metabolic process. In children with inherited CPT-1a deficiency there is a high risk for Reye syndrome-like disease and potential death [8]. In adults, CPT-1a dysfunction may be involved in the development of impaired glucose tolerance, a complex interplay of both β-cell responsiveness and peripheral glucose uptake. The link is as follows: CPT-1 dysfunction may result in an excessive build-up of acyl-CoAs in the cytoplasm of affected cells, which is a common characteristic of insulin resistance. I used a gene knockout mouse model that is heterozygous for CPT-1a deficiency to evaluate altered lipid metabolism in the development of insulin resistance and β-cell dysfunction. I predicted that the CPT-1a +/- mouse model would be informative in regard to further elucidating the mechanism by which these altered metabolic states occur.

In my first aim of this dissertation, I hypothesized that a mouse model with a null allele of CPT-1a would provide important phenotypic characteristics in order to understand the physiological function of CPT-1a and its role in human health and the potentially fatal pediatric disease caused by CPT-1a deficiency. The most striking finding in these studies was that CPT-1a -/- mice were not viable; this homozygous deficiency was lethal early in gestation. Given the fact that there are 30 or more human patients reported in the literature [18], this was an unexpected finding. This finding further stresses the

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pivotal nature of this enzyme's function in proper fatty acid and glucose homeostasis and cellular function.

Using FISH we confirmed that the lethality was specifically due to the Cpt-1a mutation rather than a possible integration at another locus. After genotyping embryos and fetuses from day 10 to day 21 during gestation, we concluded that the lethality was occurring before day 10 and possibly as early as blastocyst formation, which supports earlier work from our group also demonstrating the essential nature of fatty acid metabolism in embryogenesis [42].

Moreover, we did not expect the skewed inheritance patterns demonstrating that the mutant allele was preferentially transmitted on both the B6/129 and 129 congenic backgrounds. Although there have been several cases of non-Mendelian transmission patterns published in the literature [43-45], the explanation in this case remains elusive.

Our first attempt to elucidate this result was to look for sex preference in transmission of the mutant allele. That is, we wanted to investigate whether both the male and the female heterozygous parents were capable of transmitting the mutant allele. Additionally, we wanted to determine if there was a higher frequency of transmission from either parent. Our findings suggest that there is no sex preference for transmission of the mutant allele, leaving us still with this enigma.

I hypothesized that the CPT-1a +/- mice would have lower CPT-1a enzyme activity and mRNA expression in tissues where CPT-1a is the primary isoform expressed. Male CPT-1a enzyme activity was as predicted and measured at approximately 50% of CPT-1a +/+ controls. In contrast, CPT-1a +/- females demonstrated no significant difference in enzyme activity as compared to wild-type controls. This is not the first instance of

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differences in female and male gene knockout mice; however, it was surprising. The CPT-1 assays were not specific for the CPT-1a isoform. Thus, we do not know if the total enzyme measured was representative of CPT-1a activity or CPT-1a, 1b, and 1c activity combined. Further, the assay, although considered the best method available, is an *in vitro* assay based on malonyl-CoA sensitivity, thus being only an estimate of *in vivo* function.

In contrast, both male and female CPT-1a mice demonstrated lower *Cpt-1a* mRNA expression in tissues where CPT-1a is the primary isoform expressed. Tissue specific expression patterns of mouse CPT-1a were similar to those found in previous studies with rats [46,47]. Although we did find that CPT-1a was the primary isoform expressed in male white adipose tissue, CPT-1b was the primary isoform expressed in female adipose tissue.

We expected that the CPT-1a -/- mouse would demonstrate lower fasting serum glucose, higher fasting free fatty acids, and triglycerides when mice were fed SRD for 6-8 weeks. Thus we were surprised to find that these predictions were still true of CPT-1a +/- mice. I should note that the differences were modest, but indeed significant. Because this was a heterozygous mutant rather than a full knockout, we feel that the findings emphasize the importance of CPT-1 in serum metabolite homeostasis.

The goal of the large second study of this dissertation was to characterize the CPT-1a deficient mouse for markers of the development of type 2 diabetes such as impaired glucose tolerance as a result of insulin resistance or β-cell dysfunction. My hypothesis was that this mouse, with its genetically induced limitation on fatty acid oxidation and high calorie feedings, would have diabetogenic characteristics allowing us to

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elucidate, at least partially, the role for excess fatty acids in the development of type 2 diabetes. We predicted that the CPT-1a +/- mouse would demonstrate higher body weight and body fat and have elevated fasting and fed free fatty acids and triglycerides. Paradoxically, we found the opposite for most of our predictions. The most striking but consistent finding was that the CPT-1a +/- mice, fed either high-carbohydrate diet or high-fat diet, regardless of duration, were more insulin sensitive. This was a surprising result especially considering the mechanistic views for the development of insulin resistance at present. Moreover, to further support these findings, preliminary data from hyperinsulinemic-euglycemic clamp measurements also found CPT-1a +/- mutants to be significantly more insulin sensitive [48].

Because of the overwhelmingly strong association between the prevalence of obesity in our population which is concurrent with the ever so daunting increase in number of patients diagnosed with type 2 diabetes, excess fat is viewed as being linked in some way to the upsurge of people with type 2 diabetes. Current dogma holds that the development of type 2 diabetes is a result of progressing insulin resistance which eventually leads to β-cell failure. This is presumed to be a result of compensatory hypersecretion eventually leading to complete β-cell failure and hyperglycemia [49].

Shulman [49] and several others have demonstrated that a build-up of LCCoAs or other fatty acid metabolites, rather than triglyceride, as a result of mitochondrial dysfunction in skeletal muscle causes dysregulated insulin signaling with a primary end result of decreased glucose uptake. The high insulin (relative) often seen in patients in the pre-diabetic state has been explained by compensatory hyperinsulinemia which is thought to lead to β-cell failure as a result of exhaustion.

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In contrast, there are those who believe we need to reassess the current dogma and at least acknowledge that there have been several major studies finding β-cell dysfunction in patients even before the development of impaired glucose tolerance [50]. Thus, there are those who believe that we need to investigate the molecular mechanisms behind β-cell dysfunction as a primary affecter leading to disease rather than a result of insulin resistance. Although these two views are in opposition, they generally agree that excess fat or fatty acid metabolities contribute to these metabolic derangements either as a result of genetic deficiencies or excess intake and limited expenditure.

In stark contrast, there is also the view that it is excess FAO and not excess storage that is playing a pivotal role in the development of type 2 diabetes. This view may stem from earlier findings of Randle *et. al.* [51], who suggested that an increase in FAO leads to a decrease in glucose oxidation which results in impaired glucose tolerance and eventually hyperglycemia [51]. Surprisingly, there is support for this in recent studies by Daniel P. Kelly and colleagues, demonstrating that key regulators of glucose metabolism such as PDK 2 and 4 positively regulated glucose disposal as a result of inhibition of FAO [52]. The limitation with these studies explaining impaired glucose tolerance to be a result of excess FAO is that, even with the administration of FAO inhibitors, there was still excess fat in the tissues and the fact that the model used was unlikely physiological (transgenic over expression of PPAR-α). This was an interesting observation that requires further elucidation. The obvious limitation in this view is that there is not a high prevalence of lean type 2 diabetic patients, making the argument perplexing that excess fatty acid oxidation is a major contributor to the development of disease.

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In support of these caveats, Dobbins et.al. demonstrated that a decrease in FAO led to a decrease in glucose infusion rate under clamp conditions, indicating a decrease in insulin sensitivity [34]. The strength of this study was that they used normal rats and treated them with a CPT-1 inhibitor rather than over-expressing a gene and having to rule out possible unrelated effects of nonphysiological over-expression of this gene. The decrease in insulin sensitivity demonstrated after CPT-1 inhibition supported my initial hypothesis that CPT-1a +/- mice would have impaired glucose tolerance either as a result of insulin resistance or β-cell dysfunction or both.

My work both supports and conflicts with all three of these views. In support of McGarry and our original hypothesis that CPT-1a deficient mice would be less glucose tolerant and less insulin sensitive, we found *Cpt-1b* mRNA expression in liver was increased in CPT-1a +/- mice and may act in compensation for the CPT-1a deficiency and remain consistent with the McGarry work. Although we were unable to reach a significant difference in the high-fat group, the HCD-fed CPT-1a +/- mice demonstrated higher *Cpt-1b* mRNA expression than wild-type controls. Consistent with these diet differences in *Cpt-1b* mRNA expression were the results that demonstrated CPT-1a +/- mice fed the HCD were more glucose tolerant than wild-type controls, whereas HFD-fed CPT-1a +/- mice were less glucose tolerant than wild-type controls. However, this result was attributed to β-cell dysfunction rather than a decrease in insulin sensitivity because CPT-1a +/- mice were consistently found to be more insulin sensitive regardless of diet. In further support of this deduction, CPT-1a +/- mice fed the HCD for both diet durations demonstrated higher insulinogenic indexes than CPT-1a +/+ mice. In contrast, CPT-1a +/- mice fed the HFD showed no significant differences in insulinogenic indexes as com-

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pared to wild-type. The trend, however, indicated lower insulingenic indexes in the CPT-1a +/- mice fed the HFD. I speculate that the conflicting findings in glucose tolerance in the CPT-1a +/- mice on the different diets may be explained by the differences in regulation of the two CPT-1 isoforms and the importance of the milieu in which each is expressed. We suspect that the increase in *Cpt-1b* mRNA expression was not sufficient to compensate for the high-fat diet stress. Alternatively, we suspect that the increase in *Cpt-1b* expression in liver of CPT-1a +/- mice may not have been as sensitive to inhibition in this milieu. More specifically, the HCD which was meant to increase stress by elevating malonyl-CoA inhibition may not have been as potent in these circumstances.

In support of the view that β-cell responsiveness may decline before or in the absence of insulin resistance rather than as a result, we found impaired glucose tolerance in the HFD fed CPT-1a +/- mice in the absence of insulin resistance. We speculated from the trend of lower insulinogenic indexes, as well as the finding that they were more insulin sensitive than wild-type controls, that CPT-1a +/- mice may have experienced some levels of β-cell dysfunction as a result of high-fat feeding combined with the CPT-1a deficiency.

Islet per pancreas area and total islet area was consistently lower in the CPT-1a +/- mice fed either challenge. In support of Shulman's view on the HCD, we speculated that the lower islet area was a result of the CPT-1a +/- mice being more insulin sensitive and not having to undergo diet induced β-cell hyperplasia or hypertrophy. However, on the HFD, where there was evidence of β-cell dysfunction, we interpreted the lower islet area findings as representative of apoptosis or HFD-induced pre-pro-insulin processing deficiencies.

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There are limitations with the interpretations of increased *Cpt-1b* expression being compensatory, and those are consistent with Kelly's findings. These include the result of significantly greater microvesicular fat content in livers of CPT-1a +/- mice on either diet. This can be considered consistent with the Kelly view because there is an abundance of published literature associating liver fat content with decreased insulin sensitivity. However, CPT-1a +/- mice were more insulin sensitive concomitant with higher microvesicular fat content. Furthermore, a recent CPT-1a inhibitor study also demonstrated significant higher microvesicular fat content; however, they found no differences in insulin sensitivity. Moreover, the studies associating liver fat with insulin sensitivity may be grouping both macrovesicular and microvesicular fat content. This may lead to misinterpretation because macrovesicular fat is thought to be diet induced in contrast to microvesicular fat content.

However, both in contrast to and consistent with Kelly *et al.,* our results showed significantly higher *Pdk* 4 expression in liver of HCD fed CPT-1a +/- mice. This finding supports the hypothesis that lower fatty acid oxidation leads to higher glucose metabolism. However, in contrast, *Pdk* 4 was significantly lower in skeletal muscle of HFD fed CPT-1a +/-mice. The overall trend in both diet groups was that CPT-1a +/- mice had higher *Pdk* 2 and 4 expression in liver and overall lower *Pdk* 2 and 4 expression in skeletal muscle, which is the opposite of the results from Kelly *et. al.*, who found higher *Pdk* 4 in skeletal muscle was associated with a decrease in insulin sensitivity.

Overall our results both support and contrast all three controversies in some way. I believe that the characterization of CPT-1a +/- mice for the development of type 2 diabetes, or markers thereof, has provided substantial contributions to our understanding of

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metabolic influence on disease progression. Further, I think our studies may be complimented by future studies of *in vivo* islet function in CPT-1a +/- mice and mRNA expression studies in isolated β-cells of these mice. Moreover, a complete assessment of differences in these mice within the insulin signaling cascade of skeletal muscle and liver would support or contrast current dogma for our understanding of the mechanisms involved in the development of type 2 diabetes.

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APPENDIX

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE ANIMAL RESEARCH APPROVAL FORMS

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THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Office of the Provost

NOTICE OF APPROVAL

DATE: September 8, 2004

TO: Philip A. Wood, D.V.M., Ph.D.
KHGB-620A 0024
FAX: 975-4418

FROM: Suzanne M. Michalek, Ph.D., Vice Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: A Mouse Model for Human Inherited Diseases
Sponsor: NIH
Animal Project Number: 040903732

On September 8, 2004, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	B	500
Mice	A	1500

Animal use is scheduled for review one year from September 2004. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 040903732 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

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GRADUATE SCHOOL
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DOCTOR OF PHILOSOPHY

Name of Candidate Lara R. Nyman

Graduate Program Cellular and Molecular Physiology

Title of Dissertation A Mouse Model With Carnitine Palmitoyltransferase-1a Deficiency Reveals its Crucial Roles in Gestation and Glucose Tolerance

I certify that I have read this document and examined the student regarding its content. In my opinion, this dissertation conforms to acceptable standards of scholarly presentation and is adequate in scope and quality, and the attainments of this student are such that she may be recommended for the degree of Doctor of Philosophy.

Dissertation Committee:

Name		Signature
Philip A. Wood	**, Chair**	
Marcus M. Bamman		
William A. Bradley		
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Director of Graduate Program

Dean, UAB Graduate School

Date JAN 0 6 2006

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