Characterization of mutant carnitine palmitoyltransferase I gene expressed in yeast and mice

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Presented to Division of Environmental & Biomolecular Systems within
The Department of Science & Engineering
and the Oregon Health & Science University
School of Medicine
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy
in
Biochemistry and Molecular Biology

December 2008

Department of Science & Engineering

School of Medicine

Oregon Health & Science University

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ACKNOWLEDGMENTS

First of all I would like to express my sincere gratitude to my advisor Dr. Gebretateos Woldegiorgis for his continuous encouragement, support and guidance throughout my graduate studies. I am very thankful for his patience and always believing in me during the ups and downs of my Ph.D. research.

I am deeply indebted to Dr. Michiko Nakano and Dr. James Whittaker and Dr. Matthew S. Sachs for their insight and guidance throughout my Ph. D studies. A special thanks to Dr. Peter Zuber and Dr. Pierre Moënne Loccoz and Dr. Ninian Blackburn for their unconditional support, tutoring and seminar discussions. I also wish to thank Dr. David Koeller for his efforts and time to serve on my Ph.D. committee.

I would like to thank all the members in my lab and Oregon National Primate Research Center small animal unit and Dr. Patricia D. Hurn Lab. I am also grateful to the people in EBS division for their immense help and friendship during my PhD studies.

Finally, special thank goes to my parents and many former teachers for providing me with love, support, and encouragement during my long period of studies. I would like to express my deepest gratitude for the constant support, understanding and unconditional love that I received from my family and my wife Xiaojian.

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ABSTRACT

Characterization of mutant carnitine palmitoyltransferase I gene expressed in yeast and mice

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Carnitine palmitoyltransferase I (CPTI) catalyzes the conversion of long-chain fatty acyl-CoAs to acylcarnitines in the presence of L-carnitine, the first step in the transport of long chain fatty acids from the cytosol to the mitochondria matrix, a rate-limiting step in β-oxidation. To determine the role of the N-and C-terminal domains of L-CPTI on malonyl-CoA sensitivity, a series of double site-specific N-and C-terminal mutants were constructed. The mutants that decrease malonyl-CoA sensitivity (Δ18 and M593A) were counteracted by those that increase malonyl-CoA sensitivity (Δ19-30 and E590A) resulting in an increase in inhibitor sensitivity higher than either the Δ18 or M593A mutant. Furthermore, combining the mutants that decrease malonyl-CoA sensitivity (Δ18 + M593A) further decreases the sensitivity of the enzyme to the inhibitor, while combining the mutants that increase malonyl-CoA sensitivity (Δ19-30 + E590A) increased the sensitivity of the enzyme to the inhibitor to a level 100-fold higher than that observed with the wild-type enzyme. Mutant Δ19-30+M593A and E590A+M593A exhibited malonyl-CoA sensitivity similar to the wild-type. This data supports the hypothesis that the N-terminal end interacts with a region of the C-terminal end far from

the catalytic site. Since the data shows that the mutations are compensatory or additive, we can hypothesize that malonyl-CoA binding could change the interaction of the N-terminal domain with the C-terminal domain resulting in a conformation with less catalytic activity.

To address the pathophysiological effect of diminished M-CPTI expression on fatty acid metabolism in vivo, we developed a mouse M-CPTI (muscle isoform) gene knockout heterozygous model. We used a gene targeting strategy in ES cells that resulted in the deletion of exon 6, 7 and part of 5 of M-CPTI, a null allele. Homozygous deficient mice (M-CPTI -/-) were not viable. We conclude that targeted deletion of M-CPTI results in embryonic lethality. However heterozygous (M-CPTI +/-) pups survived. M-CPTI +/- mice became more weak, immobile and inactive than wild-type mice after fasting 48h. The heterozygous mice heart had half of CPTI protein expression and CPT activity of the wild-type mice heart. There was no sex difference on the CPTI activity. In heart and skeletal muscle the heterozygous (M-CPTI +/-) mice showed half CPT mRNA level of the wild-type mice, but the heterozygous (M-CPTI +/-) mice showed more increase in CPT mRNA level than the wild-type mice after fasting for 48h.

To gain a further understanding of the role of CPTI in regulating fatty acid metabolism in vivo, we generated a transgenic mouse model that over-expresses human heart malonyl-CoA insensitive mutant M-CPTI in heart. The purpose of this study was to characterize the physiological and metabolic phenotype of localized M-CPTI over-expression in heart, especially the effect of M-CPTI over-expression on obesity and diabetes in vivo. In this study, mice with cardiac-specific expression of human heart wild-type M-CPTI was not found, while mice over-expressing human heart malonyl-CoA insensitive M-CPTI in heart were obtained. An increase in M-CPTI content can upregulate fatty acid metabolism in vivo under basal conditions and stress burden. M-CPTI plays an important role in body weight control, especially for the high fat diet fed mice. Our data set the basis for M-CPTI activation to treat obesity. The over-expression of CPTI leads to increased CPTI activity in isolated mitochondria and may play an important role in control of lipid storage in vivo. We also found that transgenic mice that over-express the mutant cardiac-specific M-CPTI were more susceptible to diabetes.

CHAPTER 1

INTRODUCTION

1.1 CARNITINE PALMITOYLTRANSFERASE SYSTEM

Fatty acids, as a major energy source for muscle and liver in mammals, are degraded mainly by β-oxidation, which in animal cells takes place in both mitochondria and peroxisome. Mitochondria are the primary site of fatty acid β-oxidation, while the peroxisome is involved in the degradation of complex fatty acids, such as very long chain fatty acyl-CoA, long chain dicarboxylyl-CoA.

Mitochondrial fatty acid β-oxidation play an essential role in energy homeostasis in situations that require simultaneous glucose sparing and major energy supply, such as prolonged fasting and exercise (Schulz, 1985). While short-chain fatty acids (less than 8 carbons) and medium-chain fatty acids (8 to 14 carbons) can enter the mitochondria by simple diffusion, long-chain fatty acids (16 carbons and more; LCFA), the major fraction of fatty acids delivered to target tissues, can not enter the mitochondria by simple diffusion. After being activated by a long-chain fatty acyl-CoA synthetase on the outer mitochondrial membrane, LCFA are imported into the mitochondrial matrix by the carnitine palmitoyltransferase (CPT) system (EC 2.3.1.21).

Once into the mitochondrial matrix, LCFA will go into the β-oxidation process, which includes multiple cycles leading to the formation of acetyl-CoA, with the first of each cycle consisting of dehydrogenation, followed by hydration, a second dehydrogenation, and finally thiolysis. The acetyl-CoA will go into the citic acid cycle to be oxidized to CO_2, while one GTP (guanine triphosphate) is generated in each citric acid cycle, there are three NADH (nicotinamide adenine dinucleotide) and one $FADH_2$ (flavin adenine dinucleotide) are produced. These reduced electron carriers, NADH and $FADH_2$, are subsequently oxidized by the electron transfort chain to generate ATP to meet the body need.

In 1963, Irving B. Frits and Kenneth T. N. Yue postulated that the CPT system consisted of two pools of CPT. CPTo, on the outside of the inner mitochondrial membrane, transfers the acyl group from CoA to carnitine, and CPTi, on the inside of the inner membrane, transfers the acyl group back to CoA from carnitine (Irving *et al.*, 1963). Gradually it is recognized that the carnitine palmitoyltransferase enzyme system, in conjunction with acyl-CoA synthetase and carnitine/acylcarnitine translocase, provides the mechanism whereby long-chain fatty acids are transferred from the cytosol to the mitochondrial matrix to undergo β-oxidation for energy production. The carnitine palmitoyltransferase enzyme I (CPTI) isozymes are located in the mitochondrial outer membrane and are detergent-labile, whereas carnitine palmitoyltransferase enzyme II (CPTII) is located in the inner mitochondrial membrane and is detergent-stable as shown in Fig. 1.1. (Murthy *et al.*, 1987; Bieber *et al.*, 1988). Compare with CPTII, CPTI contains an N-terminal extension of about 140 residues that are important for attachment to the mitochondrial membrane and other functions. In addition, the catalytic activity of CPTI is inhibited by malonyl-CoA synthesized from excess acetyl-CoA; whereas CPTII is not inhibited by malonyl-CoA. There are 3 isoforms of CPTI: L-CPTI or CPTIa (liver), M-CPTI or CPTIb (muscle), CPTIc (brain) (Price *et al.*, 2002; Wolfgang *et al.*, 2006; Wolfgang *et al.*, 2008), their characteristcs are summarized in Table 1.1. Briefly, CPTIc can bind malonyl-CoA but does not catalyze acyl transfer from various fatty acyl-CoAs to carnitine. The function of CPTIc is not clear, recent data suggested it regulates energy homeostasis (Wolfgang *et al.*, 2008). It is known that L-CPTI and M-CPTI, located on the outer membrane of the mitochondrion, mediate the transfer of the acyl-chain of the cytosolic long-chain acyl-CoA to carnitine. The acylcarnitine thus formed crosses to the inner mitochondrial membrane through the carnitine-acylcarnitine translocase, and is reconverted to acyl-CoA and carnitine by carnitine palmitoyltransferase II (CPTII) located on the matrix side of the inner mitochondrial membrane. Through these steps, the acyl moiety of long-chain acyl-CoA can move into the matrix space and undergo β-oxidation to produce acetyl-CoA. Therefore, the conversion of acyl-CoA to acylcarnitine by CPTI plays an important role in regulation of mitochondrial β-oxidation. For L-CPTI and M-CPTI, although their primary structures are similar, their affinities for carnitine and susceptibilities to malonyl-CoA inhibition are quite different. In addition, L-CPTI

and M-CPTI are encoded by different genes, and their tissue distributions are quite different. L-CPTI, which is dominant in liver, kidney, and intestine, is expressed in all tissues except skeletal muscle and brown adipose tissue; whereas M-CPTI is more abundant in heart and skeletal muscle and brown adipose tissue (BAT). Both L-CPTI and M-CPTI have different expression levels depending on tissue, developmental stage, hormonal regulation and species (Naoshi *et al.*, 2003).

In 1997, Woldegiorgis lab cloned the human heart M-CPTI cDNA and set the expression system devoid of endogenous CPT activity and the functional characterization of a human heart M-CPTI in the absence of L-CPTI and CPTII. Woldegiorgis lab also cloned the rat liver CPTI cDNA and CPTII cDNA and generated anti-CPTI and anti-CPTII rabbit polyclonal antibodies (Zhu *et al.*, 1997; de Vries *et al.*, 1997).

There are great physiological and patho-physiological significance to study the CPT system (CPTs). By regulating long-chain fatty acids oxidation, CPTI is involved in other vital functions such as control of food intake (Obici *et al.*, 2003), insulin secretion (Rubi *et al.*, 2002; Lehtihet *et al.*, 2003), and gluconeogenesis (Foster 2004). CPTI has also been involved in cardiac reperfusion injury (Lopaschuk *et al.*, 1988), sepsis (Eaton *et al.*, 2003) and apoptosis (Mutomba *et al.*, 2000). Since dysregulation of the CPTs are linked to many serious human diseases, CPTs become an attractive target for the development of new therapeutic agents for the treatment of diabetes, obesity, heart failure and other human diseases (Anderson *et al.*, 1998).

Modulation of the catalytic activity of the CPT system is currently used to develop novel drugs against diabetes mellitus. Specific inhibitors of L-CPTI become a target for the treatment of type 2 diabetes mellitus, for example, the class of non-hydrolyzable acylaminocarnitine analogs of the CPTI product acylcarnitine, ST1326, is the best characterized and has bee used in clinical trial (Rufer *et al.*, 2006).

By now tere are 24 L-CPTI mutations that have been identified. M-CPTI and CPTIc deficiencies have not been hitherto reported. L-CPTI deficiency is characterized by recurrent attacks of fasting hypoketotic hypoglycemia. Around 40 CPTII mutations (missense or truncating mutations) have been detected to date. CPTII deficiency has several clinical presentations. There are 2 forms of CPTII deficiency based on the time of onset, the early-onset (infantile) form and the adult form. The early-onset form is

characterized by cardiomyopathy and hypoketotic hypoglycemia. The adult form is characterized by episodes of rhabdomyolysis triggered by prolonged exercise. Treatment includes avoidance of fasting and/or exercise, a low fat diet enriched with medium chain triglycerides and carnitine. Prenatal diagnosis can be provided for pregnancies at a 25% risk of infantile CPTII deficiency [see review (Bonnefont *et al.*, 2004)].

1.2 CPT II STRUCTURE AND CATALYTIC MECHANISM

The family of carnitine acyltransferases includes carnitine acetyltransferase (CAT, with a substrate preference for short-chain acyl groups), carnitine octanoyltransferase (COT, with a substrate preference for medium-chain acyl groups) and carnitine palmitoyltransferase. The crystal structures of rat CAT and rat COT was reported earlier than that of CPTII (Wu *et al.*, 2003; Govindasamy *et al.*, 2004; Jogl *et al.*, 2003; Hsiao *et al.*, 2004; Jogl *et al.*, 2005). The structures of CAT and COT contain two domains, N and C domains. For CAT and COT, the active site is located at the interface between the two domains, and the carnitine and CoA substrates are bound at opposite sides of the catalytic His residue. CPTII shares about 35% amino acid sequence identity with these two enzymes, and the overall structure of rat CPTII is similar to that of rat CAT and rat COT. Human L-CPTI and rat CPTII share about 27% identity in the amino acid sequence of their catalytic cores, and the catalytically important residues are fully conserved as shown in Fig. 1.2 (Rufer *et al.*, 2006). While rat liver CPTI and human liver CPTI share 86% identity in the amino acid sequence, their catalytic core maybe fully conserved, thus the CPTII structure can shed light into the understanding of rat liver CPTI catalytic mechanism and regulation.

1.2.1 Overall structure

Rufer lab reported the 1.6 Å resolution structure of the full-length mitochondrial membrane protein rat CPTII and elucidated the binding mode of ST1326 by solving the crystal structure of full-length rat CPTII in complex with ST1326 at 2.5 Å resolution. ST1326 is a noncleavable analog of palmitoyl-carnitine, the physiological substrate of CPTII. Based on the CPTII structure, N-terminal (residues 111–440) and C-terminal

(residues 441–658) domains can be determined, which consist of a six-stranded, central antiparallel β sheet and surrounding α helices as shown in Fig. 1.3. Two of these β strands (β1, β16) mediate the major domain contact and line the hydrophobic acyl-tunnel. The ST1326 complex structure has similar conformation as the uninhibited enzyme.

The crystal structure of rat CPTII in complex with ST1326 really shed light in the molecular details of protein-substrate interactions of CPTII. The key residues of the active site as shown in Fig. 1.4 are fully conserved between rat CPTII and L-CPTI. The catalytic core of CPTII is located in a Y-shaped tunnel at the domain interface (Fig. 1.3). The Y-shaped tunnel consists of binding sites for the acyl, carnitine, and CoA moieties (Nic a' Bhaird *et al.*, 1993). The acyl and carnitine tunnels of the active site of CPTII are occupied by ST1326, whereas the CoA tunnel can be assigned by homology modeling (Fig. 1.3). The hydrophilic aminocarnitine head group of ST1326 is tightly bound in a hydrogen bond network. The key residues of catalytic core form an extensive hydrogen network with the aminocarnitine head group of ST1326 (Fig. 1.4). For example, the catalytic base His-372 forms a hydrogen bond with the amino-nitrogen (N11) of ST1326, which substitutes the ester oxygen of the native ligand palmitoylcarnitine. The structural information above is fully consistent with the important catalytic role of His-372 residue as shown in Fig. 1.4 (Rufer *et al.*, 2006). It suggests that the correspondent reserved residue His-473 of rat L-CPTI maybe the candidate catalytic base in CPTI catalysis. In a word, the amino terminal residues works together with the carboxy terminal residues to facilitate the catalysis occur. While the overall topology of rat CPTII is retained in the inhibited and uninhibited states, the superimposition of these two states suggests a rearrangement of active site residues occurring unpon ligand binding (Rufer *et al.*, 2007).

1.2.2 Membrane association

It was reported that rat CPTII has a unique amino acid sequence according to a BLAST database search as compared to CPTI isoforms, CAT, and COT (Fig. 1.2). Based on the crystal structure of rat CPTII, a model for membrane attachment of CPTII mediated by this insert was proposed. The crystal structure of rat CPTII shows that there is a pair of antiparallel helices (α9 and α10) that protrude from the catalytic core (Fig. 1.3). Helix α9 is clearly amphipathic, whereas helix α10 is predominantly hydrophobic.

As rat CAT and COT lack the insert and are soluble proteins, the insert most likely confers the membrane association of CPTII (Rufer *et al.*, 2006). It is reported that rat CPTII interacts with the mitochondrial membrane as a monomer (Rufer *et al.*, 2007).

1.3 M-CPTI GENE STRUCTURE AND CHARACTERIZATION

The rat M-CPTI cDNA was first identified in 1995 (Yamazaki *et al.*, 1995). The human M-CPTI was subsequently isolated using the rat cDNA as a probe for screening human heart cDNA libraries (Yamazaki *et al.*, 1996). Both the nucleotide sequences and predicted primary structures of the rat and human proteins are very homologous (85% and 86% identity, respectively). In both human and rat, the M-CPTI transcript size (3 kb) is smaller than that of L-CPTI (4.7 kb).

Mouse M-CPTI was reported to be located on chromosome 15 (Cox *et al.*, 1998). The mouse M-CPTI gene seemed to have multiple initiation sites. Furthermore, the response element for peroxisome proliferators was found to exist in the upstream of the mouse M-CPTI gene as well as in the upstream of the rat and human M-CPTI genes. The mouse M-CPTI consisted of 772 amino acids, and had a predicted size of 88.2 kDa, similar to that of rat (88.2 kDa) and human M-CPTI (87.8 kDa). The amino acid sequence of mouse M-CPTI was highly homologous with those of the rat and human proteins (about 95% and 87%, respectively) (Yamazaki *et al.*, 2003).

1.4 REGULATION OF CPTI BY FATTY ACIDS

CPTI can be regulated at different levels including allosteric, transcriptional, or pre-mRNA splicing levels (Yu *et al.*, 1998). Mitochondrial fatty acid β-oxidation enzyme mRNAs increased when fatty acid was supplemented in medium of cell culture indicate that fatty acid play a regulatory role in faty acid oxidation gene transcription (Gulick *et al.*, 1994). There is enzyme substrate-responsive regulated expression of alternative M-CPTI transcripts and alternative pre-mRNA splicing variants in tissues (Yu *et al.*, 1998). It has been assumed that fatty acids regulate CPTI gene transcription via a PPAR-mediated process (Kliewer *et al.*, 1997; Duplus *et al.*, 2000), PPAR is a member of the

nuclear receptor superfamily that was originally cloned from a liver cDNA library derived from rodents fed peroxisome proliferator agents (Issemann *et al.*, 1990).

Long-chain fatty acids have been shown to activate M-CPTI transcription in rat primary cardiac myocytes, and the response element has been mapped for both the human and rat promoters. This fat-activated/fatty acid response element (FARE) is activated by PPARα/retinoid X receptor (RXR) heterodimers (Yu *et al.*, 1998). In PPARα null mice, the CPTI inhibitor etomoxir fails to induce cardiac expression of the M-CPTI gene, whereas in wild type mice it does (Brandt *et al.*, 1998). In addition, PPARα ovexpression in mice heart increases fatty acid oxidation including increased CPT activity (Finck *et al.*, 2002). All above lines support that intracellular fatty acid and its metabolites comprise a signaling pathway mediated via PPARα.

It was reported that the PPARα-mediated regulation was enhanced by the PPARγ coactivator-1 (PGC-1) (Vega *et al.*, 2000). PGC-1α stimulates M-CPTI gene expression through myocyte enhancer factor-2 (MEF2) (Czubryt *et al.*, 2003). Upstream stimulatory factors (USF) proteins have a novel role in repressing the expression of the M-CPTI gene and modulating the induction by PGC-1α (Moore *et al.*, 2003). Several lines of evidence support that the PGC-1 family is a group of inducible transcriptional coactivators orchestrating control of cellular energy metabolism [see review (Finck BN and Kelly DP, 2006; Duncan JG and Finck BN, 2008)]. Since the identification of PPAR as a PGC-1α transcription factor target, a variety of additional PGC-1α target nuclear receptors (NRs) have been identified. Through these transcription factor partners, PGC-1α exerts strong effects on many aspects of mitochondrial energy metabolism. A model that depict the mechanisms of PPARα activation and PGC-1α coactivator activity was shown in Fig. 1.5 [see review (Duncan JG and Finck BN, 2008)]. In one hand, overexpression of PGC-1α in transgenic mouse heart resulted in abnormal mitochondria metabolism and mitochondrial proliferation in cardiac myocytes (Lehman *et al.*, 2000). In another hand, PGC-1α–deficient mouse lines are viable and exhibit multisystem energy metabolic abnormalities [see review (Finck *et al.*, 2006)]. Recent report suggests that fatty acid induced decreases in PGC-1 expression in myocyte cell line are dependent on fatty acid structure or its specific oxidation products (Crunkhorn *et al.*, 2007).

1.5 EXPRESSION OF CPTI IN VIVO

1.5.1 Human heart CPTI over-expression in skeletal muscle

To investigate CPTI role in regulating fatty acid metabolism in skeletal muscle, Bruce lab used in vivo electrotransfer (IVE) technique to locally over-express huan M-CPTI in muscle of rat. A vector expressing the human M-CPTI was electrotransferred into the right lateral muscles of the distal hindlimb of rats; whereas a control vector expressing GFP (green fluorescent protein) was electrotransferred into the left muscles. The over-expression of CPTI leads to increased CPTI function in isolated mitochondria, such as increased mitochondrial oxygen consumption rate. In an intact isolated muscle preparation, M-CPTI over-expression shunted fatty acid away from esterification and toward oxidation, resulting in decreased triacylglycerol content in muscle. These studies demonstrate that CPTI plays an important role in regulating muscle fatty acid metabolism (Bruce *et al.*, 2007).

1.5.2 CPTI over-expression in cell lines

To test whether L-CPTI over-expression can inreases fatty acid oxidation, Human embryonic 293T kidney cells were transfected and the expression of the L-CPTI transgene in the tet-on vector was achieved with doxycycline. L-CPTI over-expression in 293T cells increases mitochondrial long-chain fatty acid oxidation rate about 6-fold. Addition of palmitic acid decreased viability of L-CPTI over-expressing cells in a concentration-dependent manner (Jambor de Sousa *et al.*, 2005). Another lab reported that L-CPTI over-expression in primary rat hepatocytes by adenovirus vector increased the rate of fatty acid β-oxidation and ketogenesis. Furthermore, increased L-CPTI was associated with decreased accumulation of triglyceride in hepatocytes (Stefanovic Racic *et al.*, 2008). These findings that transgenic manipulation of L-CPTI can increase fatty acid oxidation in living cells will encourage transgenic animal to be used to test the role of CPTI in the regulation of metabolism.

Lipid metabolism in the β-cell is critical for the regulation of insulin secretion (Lehtihet *et al.*, 2003). The over-expression of L-CPTI in pancreatic β-cells increases the fatty acid oxidation rate at high and low glucose concentrations. The effect of CPTI was

reverted by the use of CPTI inhibitor etomoxir and by the exogenous addition of fatty acids. Furthermore up-regulation of CPTI contributes to the early loss of response to high glucose in β-cells (Rubi *et al.*, 2002). These results showed that CPTI plays a role in regulation of insulin secretion in the β-cell.

1.6 CHARACTERIZATION OF CPTI GENE KNOCKOUT

There was a report that L-CPTI gene knockout heterozygous mouse model was constructed by a replacement gene targeting strategy in ES cells. Homozygous L-CPTI deficiency is lethal at very early embryonic stages. Heterozygous matings produced an unexpectedly high percentage of L-CPTI +/− offspring on both an inbred 129 and mixed B6/129 genetic background, and there was no sex preference with regard to germ-line transmission of the mutant allele. The CPTI activity in liver from L-CPTI +/− males was about half of that wild-type control, but there was no significant difference in females as compared to wild type control. There were modest changes in glucose and fatty acids during fasting of young L-CPTI +/− mice, and all show normal cold tolerance (Nyman *et al.*, 2005).

It was reported that M-CPTI knockout resulted in no homozygous fetuses found at any stages examined (Ji *et al.*, 2008). The allelic transmission mode from the M-CPTI +/- male and female mice in breeding pairs with wild-type mates was normal. In contrast, the number of M-CPTI +/- pups produced from M-CPTI +/- breeding pairs was under-represented. Surprisingly, heterozygous M-CPTI mice had a normal phenotype, including normal body weight, normal fasting blood glucose levels. Following an extended time of cold challenge, there was a significant increase in the percentage of M-CPTI +/− mice developing lethal hypothermia compared to M-CPTI +/+ mice (Ji *et al.*, 2008). These findings that M-CPTI +/− mice were more susceptible to cold challenge than wild type mice, while L-CPTI +/− mice were not, indicated that M-CPTI may play a more important role in body temperature control than L-CPTI does.

TABLE 1.1 Characteristics of 3 CPTI isoforms

Isoforms	L-CPTI (CPTIa)	M-CPTI (CPTIb)	CPTIc
Tissue distribution	liver, kidney, white adipose tissue (WAT), testis, ovary, pancreatic islet, lung, spleen, brain, intestine	brown adipose tissue (BAT), heart, skeletal muscle, testis and WAT	brain (hypothalamus)
IC50 for malonyl-CoA	2 μM	70 nM	?
Km for carnitine	200 - 400 μM	1.5 - 2 mM	?
Function	FA metabolism, food intake, insulin secretion, gluconeogenesis		?

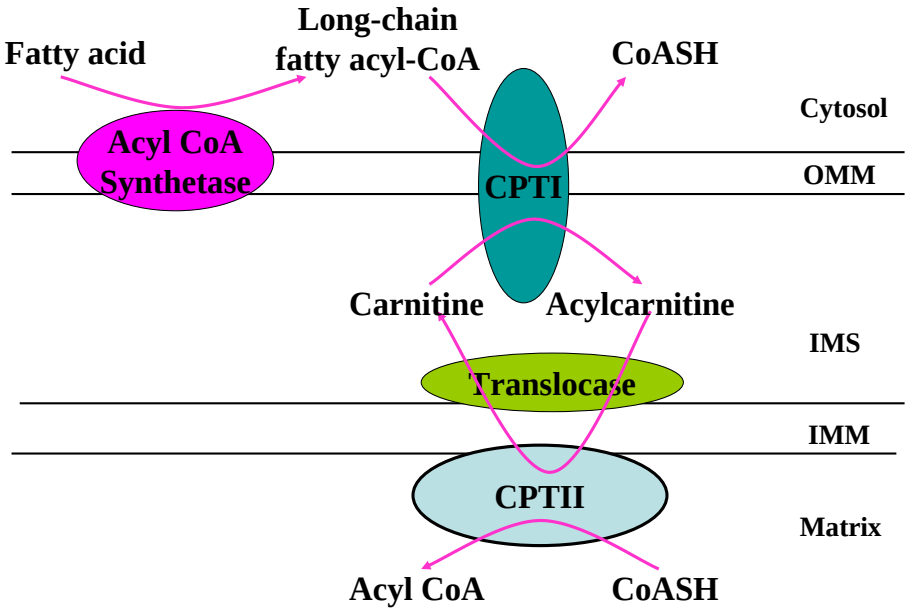

FIG. 1.1. Schematic view of CPT-meciated fatty acid import. OMM, outer mitochondrial membrane; IMS, intermembrane spce; IMM, inner mitochondrial membrane (Woldegiorgis *et al.*, 2000).

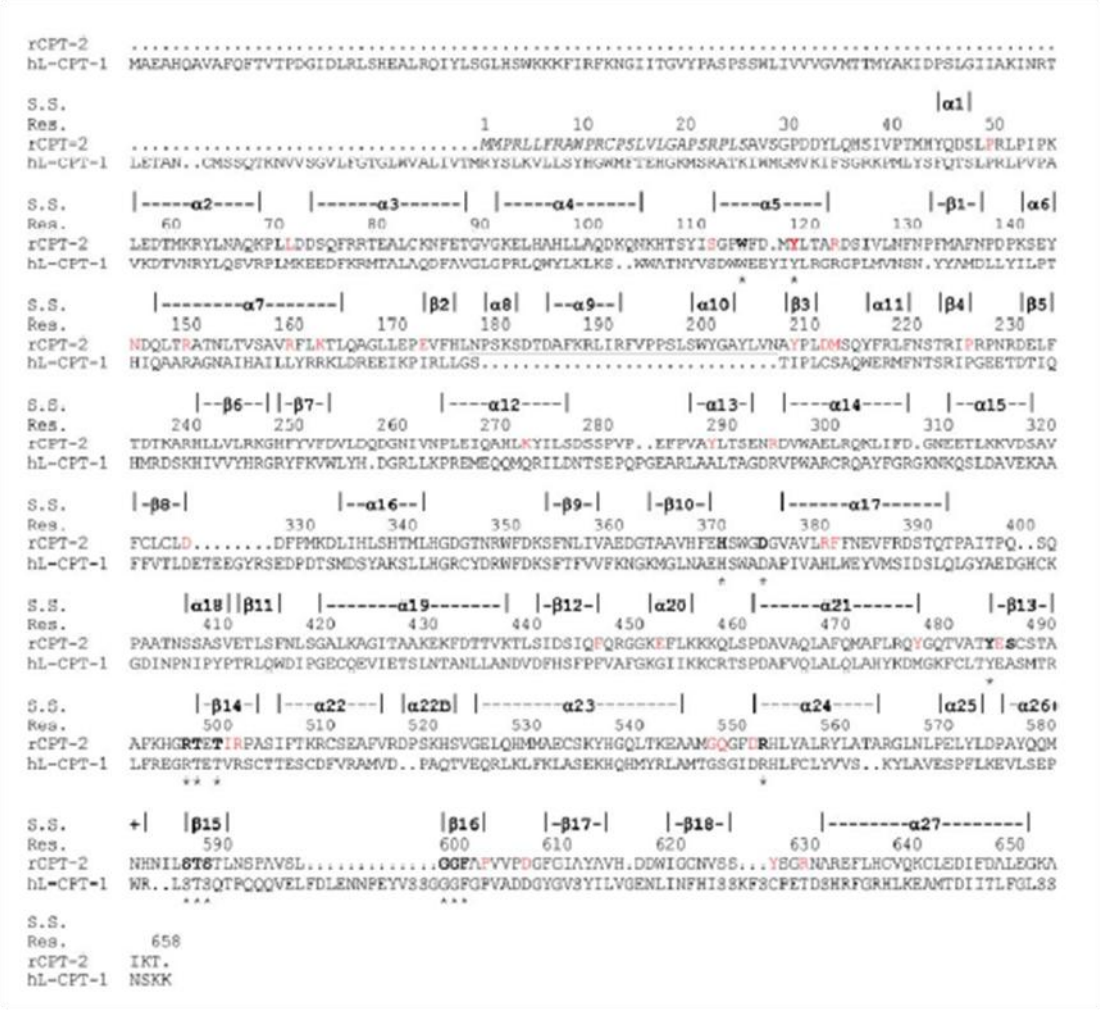

FIG. 1.2. Amino acid sequence alignment of rat CPTII and human L-CPTI. Secondary structure elements (S.S.) are indicated. The residue numbering corresponds to the rat CPTII precursor, and its mitochondrial import sequence is italicized. The CPTII specific insert (amino acids 179–208) is underlined. Key residues of the acylcarnitine binding site of rat CPTII are in bold letters and are labeled with an asterisk when fully conserved in human liver CPTI. Residues are printed in red when mutations in CPT-2 deficiency have been reported (Rufer *et al.*, 2006).

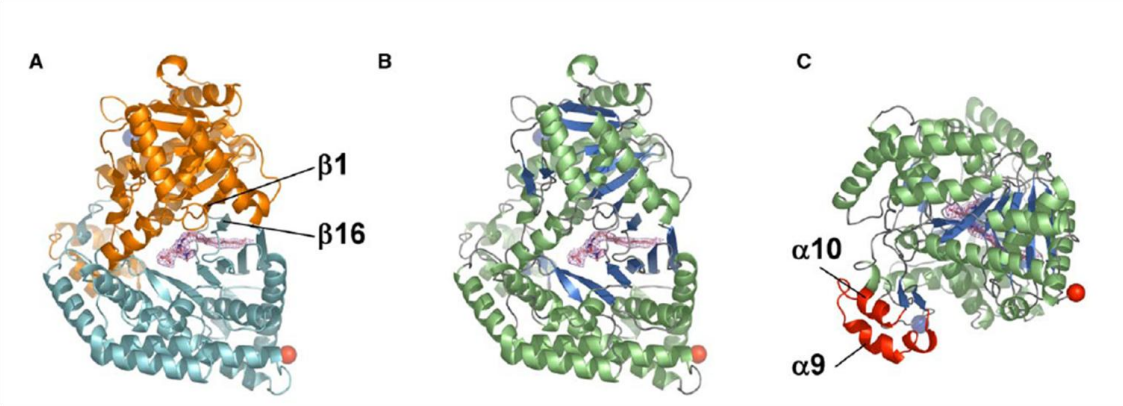

FIG. 1.3. Structure of rat CPTII with ST1326 bound to its active site. The amino and carboxy termini are labeled with blue and red spheres, respectively. A, ST1326 binds at the interface of the amino-terminal (cyan) and carboxy-terminal (orange) domains of rat CPTII. Strands β1 and β16 mediatethe major domain contact by forming an antiparallesheet. B, The central β strands (blue) are surrounded by α helices (green). C, Same as B, but rotated 90º to the back. The CPTII specific insert (red) protrudes from the amino-terminal domain (Rufer *et al.*, 2006).

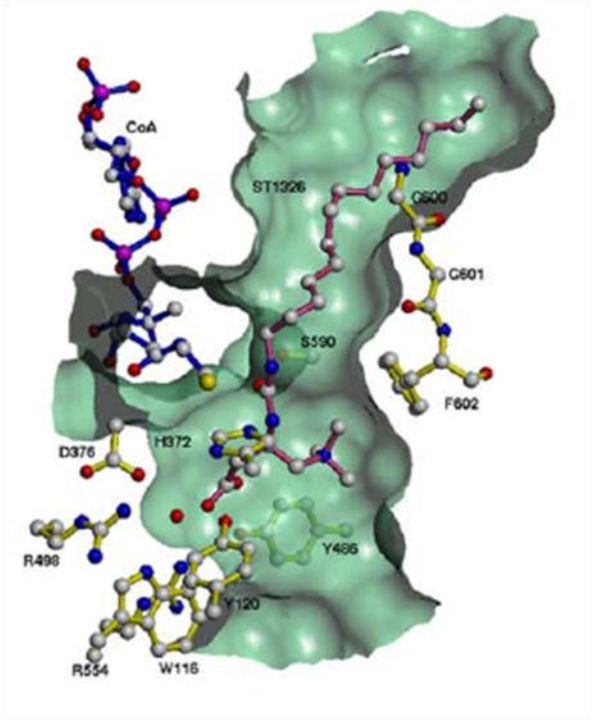

FIG. 1.4. Figure (generated with MOLOC; Gerber, 1992) of the tripartite active site tunnel with bound ST1326 viewed perpendicular to the domain interface. Key active site residues are depicted in yellow. The cocrystallized ST1326 is shown in pink. The CoA molecule (blue) was modeled based on the CoA coordinates from the rat CAT-CoA complex structure (PDB code: 1t7q). The protein environment of the modeled CoA molecule was omitted for figure clarity (Rufer *et al.*, 2006).

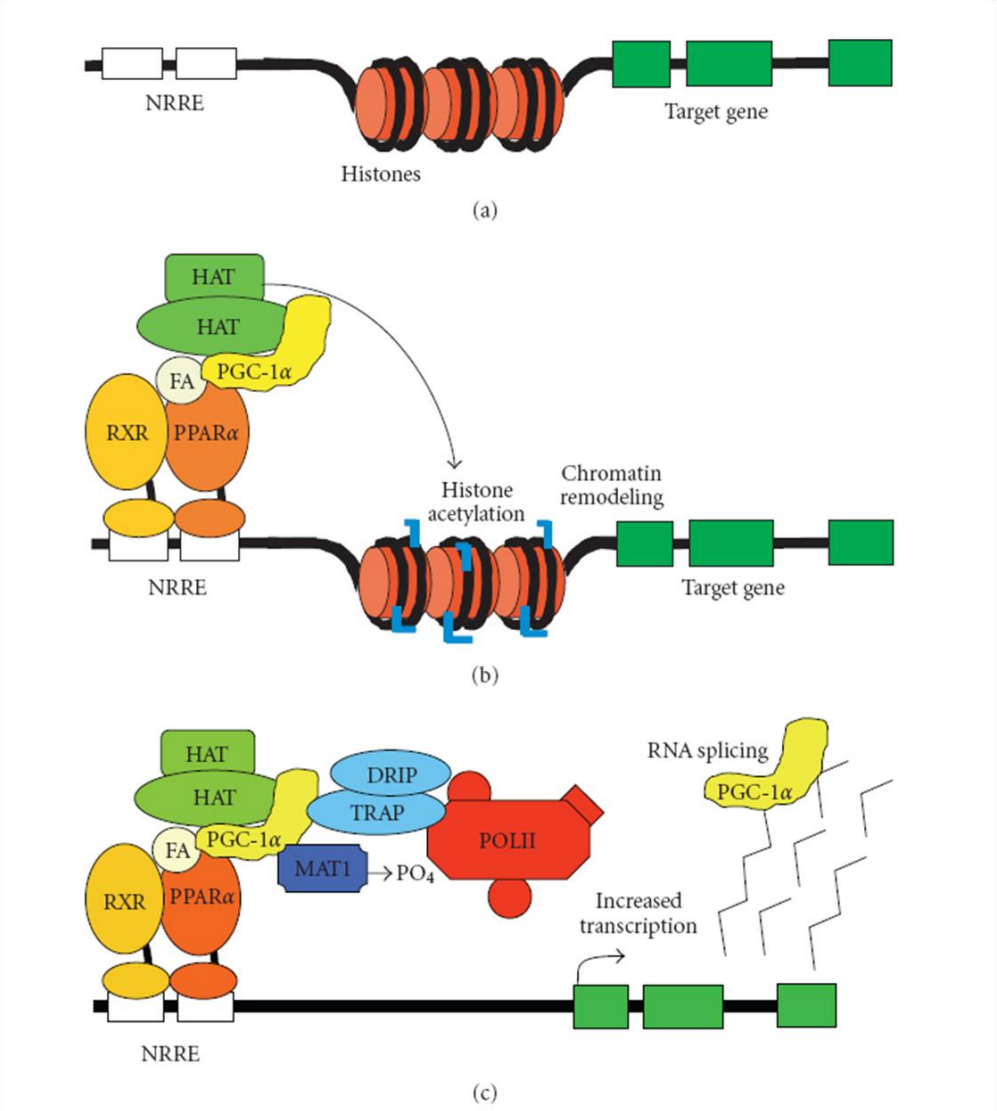

FIG. 1.5. Mechanisms of PPARα activation and PGC-1α coactivator activity. Depiction of a potential PPARα target gene and nuclear receptor response element (NRRE) within the promoter region in the nonactivated state *(top)*. PPARα activation by fatty acid (FA) ligand leads to binding to the NRRE with its heterodimeric partner RXRα; and its coactivator PGC-1α. PGC-1α recruits additional coactivators with histone acetyltransferase (HAT) activity, which promotes chromatin unwinding and increases RNA polymerase II (POL II) access to the target gene promoter *(middle)*. PGC-1α also interacts with the TRAP/DRIP complex and with ménage-à-trois 1 (MAT1) which phosphorylates POL II to increase the probability of gene transcription. In addition, PGC-1α plays a role in RNA splicing via an RNA processing domain in its C-terminus *(bottom)* (Duncan JG *et al.*, 2008).

CHAPTER 2

THE INTERACTION BETWEEN SPECIFIC DOMAINS IN THE N- AND C-TERMINAL REGIONS OF RAT LIVER CARNITINE PALMITOYLTRANSFERASE I DETERMINES MALONYL-COA SENSITIVITY

2.1 INTRODUCTION

Carnitine palmitoyltransferase I (CPTI) catalyzes the conversion of long-chain fatty acyl-CoAs to acylcarnitines in the presence of L-carnitine, the first step in the transport of long chain fatty acids from the cytoplasm to the mitochondria matrix, a rate-limiting step in β-oxidation (Bieber *et al.*, 1988; McGarry *et al.*, 1997). As an enzyme that catalyzes the first rate-limiting step in β-oxidation, CPTI is regulated by its physiological inhibitor, malonyl-CoA (Bieber *et al.*, 1988; McGarry *et al.*, 1997), the first intermediate in fatty acid synthesis, suggesting there is a coordinated control of fatty acid oxidation and synthesis. Mammalian peripheral tissues express two isoforms of CPTI, a liver isoform (L-CPTI) and a muscle isoform (M-CPTI), which are 62% identical in amino acid sequence (Weis *et al.*, 1994; Brown *et al.*, 1995; Zhu *et al.*, 1997; Yamazaki *et al.*, 1995, 1996). Previous studies by our lab and others have demonstrated that the muscle isoform of CPTI, M-CPTI, is much more sensitive to malonyl-CoA inhibition than the liver isoform, but the molecular/structural basis for the differences in malonyl-CoA sensitivity between M-CPTI and L-CPTI remain to be established (Weis *et al.*, 1994; Brown *et al.*, 1995; Zhu *et al.*, 1997; Yamazaki *et al.*, 1995, 1996). Because of its central role in fatty acid metabolism, understanding the molecular mechanism of the regulation of the CPT system is an important first step in the development of treatments for diseases,

such as myocardial ischemia, diabetes, obesity and human inherited CPTI-deficiency diseases (Prentki *et al.*, 1996; Corr *et al.*, 1995; Bennefont *et al.*, 1996).

Woldegiorgis lab developed a high level expression system for human heart M-CPTI, rat L-CPTI, and CPTII in the yeast *Pichia pastoris*, an organism devoid of endogenous CPT activity (Zhu *et al.*, 1997; de Vries *et al.*, 1997; Woldegiorgis *et al.*, 2000). Woldegiorgis's group found that glutamic acid 3 and histidine 5 in L-CPTI were necessary for malonyl-CoA inhibition and high affinity binding but not for catalysis (Shi *et al.*, 1998; Shi *et al.*, 1999). In addition, the mutagenesis studies showed that a change of the highly conserved C-terminal Glu-590 residue in L-CPTI to alanine, glutamine, or lysine significantly increased its sensitivity to malonyl-CoA inhibition with little change for catalysis (Napal *et al.*, 2003). It has been generally predicted that the catalytic and substrate binding sites in both L-CPTI and M-CPTI reside in the C-terminal region of the enzymes (MaGarry *et al.*, 1997; Woldegiorgis *et al.*, 2000).

For M-CPTI, the mutagenesis studies showed that in addition to Glu-3 and His-5, Val-19, Leu-23, and Ser-24 are necessary for malonyl-CoA inhibition and high affinity binding, in agreement with the differences in malonyl-CoA sensitivity observed between M-CPTI and L-CPTI (Zhu *et al.*, 2003). Furthermore, the cysteine-scanning mutagenesis demonstrates that a single substitution mutation of Cys-305 to alanine abolishes M-CPTI catalytic activity (Liu *et al.*, 2005).

To determine the role of the interaction of the N-and C-terminal domains on malonyl-CoA sensitivity in L-CPTI, a series of combined N-and C-terminal residue site-specific mutants as well as a C-terminal site-specific double mutant were constructed from deletion and site-specific mutants that are known to either decrease or increase malonyl-CoA sensitivity in L-CPTI.

2.2 RESULTS

CPTI is controlled by the steady state level of malonyl-CoA and the enzyme's sensitivity to inhibition by malonyl-CoA. The former is established by the activities of

acetyl-CoA carboxylase 2 and malonyl-CoA decarboxylase, while the latter is influenced by post-translational modification (phosphorylation) of CPTI and /or by changes in the lipid environment of CPTI (Distler *et al.*, 2007). It has previously been demonstrated that deletion of the first 18 N-terminal amino acids in L-CPTI decreased malonyl-CoA sensitivity, whereas deletion of the N-terminal amino acids 19-30 increased malonyl-CoA sensitivity of the enzyme (Shi *et al.*, 1998; Jackson *et al.*, 2000). Furthermore, mutation of the C-terminal residue M593 to Ala decreased, whereas mutation of E590 to Ala increased malonyl-CoA inhibition of L-CPTI (Morillas *et al.*, 2003; Napal *et al.*, 2003). Measurement of CPTI activity in mitochondria from the yeast strains that expressed the wild-type (WT) and the combined mutant L-CPTIs demonstrated that both were active. For the WT and all the mutants, proteins of predicted sizes were synthesized and expressed at similar steady state levels (Fig. 2.1). As shown by the IC_{50} values in Table 2.1 and Fig. 2.2, the double mutant Δ18 + M593A showed a complete loss in malonyl-CoA sensitivity, whereas the Δ18+E590A mutant exhibited a 15-fold increase in malonyl-CoA sensitivity compared to the Δ18 mutant alone, but still a significant loss in malonyl-CoA sensitivity compared to the WT. The triple mutant Δ18+E590A+M593A was less sensitive than either the Δ18 or M593A mutants alone but more sensitive to malonyl-CoA inhibition than the double mutant Δ18 + M593A. In a similar study with the Δ19-30 mutant, the double mutant Δ19-30+M593A had malonyl-CoA sensitivity similar to the WT (Table 2.1, Fig. 2.2), whereas the Δ19-30+E590A mutant exhibited a 100-fold increase in malonyl-CoA sensitivity compared to the WT. The C-terminal double mutant, E590A+M593A had malonyl-CoA sensitivity similar to the WT, whereas the triple mutant Δ19-30+E590A+M593A showed a 6-fold increase in malonyl-CoA sensitivity compared to the WT. The data suggest there is an interaction between mutant residues in the N-and C-terminal domains with the same or opposite effect on malonyl-CoA sensitivity of L-CPTI. But the observed effect on the inhibitor sensitivity could also be a reflection of the net change in malonyl-CoA inhibition as a result of the independent positive and negative response elicited by the mutations. If interaction between the N-and C-terminal residue domains is the main driving force for the observed changes in malonyl-CoA sensitivity, it remains to be established.

In summary, the mutants that decrease malonyl-CoA sensitivity (Δ18 and M593A) were counteracted by those that increase malonyl-CoA sensitivity (Δ19-30 and E590A) resulting in an increase in inhibitor sensitivity higher than either the Δ18 or M593A mutant. Furthermore, combining the mutants that decrease malonyl-CoA sensitivity (Δ18 + M593A) further decreases the sensitivity of the enzyme to the inhibitor, while combining the mutants that increase malonyl-CoA sensitivity (Δ19-30 + E590A) increased the sensitivity of the enzyme to the inhibitor to a level 100-fold higher than that observed with the WT enzyme. Mutant Δ19-30+M593A and E590A+M593A exhibited malonyl-CoA sensitivity similar to the WT.

For the conserverd residue mutations in L-CPTI, while mutant E3A of L-CPTI abolishes malonyl-CoA inhibition and high affinity binding but has 70% CPT activity of wild-type, mutant E590A of L-CPTI increases its malonyl-CoA sensitivity close to that observed with the muscle isoform of the enzyme, and has 80% CPT activity of wild-type, we found that double mutant E3A+E590A inactivated L-CPTI. It indicates that both the residues in the N-terminal and C-terminal region combine to play an important role in the catalytic activity. In addition, mutation of Tyr-589 to His inactivated L-CPTI, suggesting that nitration of Tyr-589 plays an important role on regulation of L-CPTI catalysis. For M-CPTI mutagenesis, substitution of Glu-590 with neutral uncharged residue Ala significantly decreased its catalytic activity with a little increase of its malonyl-CoA sensitivity. This suggested that the negatively charged longer side chain of glutamate is essential for catalysis and malonyl-CoA sensitivity. Glu-590 may be required for M-CPTI stability and positioning of the imidazole ring of His-473 for efficient catalysis and inhibition, thus facilitating productive interaction with the substrates and the inhibitor. Our cysteine-scanning mutagenesis and the His-473 and Asp-454 mutation studies with CPTI suggest that there are at least two conserved residues, namely, Cys-305 and His-473, at the active site pocket of CPTI that are essential for catalysis because separate mutation of these residues to Ala inactivates CPTI. In addition, Asp-454 may interact with His-473 and indirectly facilitate catalysis because a mutation of this conserved residue that is located close to the active site His-473 caused a significant loss in CPTI activity. The CPTI protein expression was shown in Fig. 2.3.

2.3 DISCUSSION

The data suggest there is an interaction between mutant residues in the N-and C-terminal domains with the same or opposite effect on malonyl-CoA sensitivity of L-CPTI. It suggested that there are two components important for the peculiar (allosteric) malonyl-CoA sensitivity of the enzyme: 1) amino acids close to catalytic site, since E590 and M593 are affecting IC_{50}; and 2) the interaction of the amino-end with the carboxyl component of the protein, since Δ18 and Δ19-30 also affect IC_{50}. The effect of the double mutants indicates that none of these mutations have a dominant effect. Since the data shows that the mutations are compensatory or additive, we can hypothesize that malonyl-CoA binding could change the interaction of the N-terminal domain with the C-terminal domain resulting in a conformation with less catalytic activity.

The question is now if we can support the existence of different forms of the enzyme depending on how the amino-end interacts with the carboxyl part of the protein, which in turn will depend on the presence of malonyl-CoA. We already know that the proteolysis map of Δ18E590A and Δ18M593A are similar, with perhaps some difference in kinetics of proteolysis fragment generation, more experiments need to be performed (Marrero, unpublished).

We suggest that the observed compensatory and/or additive malonyl-CoA sensitivity of the enzyme is due to amino acids close to the catalytic site like E590 and M593, and the interaction of the N-and C-terminal domains of the protein as shown by the Δ18 and Δ19-30 mutants.

Based on this result, the mutations can be classified into two components, the ones that favour a low catalytic state and a second class that favours a high catalytic state on malonyl-CoA binding. The data also indicate that the amino-end interaction can be divided into two parts that should be accommodated into the carboxyl component to "sense" malonyl-CoA levels and dictating a poor catalytic enzyme conformation in its presence. The in silico or crystallographic accommodation of the amino-end into the carboxyl-component will be a breaktrough in CPTI malonyl-CoA sensitivity study.

It suggested that it is the amino-end interaction with the carboxy-end, not interaction between different regions or residues of the N-terminal end that is the main determinant of malonyl-CoA sensitivity, since the L-CPTI Glu3Ala mutant exhibited complete loss in malonyl-CoA inhibition in the presence or absence of the Δ19-30 or Ser24AlaGln30Ala mutant (Jackson *et al.*, 2001). Allosteric proteins are usually not monomeric, but amino-carboxyl end interaction of CPT1 could render the surface interaction needed to generate different forms with different affinities for the substrate, and malonyl-CoA could regulate the equilibrium of these forms.

2.4 MATERIALS AND METHODS

2.4.1 Construction of plasmids

The Δ18 and E590A mutants were constructed as described previously (Shi *et al.*, 1998; Napal *et al.*, 2003). The Δ19-30 and M593A mutant were constructed as described previously (Jackson *et al.*, 2000; Morillas *et al.*, 2003). Double mutant E590A+M593A was constructed by the overlap extension PCR procedure using the primers shown in Table 2.2 with a template, a plasmid containing the full-length rat L-CPTI in pUC119 to generate pYGW12 (Shi *et al*., 1999). The primers f-GWW3·r-E590AM593A and r-MDR2·f-E590AM593A were used to generate 900 bp and 600 bp PCR products, respectively, using the wild-type L-CPTI cDNA as a template. The two PCR products were purified, mixed, and used as a template for a second-round PCR with the primers f-GWW3·r-MDR2. The 1.5-kb PCR product was digested with *Ava*I-*Sac*I, and the DNA fragment was subcloned into *Ava*I-*Sac*I-cut wild-type L-CPTI cDNA in the pGAP expression vector. Bacterial colonies obtained upon transformation of the mutagenesis reactions were screened by PCR using the primer pair f-GWW3-r-E590AM593ACK for Ala.

For double mutant Δ18+E590A construction, the *Ava*I-*Sac*I fragment (1067-2087 of L-CPTI) was excised from the L-CPTI encoding E590A mutant pHWO10 plasmid and was ligated into the rat liver CPTI Δ18 mutant pHWO10 plasmid without *Ava*I-*Sac*I

fragment to get Δ18+E590A double mutant. Similar construction was done for double mutant Δ18+M593A, Δ19-30+E590A and Δ19-30+M593A.

For triple mutant Δ18+E590A+M593A construction, the *Ava*I-*Sac*I fragment (1067-2087 of L-CPTI) was excised from the E590A+M593A double mutant pHWO10 plasmid and was ligated into the rat liver CPTI Δ18 mutant pHWO10 plasmid without *Ava*I-*Sac*I fragment to get Δ18+E590A+M593A triple mutant. Similar construction was done for triple mutant Δ19-30+E590A+M593A. The primers used above were listed in Table 2.2.

The L-CPTI and M-CPTI conserved residue mutant was constructed by the overlap extension PCR method using the primers listed in Table 2.3. The mutations were confirmed by DNA sequencing.

The expression plasmids were linearized by digestion with the restriction enzyme *Bsp*EI and integrated into the His4 locus of *P. pastoris* GS115 by electroporation (Shi *et al.*, 1999). Histidine prototrophic transformants were selected on YND (yeast nitrogen base with dextrose) plates and grown on YND medium.

2.4.2 CPT assay

Mitochondria were isolated by disrupting the yeast cells with glass beads and used to monitor activity and malonyl-CoA sensitivity. CPT activity and malonyl-CoA sensitivity was assayed by the forward exchange method using L-[methyl-^{3}H] carnitine (de Vries *et al.*, 1997; Bremer *et al.*, 1985). Briefly, in a total volume of 0.5 ml, the standard enzyme assay mixture contained 1.0 mM L-[^{3}H]carnitine (10,000 dpm/nmol), 50 mM palmitoyl-CoA, 20 mM Hepes (pH 7.0), 1% fatty acid-free albumin, and 75 mM KCl with or without 10–100 mM malonyl-CoA. Reactions were initiated by addition of mitochondria. The reaction was linear up to 12 min, and all incubations were performed at 30°C for 5 min. Reactions were stopped by addition of 6% perchloric acid and centrifuged at 2000 rpm for 7 min. The resulting pellet was suspended in water, and the product, [^{3}H] palmitoylcarnitine, was extracted with butanol at low pH. After centrifugation at 2000 rpm for 2 min, an aliquot of the butanol phase was transferred to a vial for radioactive counting.

2.4.3 Western blot

Proteins were separated by SDS-PAGE in a 10% gel and transferred onto nitrocellulose membranes. For L-CPTI, immunoblots were developed by incubation with the rat L-CPTI-specific polyclonal antibodies (1:3000 dilution), followed by an antirabbit IgG conjugated to horseradish peroxidase (1:10000 dilution) as described previously (Shi *et al.*, 1999). For M-CPTI, Immunoblots were developed by incubation with the mice M-CPTI polyclonal antibodies (1:1500 dilution), followed by incubation with peroxidase conjugate anti-rabit IgG (1:10000 dilution) as described previously (Zhu *et al.*, 1997). The antigen-antibody complex was detected using an ECL-enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). Sources of other materials and procedures were as described in the previous publication (Shi *et al.*, 1999).

TABLE 2.1 CPTI activity and malonyl-CoA sensitivity of wild-type and mutant L-CPTI. Mitochondria (150 µg of protein) from the yeast strains expressing wild-type and mutant rat liver L-CPTI were assayed for CPT activity and malonyl-CoA sensitivity as described previously (de Vries *et al.*, 1997; Bremer *et al.*, 1985). The results are the means ± S.D. of at least two independent experiments with different mitochondrial preparations. ND= not determined.

Strains	Activity	IC_{50} Malonyl-CoA
L-CPTI Strains	$nmol.min^{-1}.mg^{-1}$	μM
Wild-Type	12.5±0.5	2.0±0.2
Δ18	8.5±0.6	380.0± 30
Δ19-30	14.3±0.8	0.3±0.1
M593A	8.3±0.5	11.0±0.3
E590A	9.9±1.7	0.21±0.08
Δ18+M593A	9.8±1.1	ND (>>600)
Δ18+E590A	9.4±0.8	25.0±1.1
Δ18+E590A+M593A	8.7±0.6	ND (>>400)
Δ19-30+M593A	16.9±1.2	2.63±0.2
Δ19-30+E590A	16.0±1.1	0.02±0.01
Δ19-30+E590A+M593A	17.3±0.8	0.33±0.02
E590A+M593A	14.2±0.5	2.4±0.2

TABLE 2.2 Oligonucleotide primers used for construction of the L-CPTI mutants

Primer	Sequence (5' to 3')
E590AM593A F	GTTCTGCCTCACATAT**GCG**GCCTCC**GCG**ACCCGGC
E590AM593A R	GCCGGGT**CGC**GGAGGC**CGC**ATATGTGAGGCAGAAC
E590AM593A CK	GTTCTGCCTCACATAT**GC**
GWW3	ATCACCCCAACCCATATC
MDR2	GGCCGCTCGAGCTATTACTTTTTAGAATTGATGGT
E590AF	GTTCTGCCTCACATAT**GCG**GCCTCCATGACCCGGC
E590AR	GCCGGGTCATGGAGGC**CGC**ATATGTGAGGCAGAAC
M593AF	CCTCACATATGAGGCCTCC**GCG**ACCCGGCTCTTCCGAGAAGG
M593AR	CCTTCTCGGAAGAGCCGGGT**CGC**GGAGGCCTCATATGTGAGG
Δ18	CTTCACAAGCTTGAATTC**ATG**ATTGACCTCCGCCTGAGC
RL665	CCACCAGGATTTTAGCT
Δ19-30A	TT*GCGGCCGC*AATAGGTCCCCACT
Δ19-30B	GCAGCAC*CTTAAG*CGAGTAGCG
Δ19-30C	GCTACTCG*CTTAAG*GTGCTGC
Δ19-30D	TCCTCGAGGCCTTACAGATTCCAG

TABLE 2.3 Oligonucleotide primers used for construction of CPTI mutants

Primer	Sequence (5' to 3')
RL665	CCACCAGGATTTTAGCT
RLE3A	CCCAAGCTTGAATTCATGGCA**GCG**GCTCACCAAGCTGTGGC
GWW1	TCGTGGTGGTGGGTGTG
C305AR	CTCCCACTGGGCCGA**GGC**GAGTGGAATGGTGGA
C305AF	TCCACCATTCCACTC**GCC**TCGGCCCAGTGGGAG
GWWR3	TATGCCTATCTTGCTGTTTTTGAA
C305A CK	TCCACCATTCCACTC**GC**
Y589HF	AGTTCTGCCTCACA**CAC**GAGGCCTCCATGACCCG
MDR2	GGCCGCTCGAGCTATTACTTTTTAGAATTGATGGT
Y589HR	CGGGTCATGGAGGCCTC**GTG**TGTGAGGCAGAACT
GWW3	ATCACCCCAACCCATATC
Y589H CK	CATGGGCAAGTTCTGCCTCACA**CAC**
rL-CPTI his FWD	GAATTCATGGCAGAGGCTCACCAAGCTG
rLCPT his REV	GAATTCTTAATG**ATGATGATGATGATGATG**TCCAGTAGG
F2	TCATGTATCGCCGTAAAC
rE590A	GGTCATTGAGGC**CGC**ATAGGTCAGGCA
fE590A	TGCCTGACCTAT**GCG**GCCTCAATGACC
C1	CTTTGCGGATGTGGTTTCCA
cE590A	GGTCATTGAGGC**CG**
H473AF	CTCAATGCAGAG**GCC**GCGTGGGCAGAT
H473AR	ATCTGCCCACGC**GGC**CTCTGCATTGAG-3
H473ACK	CTCAATGCAGAG**GC**
D454AF	AACAGGTGGTTT**GCC**AAATCCTTCACT
D454AR	AGTGAAGGATTT**GGC**AAACCACCTGTT
D454ACK	AACAGGTGGTTT**GC**

FIG. 2.1. Immunoblot showing L-CPTI expresion of wild-type and mutants. Wild type (*lane*1); control without insert (*lane*2); M593A (*lane*3); Δ18+E590A (*lane*4); Δ18+M593A (*lane*5); Δ18+E590A+M593A (*lane*6*)*; wild type (*lane7*); E590A+ M593A (*lane8*); Δ19-30 (*lane9*); Δ19-30+ E590A (*lane10*); Δ19-30+ M593A (*lane11*); Δ19-30+ E590A+ M593A (*lane12*). Mitochondria (20 µg) from the yeast strains expressing the wild type and each of the mutants were separated on a 10% SDS-PAGE and blotted onto a nitrocellulose membrane. The immunoblot was developed using L-CPTI-specific antibodies as described previously (Shi *et al.*, 1999).

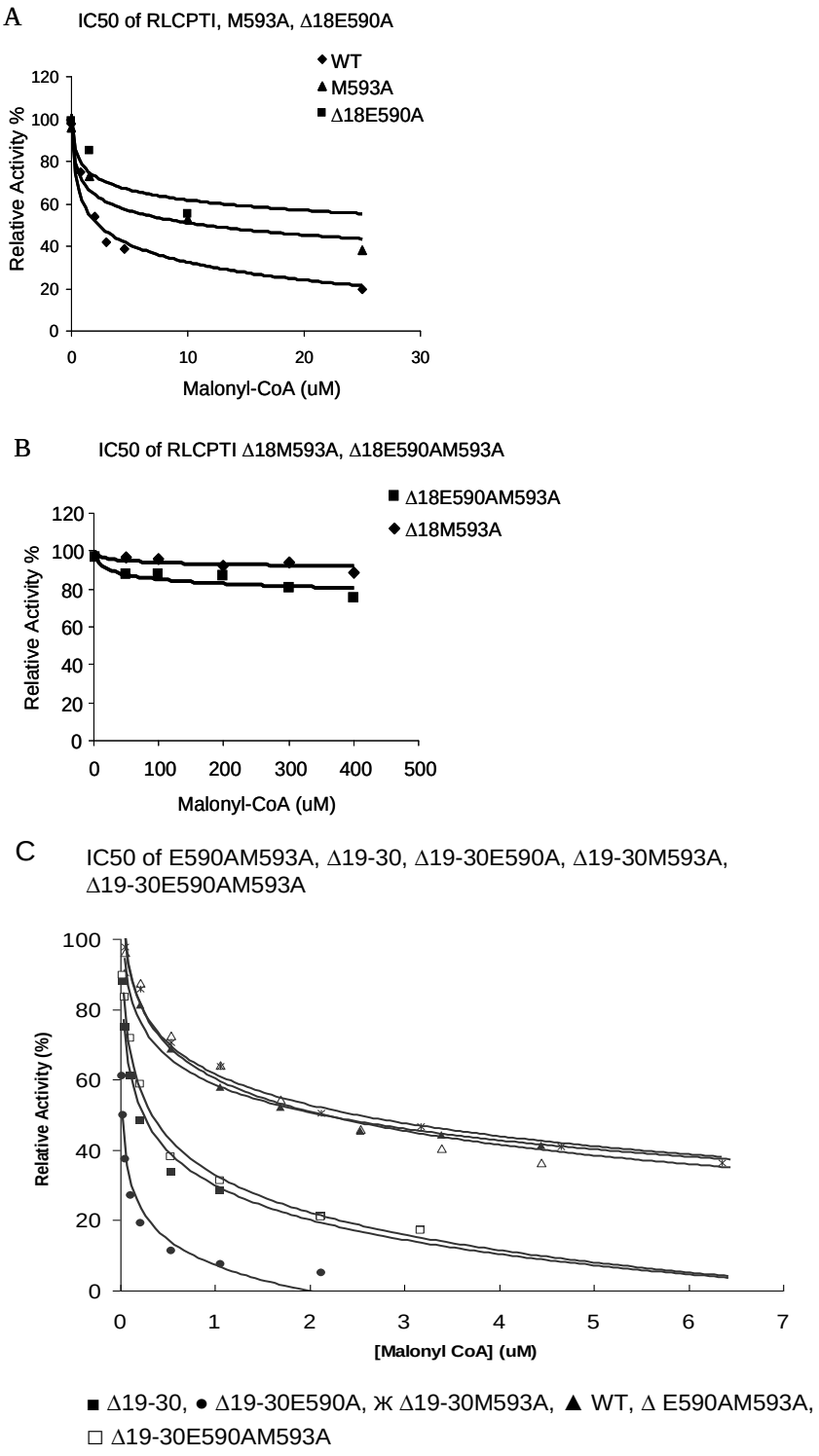

FIG. 2.2. Effect of increasing concentrations of malonyl-CoA on the activities of yeast-expressed wild-type and mutant L-CPTIs. Approximately 150 µg of mitochondrial protein was used for the assay. A, wild type (◆), Δ18+E590A (■), M593A (▲); B, Δ18+M593A (◆), Δ18+E590A+M593A (■); C, WT (▲), Δ19-30 (■), Δ19-30+E590A (●), Δ19-30+M593A (*), E590A+M593A (Δ), Δ19-30+E590A+M593A (□).

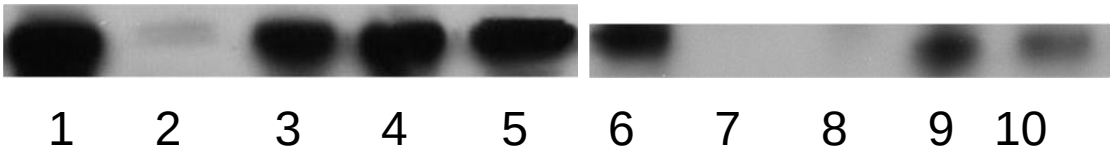

FIG. 2.3. Immunoblot showing expresion of CPTI. L-CPTI wild type (*lane*1); L-CPTI control without insert (*lane*2); L-CPTI E3A+E590A (*lane*3); L-CPTI C305A (*lane*4); L-CPTI Y589H (*lane*5); M-CPTI wild type (*lane*6*)*; M-CPTI control without insert (*lane7*); M-CPTI H473A (*lane8*); M-CPTI E590A (*lane9*); M-CPTI D454A (*lane10*). Mitochondria (20 µg) from the yeast strains expressing the wild type and each of the mutants were separated on a 10% SDS-PAGE and blotted onto a nitrocellulose membrane. The immunoblot was developed using L-CPTI-specific antibodies and M-CPTI-specific antibodies as described (Shi *et al.*, 1999; Zhu *et al.*, 1997).

CHAPTER 3

GLOBAL KNOCKOUT OF M-CPTI CAUSES EMBRYONIC LETHALITY IN MICE

3.1 INTRODUCTION

CPTI has a pivotal regulatory role in mitochondrial flux of fatty acid, as well as functioning as a key point for regulation of mitochondrial β-oxidation. There are 3 isoforms of CPTI: CPTIa or L-CPTI (liver), CPTIb or M-CPTI (muscle), CPTIc (brain). Mitochondrial L-CPTI and M-CPTI, located on the outer membrane of the mitochondrion, mediates the transfer of the acyl-chain of the cytosolic long-chain acyl-CoA to carnitine. CPTI isoforms has different tissue distributions. L-CPTI is dominant in liver, kidney, and intestine; whereas M-CPTI is more abundant in heart and skeletal muscle and in brown adipose tissue (BAT). It is worth noting that M-CPTI is expressed in tissues that consume fatty acids as their major energy source. CPTIc can bind malonyl-CoA, but does not catalyze acyl transfer from various fatty acyl-CoAs to carnitine, it is expressed predominantly in brain, its function is not clear (Price *et al.*, 2002; Wolfgang *et al.*, 2006; Wolfgang *et al.*, 2008). L-CPTI and M-CPTI have different expression levels depending on species, tissue, developmental stage and hormonal regulation (Naoshi *et al.*, 2003). As an enzyme that catalyzes the first step in fatty acid oxidation, CPTI is regulated by its physiological inhibitor, malonyl CoA (Bieber *et al.*, 1988; Zhu *et al.*, 1997; Woldegiorgis *et al.*, 2000; de Vries *et al.*, 1997).

The mechanisms involved in regulation of cellular fatty acid oxidation at the transcriptional level are largely unknown. It is suggested that the peroxisome proliferator-activated receptor (PPAR) regulates mitochondrial fatty acid oxidative enzyme gene expression (Kliewer *et al.*, 1997; Duplus *et al.*, 2000). It was reported that administration of carnitine palmitoyltransferase I inhibitors etomoxir to rats resulted in increase in lipid

accumulation (Dobbins *et al.*, 2001). Similarly, CPTI inhibitor etomoxir decrease whole body fat oxidation in humans (Hinderling *et al.*, 2002). It indicates that diminished CPTI will result in decreased fatty acid β-oxidation.

Conventional gene knockout in mice has yielded animal models for human disease, thereby expanding our understanding of the underlying pathophysiological mechanisms (Kwan, 2002; Sanbe *et al.*, 2003). To explore the pathophysiological effect of diminished M-CPTI expression on fatty acids metabolism in vivo, we generated a mouse M-CPTI gene knockout model by a gene target strategy.

3.2 RESULTS AND DISCUSSION

As shown in Fig. 3.1, The M-CPTI knockout mice were generated by deleting exon 6 and 7 and part of exon 5. 15 chimeric offspring mice (8 males and 7 females) with the disrupted M-CPTI allele were identified by PCR analysis of tail DNA and confirmed by southern blot analysis of *Xho*I digested tail DNA of the F_1 offspring. The M-CPTI +/- heterozygote F_1 male mice were mated with the M-CPTI +/- heterozygote F_1 female mice, and the chimeric offspring were genotyped by PCR and Southern blotting. After more than 3 generations of inbreeding the M-CPTI +/- heterozygous male and female mice, no homozygous M-CPTI -/- mice were found, indicating M-CPTI expression is essential for the development and survival of the embryo. We concluded that targeted deletion of M-CPTI caused embryonic lethality. These data are in parallel with other lab's reporting that homozygous M-CPTI deficiency is lethal in the mouse (Ji *et al.*, 2008). We got 48 heterozygous M-CPTI +/- mice among of 216 pups from inbreeding heterozygous M-CPTI +/- male and female parent, the percentage of heterozygous M-CPTI +/- is 22%. The underlying mechanism that the transmission rate is lower than expected 67% may be related to the frequent loss in M-CPTI +/- gestation stage. There was no sex preference in heterozygous M-CPTI +/- mice.

3.2.1 PCR results of neo gene in ES clones and Southern blot analysis of ES cell and genotyping

The expected PCR product (492bp) was seen among ES clones 2C4, 2D5, 2F9, and 2H12 (Fig. 3.2), Both clones 2F9 and 2H12 in Southern blotting showed a band at around 11 kb and a band around 7 kb, indicating these two clones were MCPTI+/- heterozygous clones (Fig. 3.3). Southern blot was carried out by using probe 2 (Fig. 3.1).

3.2.2 Characterization of the mutant M-CPTI +/- heterozygous mice

Fasting in animal speeds up the mobilization of stored lipid and increases fatty acid flux through the mitochondrial β-oxidation cycle. These processes are characterized by the shunting of peripherally stored lipids to liver and by counterregulatory hormone-mediated inhibition of acetyl-CoA carboxylase (Gulick *et al.*, 1994; Schulz, 1985). When the M-CPTI +/- heterozygous mice were fasted for 48 hours, the mice became extremely weak, immobile and inactive compared to the starved wild-type mice. Such a characteristic phenotype may be due to that the M-CPTI +/- heterozygous mice can not use the fatty acid as efficiently as the wild-type mice. Interestingly, there was a report that the M-CPTI +/- mice were more vulnerable to cold challenge than wild-type mice (Ji *et al.*, 2008).

3.2.3 Western blot analysis

We subjected the M-CPTI +/- heterozygous and wild-type mice to dietary treatment, including fasting for 48 hours to detect the change in the M-CPTI gene expression by western blotting and CPT activity assay. Western blotting showed that CPTI expression in heart of M-CPTI +/- heterozygous mice was about half of that in the wild-type mice heart (Fig. 3.4). After fasting, both heterozygous and wild-type mice had increased CPT expression, however, the heterozygous mice responded to fasting more efficiently than the wild-type (Fig. 3.4). To the best of our knowledge, no report indicated that mouse ATPase β-subunit was induced by fasting, so the ATPase β-subunit was used as an internal control for normalization of the mitochondrial membrane protein, make the CPTI expression more comparable between different samples.

3.2.4 Heart mitochondria CPT activity assay

The female M-CPTI +/- heterozygous mice (57.1±2.2, n=4) had similar CPT activity of the male heterozygous mice (60±2.6, n=5). The CPT activity of M-CPTI +/- heterozygous mice were about half of the control mice (103.8±1.6, n=4). After fasting 48 hours, CPT activity of both M-CPTI +/- heterozygous mice and wild-type mice increased by 30-50% of that of the fed littermate as shown in Table 3.1 and Fig. 3.4. There was no sex difference on the CPTI activity. This result is consistent with the western blot findings. After fasting, both heterozygous and wild-type mice had increased CPT activity, however, the heterozygous mice responded to the treatment more efficiently than the wild-type (Fig. 3.4). There seems no difference for the malonyl-CoA sensitivity between the heterozygous and wild-type mice heart mitochondria (data not shown).

3.2.5 Semi-quantitative RT-PCR

To assay the M-CPTI mRNA level of M-CPTI +/- heterozygous mice, we run semi-quantitative RT-PCR. In skeletal muscle, the heterozygous mice showed half CPT mRNA of the wild-type mice (Fig. 3.6). We subjected the heterozygous and wild-type control to fasting for 48h to detect the change in the M-CPTI gene expression by semi-quantitative RT-PCR. The heterozygous mice showed 50% mRNA expression in heart and skeletal muscle of the wild-type mice and were more sensitive to fasting than the wild-type mice as shown in Table 3.2. The M-CPTI gene transcription in heart and skeletal muscle is enhanced by fasting, especially for the heterozygous mice (Fig. 3.5, Fig. 3.6).

The result suggests that M-CPTI plays an important role in the development of mouse embryos may be through the control of the oxidation of long-chain fatty acids. The lower mRNA level of heart M-CPTI in the M-CPTI +/- mice results in lower levels of protein expression. These data are in parallel with the recent reports that heterozygous L-CPTI +/- mice and M-CPTI +/- mice had no compensation at the transcriptional level in heart (Liu *et al.*, 2007; Ji *et al.*, 2008; Nyman *et al.*, 2005).

Taken together, we developed a mouse model of heterozygous M-CPTI+/-. Heterozygous mice was more susceptible to fasting than wild-type mice. M-CPTI must play a critical role in metabolic function of the embryo. One possible explanation for

embryonic lethality is that the absence of M-CPTI expression in heart may be lethal. Another explanation is that M-CPTI activity may be required during mouse early embryo development, because CPTI can be detected at the 2-cell stage (Berger *et al.*, 2004). The phenotype that M-CPTI -/- mice can not be obtained is in parallel with the scarcity of human cases with M-CPTI deficiency.

3.3 MATERIALS AND METHODS

3.3.1 Classic genome alteration of mouse M-CPTI

The targeting construction was carried out using the targeting vector pKO scrambler NTKV1905 (Stratagene, La Jolla, CA). The 3.7 kb *Bam*HI fragment of mouse M-CPTI, and the 4.1 kb *Bam*HI-*Sal*I fragment of mouse M-CPTI (Fig. 3.1.a) were used (Miller *et al.*, 1988; Nagy, 2000; Kwan, 2002; Sanbe *et al.*, 2003). The generated plasmid pKO-BBS is a derivate of pKO1905 that has the 2.7 kb *Bam*HI-*Hind*III fragment inserted in scramble A and the 4.1 kb *Bam*HI-*Sal*I fragment cloned in scramble B (Fig. 3.1.b). The orientation of the inserts was determined by DNA sequencing. Plasmid pKO-BBS DNA was linearized by *Not*I, separated on 1% agarose gel, and purified by using the QIAEX II Gel Extraction Kit. Injecting, picking, replicating, and freezing colonies of embryo stem cell were carried out by the Transgene Facility at Oregon Health & Science University. We prepared genomic DNA from ES cells (96-well plate, the first round) and carried out genotyping.

3.3.2 PCR analysis of *neo* gene in ES clones

The *neo* primers-pair were used to test the presence of the neomycin gene. The neo forward: F (5'-AGGATCTCCTGTCATCTCACCTTGCTCCTG-3') and the neo reverse: R (5'-AAGAACTCGTCAAGAAGGCGATAGAAGGCG-3').

3.3.3 PCR and Southern blot analysis to genotype M-CPTI knock-out

Both ES clones of 2F9 and 2H12 were used to inject into C57BL/6 blastocysts and then were implanted into the uterus of foster mother. After pups were born, chimeric offspring were identified by the coat color. These chimers were the mouse M-CPTI

knockout founders. When the chimeric male mice were sexually mature (~ 8 weeks old), they were mated with the wild-type C57BL/6 female mice, resulting in F_1. The F_1 pups were subjected to PCR analysis of tail DNA to determine the presence of *neo* gene. Neo positive mice were then genotyped by Southern-blot analysis. PCR analysis to detect the existence of neo gene in F_1 mice was carried out as described in the PCR analysis for ES cells. However, *neo* positive mice can result from random integration of the knockout construct, not from real M-CPTI knockout, since not all the *neo* positive mice demonstrate the knockout pattern in the Southern-blot as shown below. DNA derived from tail biopsies was used for genotyping. About 5μg DNA solution of each sample was cut by *Xho*I overnight and separated on 0.8% agarose gel at 30 volt for overnight. Southern blot was carried out by using probe 2 which was generated by PCR with primers KO-F1 (5'-CCCCGTCACCAGTGGTCTTCTGCCACAAT-3') and KO-R1 (5'-CACCGACTGATCTCTAGCAGCAATTC-3'). Southern blotting was used to genotype the F_2, F_3 and F_4 by probe 2.

3.3.4 Mice experiments

Animals were housed in a temperature controlled room with 12 h-light/12 h-dark cycling and were fed normal chow diet ad libitum. All experiments were performed with age (8–10 weeks old) and sex matched F_3, F_4 M-CPTI +/- heterozygous mice and wild-type littermate. Mice were either fasted for 48 h or fed a normal diet. All animal procedures were performed with the approval of the Institutional Animal Care and Use Committee at Oregon Health & Science University.

3.3.5 Heart mitochondria isolation and Western blotting analysis

Proteins were separated by SDS-polyacrylamide gel electrophoresis in a 10% gel and transferred onto nitrocellulose membranes. Immunoblots were developed by incubation with the mice M-CPTI polyclonal antibodies (1:1500 dilution), followed by incubation with peroxidase conjugate anti-rabit IgG (1:10000 dilution). To allow for standardization between blots, an in-house standard (mouse monoclonal anti-ATP synthase β antibody; 1:5000 dilution; Sigma) was also run on each gel, followed by incubation with peroxidase conjugate anti-mouse IgG (1:10000 dilution) and protein

levels were expressed relative to the standard. The bands were detected by chemiluminescence (PerkinElmer Life Sciences, Boston, MA), and band densities were quantified using Quantity 1-D analysis software (Bio-Rad).

3.3.6 CPT activity assay

Heart mitochondria were isolated from the fresh heart by a series of differential centrifugation in the sucrose buffer. CPT activity was assayed by the forward exchange method using L-[^{3}H] carnitine as described previously (de Vries *et al.*, 1997; Bremer *et al.*, 1985).

3.3.7 Semi-quantitative RT-PCR

Total RNA was isolated from skeletal muscle and heart of mice using the Trizol reagent (Invitrogen, Carlsbad, CA) and purified by RNeasy mini kit (Qiagen) according to the manufacture's instructions. cDNA was synthesized from the RNA by reverse transcription using oligo(dT)$_{20}$ primer in Super Script III first-strand synthesis system (Invitrogen). For semi-quantitative RT-PCR, we used the following primer-pair: Forward 5'-ACCGGTACTTGGATTCTGTGC-3', Reverse 5'-TCCTGCTTCGGAGGTAGACATA-3', which cover the expected knockout part of M-CPTI, and mouse β-actin was used as internal control (409 bp). β-actin FWD, 5'-GAGCTATGAGCTGCCTGACG-3', corresponding to nucleotides 776-795; β-actin REV, 5'-AGCACTTGCGGTGCACGATG-3', complementary to nucleotides 1166-1185 of the mouse β-actin cDNA. RT-PCR product was detected by Kodak Image Station 2000R. Data were acquired and processed with Kodak 1D 3.6 Software.

3.3.8 Statistical analysis

Results were reported as mean values ± SE. Student's t test and one-way ANOVA were used to test for significant differences. Results were considered significantly different with a $p<0.05$.

TABLE 3.1 Effect of fasting on CPT activity in the M-CPTI heterozygous mice (nmol/mg.min)

	Wild-type	Heterozygous female	Heterozygous male
Fed	103.8±6.2 (n=4)	57.1±4.9 (n=4)	60±5.6 (n=5)
Fasted	137.6±8.3* (n=4)	87.1±6.7* (n=4)	84.2±6.4* (n=5)

*p<0.05

TABLE 3.2 Effect of fasting on CPT mRNA normalized to β-actin (ratio of CPTI/ β-actin)

	Wild-type heart	Knockout heart	Wild-type muscle	Knockout Muscle
Fed	0.66±0.06 (n=5)	0.40±0.14 (n=4)	0.37±0.11 (n=6)	0.18±0.06 (n=4)
Fasted	0.94±0.16* (n=5)	0.68±0.07* (n=4)	0.75±0.12* (n=6)	0.42±0.04* (n=4)

*p<0.05

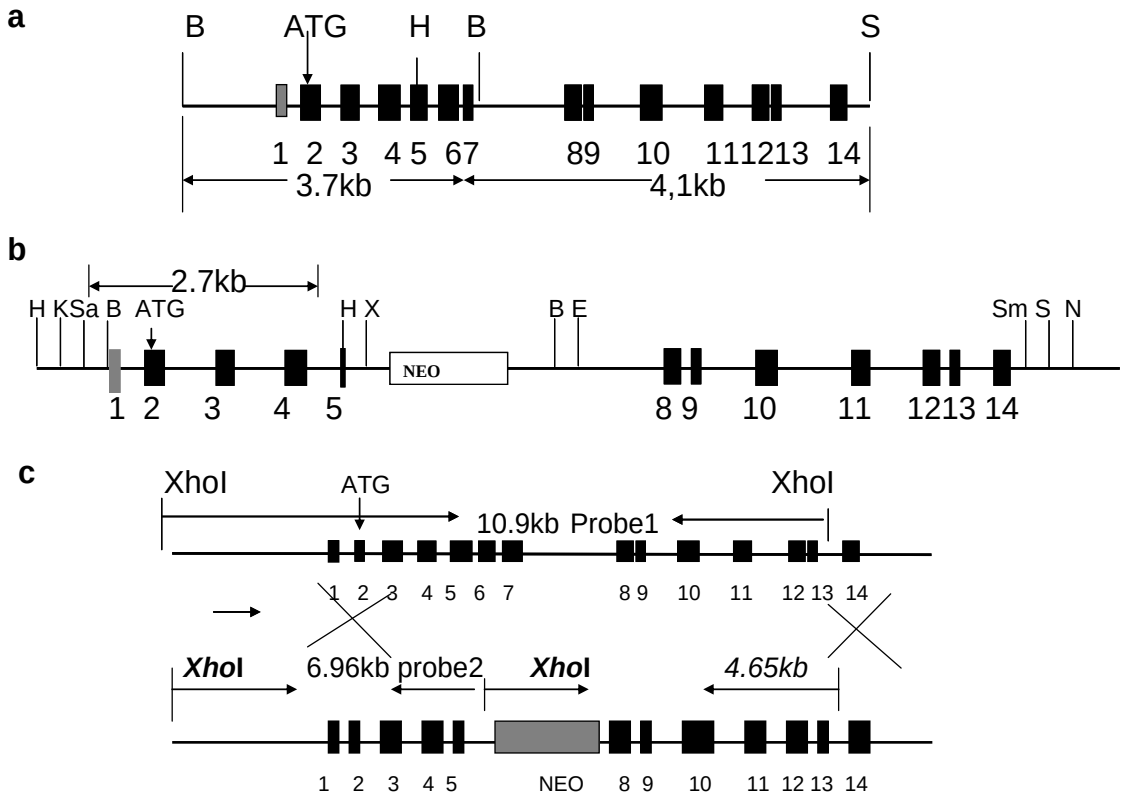

FIG. 3.1. Schematic representation of knockout mice generation. a, the MMCPT1 gene fragment used in the construction. b, the PKO-BBS construction. B:*Bam*HI; Bg:*Bgl*II; E:*Eco*RI; H:*Hind*III; K:*Kpn*I; S:*Sal*I; Sa:*Sac*I; Sm:*Sma*I; N:*Not*I; Exons 1 to 13 are indicated as solid black boxes. c, Homologous recombination.

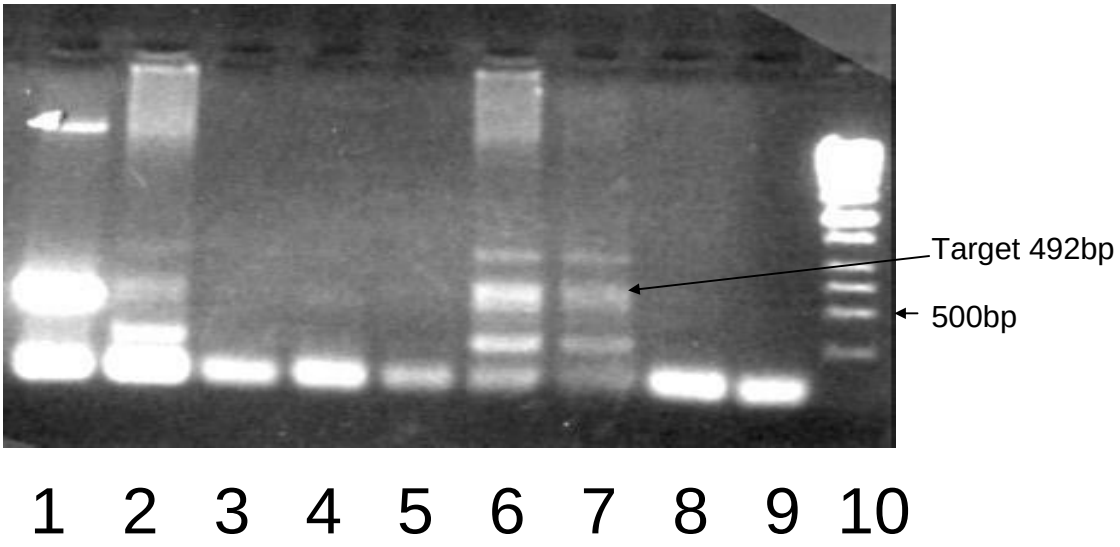

FIG. 3.2. PCR results of *neo* gene in ES clones. *lane 1*, pKO-BBS; *lane 2*, 2H12; *lane 3*, 2H11; *lane 4*, 2F9; *lane 5*, 2E11; *lane 6*, 2D5; *lane 7*, 2C4; *lane 8*, 2B2; *lane 9*, 2A2; *lane 10*, 1kb marker. Positive: 2, 4, 6, 7.

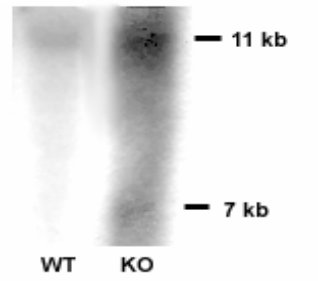

FIG. 3.3. Genotyping by Southern blot analysis. WT, wild-type; KO, heterozygous (M-CPTI +/-).

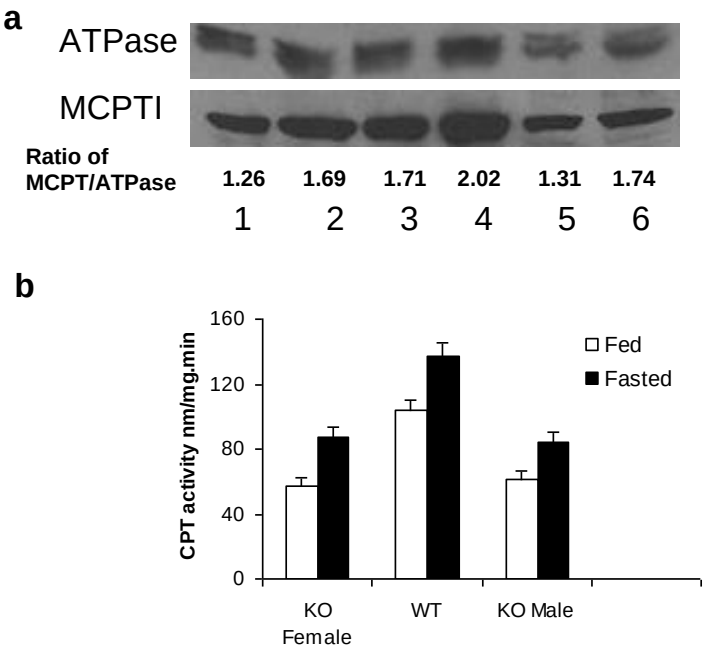

FIG. 3.4. Western blot analysis of mice heart mitochondria protein and effect of sex on CPT activity in heart of fasted and fed heterozygous and wild-type mice. a, Polyclonal antibody for MCPTI, monoclonal antibodies against β-subunit of F_1-ATPase (ATPase). *Lane1*, KO female normal diet; *lane2*, KO female fasting; *lane3*, WT female normal diet; *lane4*, WT female fasting; *lane5*, KO male normal diet; *lane6* KO male fasting. KO, heterozygous MCPTI+/-. b, CPT activity was measured in heart mitochondria protein as described in the methods section. Results shown are mean±SEM for 3 to 6 mice per group.

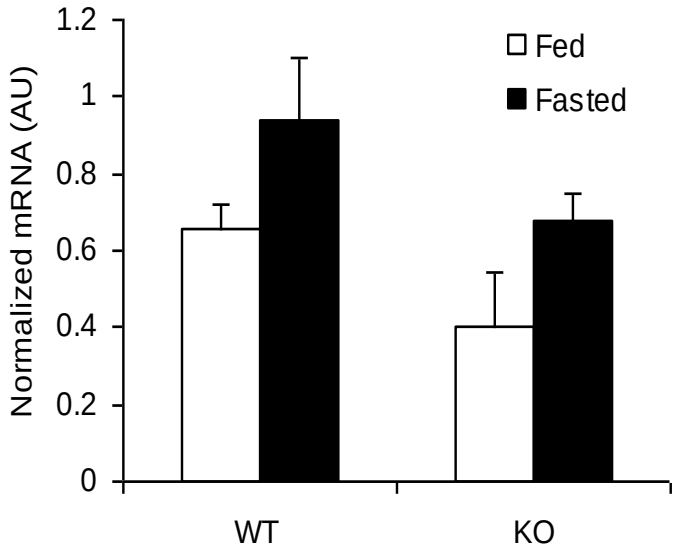

FIG. 3.5. mRNA levels in heart of fasted and fed heterozygous and wild-type mice. mRNA level was measured in total RNA extracts from heart as described in the methods section. KO, heterozygous MCPTI+/-. Results shown are mean±SEM for 3 to 6 mice per group. AU, arbitrary units normalized to β-actin.

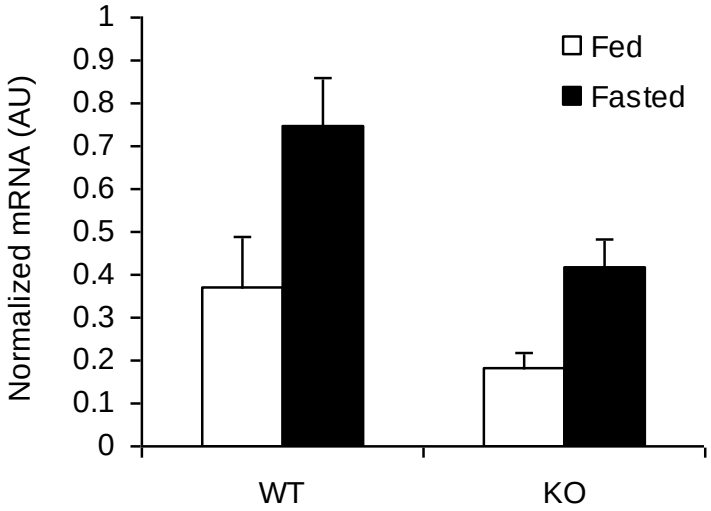

FIG. 3.6. mRNA levels in skeletal muscle of fasted and fed heterozygous and wild-type mice. mRNA level was measured in total RNA extracts from skeletal muscle as described in the methods section. KO, heterozygous MCPTI+/-. Results shown are mean±SEM for 3 to 6 mice per group. AU, arbitrary units normalized to β-actin.

CHAPTER 4

OVER-EXPRESSION OF HUMAN HEART MALONYL-COA INSENSITIVE CARNITINE PALMITOYLTRANSFERASE I MUTANT ENHANCES ENERGY UTILIZATION IN MICE

4.1 INTRODUCTION

Carnitine palmitoyltransferase I (CPTI) is a transmembraene enzyme of the mitochondrial outer membrane which catalyzes the transfer of an acyl moiety from a long-chain acyl-CoA ester to carnitine to form acylcarnitine, a rate-controlling step in fatty acid oxidation and an important site in the regulation of flux through β-oxidation. Modulation of CPTI activity has important significance on drug development to control disease such as obesity and diabetes and provides insight into the mechanism of fatty acid oxidation regulation (Zhu *et al.*, 1997; Zhu *et al.*, 2003; Liu. *et al.*, 2007; de Vries *et al.*, 1997; Yamazaki *et al.*, 2003). The muscle isoform of carnitine palmitoyltransferase I (M-CPTI) is 30- to 100-fold more sensitive to malonyl CoA inhibition than the liver isoform (L-CPTI). The site-directed mutagenesis studies demonstrate that Glu3, Val19, Leu23, and Ser24 in M-CPTI are important for malonyl CoA inhibition and binding, but not for catalysis (Zhu *et al.*, 2003).

CPTI appears to be a viable target to manipulate fatty acid metabolism. Some interventions could potentially increase fatty acid metabolism, such as exercise and a prolonged fasting. Over-expression of PPAR in mice heart causes many metabolic and biochemical changes that can increase fatty acid oxidation, an increase in CPTI activity in the process plays an important role (Finck *et al.*, 2002). Over-expression of L-CPTI in cultured primary rat hepatocytes and β-cells increases fatty acid oxidation (Stefanovic-Racic *et al.*, 2008; Blanca *et al.*, 2002) and L-CPTI over-expression increases long-chain fatty acid oxidation and reduces cell viability with incremental palmitic acid

concentration in human embryonic kidney cell line 293 (Ulrike *et al.*, 2005). In addition, over-expression of M-CPTI in skeletal muscle in vivo increases fatty acid oxidation and reduces triacylglycerol esterification (Clinton *et al*., 2007). These reports support that up-regulation of L-CPTI and M-CPTI result in more fatty acids flux into the mitochondria for β-oxidation.

The regulation of fatty acid oxidation in heart is not completely understood. The α-myosin heavy chain (αMHC) promoter has been widely used for the expression of target gene specifically in heart of mice. However, to the best of our knowledge, no studies have examined the effect of genetically manipulating CPTI expression on fatty acid metabolism in mature heart.

To gain further understanding of the direct role of M-CPTI in regulating fatty acid metabolism in vivo, we generated a transgenic mice model that specifically over-expresses malonyl-CoA insensitive mutant human heart M-CPTI in heart. Heart was selected as a target organ because there is no clear indication of the metabolic outcome of having a high activity of CPTI in this tissue. The stimuli that markedly increase the reliance of heart on mitochondrial fatty acid β-oxidation for ATP production include fasting, high-fat diet and diabetes. The transgenic (TG) and non-trangenic (NTG) mice will be treated with these stimuli. The purpose of this study was to characterize the physiological and metabolic phenotype of specifically M-CPTI over-expression in heart and to understand M-CPTI roles in the physiology and pathophysiology of mice.

4.2 RESULTS AND DISCUSSION

We have tried to over-express human heart M-CPTI and the malonyl-CoA insensitive mutant gene in the heart of mice to test the metabolic and physiological consequences. Unfortunately, no transgenic mice that specifically over-express wild-type human heart M-CPTI were identified, suggesting that M-CPTI over-expression may be lethal. However, transgenic mice that over-express the mutant malonyl-CoA insensitive human heart M-CPTI were obtained.

4.2.1 Generation of M-CPTI mutant mice

Fifteen founder lines of transgenic (TG) mice were generated using standard DNA microinjection techniques. The chimeric gene introduced into the mice contained a 5.5-kb mouce musculus alpha myosin heavy chain gene promoter linked to the 2.3-kb cDNA for mutant human heart CPTI followed by the linker and the 3' end of hGH gene (0.6-kb). The pattern of incorporation of the chimeric gene cDNA into the genome of the founder lines is shown in Fig. 4.1. From the 15 founders, 2 independent transmission lines were identified, named H and C line. The following experiments were carried out using these two lines. Each group has 3-5 mice to be treated.

4.2.2 Skewed inheritance pattern investigation

To detect the sex preference of germline transmission, breeders were set up with either the male or the female transgenic mice and paired with wild-type mates. The pups were genotyped by PCR, as shown in Fig. 4.2. There was no sex preference of transmission of the mutant human heart CPTI transgene (H line male versus female: 1/1.1; C line male versus female: 1/1.15). Approximately 10% of pups were transgenic mice. The rate of trangenesis is lower than expected 50% mye be involved in the compatibility of tansgene construct and the host gene background.

4.2.3 Tissue expression of mutant M-CPTI

Human heart CPTI expression in different tissues was detected by RT-qPCR of total RNA isolated from different tissues and Western blotting of isolated mitochondria from different tissues using the anti-His monoclonal antibodies. The data showed that the mRNA of human heart CPTI in the skeletal muscle, liver and kidney were undetectable. RT-qPCR showed that human heart CPTI is specifically expressed in the mice heart, that is in parallel with the Western blotting (data not shown). That is to say the transgene is heart specific and is over-expressed as shown in Table 4.1.

4.2.4 Copy number of M-CPTI in transgenic mice

The CPT activity of transgenic mice was almost twice that of non-transgenic mice. CPT activity assay showed that CPT expression was increased approximately twofold in

the transgenic mice heart (H line and C line) as shown in Table 4.2. Thus we think that the functional copy number of M-CPTI transgene is two.

4.2.5 Effect of CPTI over-expression on FA metabolism in fasted mice

Fasting can stimulate the mobilization of stored fat and an increase in fatty acid flux through the mitochondrial β-oxidation cycle (Schulz, 1985). Fasting increased CPT activity by 46% in normal diet fed mice, while the activity of normal diet fed transgenic mice was almost 2 times that of normal diet fed non-transgenic mice and there was no detectable change after fasting as shown in Table 4.3. This was consistent with the Western blotting results, in which ATPase β-subunit was used as the motochondrial internal standard control as shown in Fig. 4.3. RT-qPCR data showed that human heart CPTI mRNA level was high in the fed TG mice and fasting had no effect or did not increase the mRNA level. The body weight of the TG female mice after fasting for 48 hours decreased by 50% more than that of the fasted NTG female mice. It indicated that M-CPTI over-expression alone could significantly increase fatty acid metabolism in vivo and deplete fat stores in female mice as shown in Table 4.4.

4.2.6 Effect of CPTI over-expression on FA metabolism in high-fat diet fed mice

High-fat diet is a stimulus that increases the reliance of the heart on mitochondrial fatty acid oxidation for ATP production (Peters *et al.*, 1998). Feeding NTG mice high fat diet can increase CPT activity by 51% more than that of normal diet fed NTG mice, while the CPT activity of normal diet fed TG mice was almost 2 times that of normal diet fed NTG mice and there was no detectable CPT activity change after high fat diet for the TG mice as shown in Table 4.5. That was consistent with the Western blotting results as shown in Fig. 4.4. RT-qPCR data showed that human heart CPTI mRNA level before feeding a high fat diet was similar with that of after feeding a high fat diet in TG mice. The body weight data showed that after high fat diet for 6 weeks, the TG mice gain about 50% less body weight than that of NTG mice as shown in Table 4.6. The underlying mechanism may involve that fatty acid burns up in TG mice heart will interfere with lipid metabolism of the whole body, as a burden, high-fat diet will exacerbate the lipid metabolism dysfunction. Additionally, we believe that sustained activation of CPTI will

promote a state of metabolic inflexibility that lead to whole body metabolism dysfunction, which may involve reactive oxygen species generation (Dewald *et al.*, 2005).

4.2.7 Effect of diabetes and insulin administration on M-CPTI gene expression and CPT activity

To examine the effect of CPTI over-expression on metabolism in experimentally induced diabetes and insulin treat, the CPT activity and protein expression were determined in TG and NTG mice. In the uncontrolled diabetic state, the heart relies almost exclusively on fatty acid oxidation for its ATP requirements (Finck *et al.*, 2002). For the NTG mice, CPT activity assay and Western blotting showed that streptozotocin-induced diabetes increased the CPT expression by 2-fold, while insulin treatment decreased it to a normal level as shown in Fig. 4.5 and Table 4.7. However, the M-CPTI over-expression mice maintained a high CPT expression level, the same dose of streptozotocin-induced diabetes to severe hyperglycemia, and it need more insulin to minimally lower the blood glucose to the normal untreated level (data not shown). It suggested that this maybe due to the higher blood glucose level and the decreased insulin sensitivity. The body weight loss of TG mice was more than that of non-transgenic mice, that is to say, the M-CPTI over-expression mice was more vulnerable to weight loss than the non-transgenic mice as shown in Table 4.8 and Table 4.9. It is known that the diabetes induce the heart to reduce glucose uptake and utilization and to increase fatty acid utilization (Finck *et al.*, 2002). M-CPTI over-expression may further increase the high rate of fatty acid β-oxidation in diabetic TG mice, thereby exacerbate the metabolic phenotype of diabetes such as hyperglycemia and body weight loss. The M-CPTI transgenic mice depend on fatty acid for the generation of energy to significantly greater extent than NTG mice, indicating a substrate utilization profile in transgenic mice heart resembles to that of the diabetic heart.

4.2.8 RT-qPCR

Hearts from age- and sex-matched mice from 2 transgenic lines were examined for human heart M-CPTI mRNA expressions under different conditions. The data showed

that the expression of human heart M-CPTI in the transgenic mice is not regulated by fasting, high fat diet, and diabetes and insulin treatment as shown in Table 4.10.

Western blot analysis showed that the non-transgenic mice CPTI expression increases after treatment, but the expression in the transgenic mice does not change, indicating that the level of CPTI is not regulated in the transgenic mice. The ATPase β-subunit was used as an internal control for normalization the mitochondrial membrane protein. CPT activity assay indicated that the over-expression mice stays in a high level of CPT activity, while the non-transgenic mice showed an increase after fasting, high fat diet, and diabetes, and decrease after insulin treatment

Taken together, an increase in CPTI content can upregulate fatty acid metabolism in vivo under basal conditions and stress burden. M-CPTI plays an important role in body weight control, especially for mice fed a high fat diet. Here, for the first time, it set the basis for M-CPTI activation to treat obesity. Over-expression of M-CPTI leads to increased CPTI activity in isolated mitochondria and plays an important role in lipid storage control in vivo. There is a recent report that pharmacological stimulation of brain CPTI decreases food intake and body weight (Aja *et al.*, 2008). We have now developed a novel model to study the regulation of heart metabolism by M-CPTI in vivo. However, transgenic mice that over-express the cardiac-specific M-CPTI were found to be more susceptible to diabetes. It is likely that the majority of the metabolic and behavioral changes noted in the M-CPTI transgenic mice are due to metabolic alterations resulting from the heart specific over-expression of the human heart mutant M-CPTI. Over-expression of the mutant enzyme in heart made the transgenic mice insensitive to malonyl-CoA regulation, and avoided the adaptive counterregulatory responses that would probably be provoked by constitutive transgene expression.

4.3 MATERIALS AND METHODS

4.3.1 Vector construction

The full-length malonyl-CoA insensitive human heart carnitine palmitoyltransferase (M-CPTI) cDNA was amplified using the primer pair E3AFWD 5’CTCGAGATGGCGGCAG3’ and R1 5’-CTCGAGCTAATGATGATGATGA-3’ and

the 6×His tagged human heart M-CPTI E3AV19A L23A S24A cDNA as a template. The full-length human heart M-CPTI cDNA was amplified using primer pairF1-R1, F1 5'-CTCGAGATGGCGGAAGC-3'. An *Xho*I site was created at the immediate flanking 5'and 3' ends of M-CPTI. The PCR product was subcloned into the PCR2.1 TA clone vector, then it was digested with *Xho*I. The target product was subcloned into the *Sal*I cloning site of plasmid Alpha-MyHC clone26 (a gift from Dr.J.Robbins, Univ. of Cincinnati) containing α-myosin heavy chain (αMHC) promoter and human growth hormone polyA. The orientation of the construct was examined by digestion with *Hind*III.

After sequence confirmation by DNA sequencing, the fragment composed of the αMHC promoter, M-CPTI, 6 His and hGH polyA was excised by *Not*I digestion, purified, and used for production of transgenic mice by the Transgenic Mice Core Facility at Beth Israel Deaconess Medical Center and Harvard University, Boston, MA. The construct contains a C-terminal hexahistidine tag.

4.3.2 Generation of transgenic mice

His-tagged human heart M-CPTI cDNA and the malonyl-CoA insensitive M-CPTI mutant cDNA were cloned downstream of the cardiac αMHC promoter as described above. Pronuclear DNA microinjection was used to generate transgenic mice. Fertilized oocytes were removed from the oviduct of a FVB mouse, and the male pronucleus was microinjected with a solution containing the transgene. The injected eggs were cultured in vivo until the pronuclei were fused and the zygote had developed into a 2-cell embryo. The embryos were then transplanted into a surrogate mother, and pups were born 19-21 days later. Pups that developed from a zygote that successfully integrated the microinjected DNA had the transgene in every cell of their body. These heterozygous animals (called founders F_0) can then be bred to obtain homozygous mice.

4.3.3 Identification of founders and genotyping

Approximately 10-20% of pups can be expected to be transgenic, so the littermates must therefore be screened to identify founder animals. DNA for screening was isolated from mice tail and screened by PCR analysis. Genomic DNA was extracted from mice tail, PCR was run to genotype the transgenic pups, using the primer pair:

αMHC Fwd: 5'-GAATGTTTCCCCTGTAGAC-3', HHCPTR134 Rev: 5'-GTAGACGTGTTTCAGGGC-3'. The target band was 432bp. Wild-type C57BL mice DNA was used as negative control, and the M-CPTI αMHC construct plasmid DNA was used as a positive control.

4.3.4 CPT activity assay and Western blot analysis

Heart mitochondria were isolated from the fresh tissues by a series of differential centrifugation in the 0.25 M sucrose buffer. CPT activity was assayed by the forward exchange method using L-[^{3}H] carnitine as described previously (de Vries *et al.*, 1997; Bremer *et al.*, 1985).

Proteins were separated by SDS-polyacrylamide gel electrophoresis in a 10% gel and transferred onto nitrocellulose membranes. Immunoblots were developed by incubation with the polyclonal M-CPTI antibody (FP2), membranes were incubated with peroxidase conjugate anti-rabit IgG, bands were detected by chemiluminescence (PerkinElmer Life Sciences, Boston, MA), and band densities were quantified using Quantity 1-D analysis software (Bio-Rad). For anti-His monoclonal antibody, immunoblots were developed by incubation with the monoclonal anti-His antibodies (1:2000 dilution; Penta-His antibody; Qiagen), followed by goat anti-mouse IgG conjugated to horseradish peroxidase (1:10000 dilution) as described previously (Zhu *et al.*, 2003). To allow for standardization between blots, an in-house standard (anti-ATP synthase β) was also run on each gel, and protein levels were expressed relative to the standard.

4.3.5 Mice experiments

Animals were housed in a temperature controlled room with 12 h-light/12 h-dark cycling and were fed normal chow diet ad libitum. All experiments were performed with age (8–10 weeks old) and sex matched F_3, F_4 and F_5 generation mice. Transgenic mice were either fasted for 48 h or fed a normal chow diet. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ) (10 mg/100 g body weight) twice per week. Diabetes was confirmed by high blood glucose level (>250 mg/dl). Insulin was administered to diabetic mice at a combined dose of regular insulin (3 U/100 g body

weight; Lilly) intraperitoneally and Lente insulin (10 U/100 g body weight; Lilly) subcutaneously. Diabetic mice were studied 96 h following STZ treatment. Insulin-treated mice were studied 16 h following insulin treatment. For high fat diet feeding, transgenic mice were fed fish oil (200 g/kg) and corn oil (10 g/kg) based diet (calories provided by: protein 22.7%, fat 46.5%, carbohydrates 30.7%; 58NQ, TestDiet, IN) for 6 weeks. All animal procedures were performed with the approval of the Institutional Animal Care and Use Committee at Oregon Health & Science University.

4.3.6 Serum metabolite assays

Glucose concentration was measured in a drop (10 μl) of blood of the tail obtained using a Elite XL system (Bayer) after the mice were anesthetized using isoflurane

4.3.7 RT-qPCR

Total RNA from heart, skeletal muscle, liver and kidney was extracted with the Trizol reagent (Invitrogen, Carlsbad, CA) and purified by RNeasy mini kit (Qiagen) according to the manufacture's instructions. cDNA was synthesized from the RNA by reverse transcription using Taqman high capacity cDNA reverse transcription kit (Applied Biosystems). TaqMan probes and forward and reverse primers were designed with Primer Express software (Applied Biosystems). Real-time primer-pair used for human heart M-CPTI: Forward 5'-CTG CAG TGG GAC ATT CCA AA-3', Reverse 5'-CCT TGG CCA CCT GGT AGG A -3'. Taqman Probe for human heart M-CPTI: 5'-FAM –CAG TGC CAG GCG GTC ATC GAG AG-TAMRA-3'. Real-time PCR primer-pair used for mice β-actin: Forward 5'-GACGGCCAAGTCATCACTATTG-3', Reverse 5'-GAAGGAAGGCTGGAAAAGAGC-3. Probe used for mice β-actin: 5'-VIC-CAACGAGCGGTTCCGATGCCC-TAMRA-3. qPCR was run in ABI Prism 7000 sequence detector according to the manufacture's instructions and data were acquired and processed with ABI Prism 7000 SDS software.

4.3.8 Statistical analysis

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

TABLE 4.1 Tissue distribution of human heart CPTI mRNA normalized to β-actin

Tissue	Heart	Muscle	Liver	Kidney
RT-qPCR	100	0.2	0.1	0.1

TABLE 4.2 CPT activity of transgenic mice (nmol/mg.min)

	NTG female	TG female	NTG male	TG male
CPT activity	109.4±17.2	198.7 ±15.3*	106±10.7	194.6±12.8*

*p<0.05

TABLE 4.3 Effect of fasting on CPT activity in TG mice (nmol/mg.min)

	NTG female	TG female	NTG male	TG male
Fed	110.4±20.2	218.7±29.6	106.2±18.5	228.9±30.3
Fasted	160.7±35.8*	227.2±23.7	155.3±31*	231.3±41.7

*p<0.05

TABLE 4.4 Effect of fasting on body weight of TG mice

	NTG female	TG female	NTG male	TG male
Fed (g)	27.3±1.5	26±2.2	28.6±1.8	24±2.5
Fasted (g)	22.4±3.1*	20±1.8*	22±2.8*	20±2.1*

*p<0.05

TABLE 4.5 Effect of high fat diet on CPT activity in transgenic mice (nmol/mg.min)

	NTG female	TG female	NTG male	TG male
Normal	129.2±39.6	219.2±19.6	106±29.5	229±20.3
High-fat	211.0±29.3*	226.8±49.7	214±19.7*	231±40.2

*p<0.05

TABLE 4.6 Effect of high fat diet on body weight gain of TG mice after 6 weeks of feeding

	NTG female	TG female	NTG male	TG male
Normal (g)	1.75±0.3	1.6±0.22	2.33±0.3	2.0±0.17
High-fat (g)	2.25±0.21*	1.78±0.2*	3.92±0.2*	2.66±0.2*

*p<0.05

TABLE 4.7 Effect of diabetes and insulin administration on M-CPTI expression in TG mice (nmol/mg.min)

	NTG female	TG female	NTG male	TG male
Control	109.7±21.1	296.8±18.2	181.7±17.6	327.3±23.3
Diabetes	227.3±13*	300.8±21.5	340.5±11.8*	331.4±17.2
Insulin	135.4±18.3	287.6±30.7	198.8±21.6	325.9±32.3

*p<0.05

TABLE 4.8 Blood glucose level in the diabetic transgenic and nontransgenic mice

	NTG female	TG female	NTG male	TG male
Control (mg/dl)	196.7±14	192.6±20	199.7±11	185.8±17
Diabetes (mg/dl)	472±38	579.3±22*	433.5±23	588.3±14*

*$p<0.05$

TABLE 4.9 Effect of diabetes on body weight in transgenic mice

	NTG female	TG female	NTG male	TG male
Control (g)	22.7±1.3	21.3±1.2	24.1±1.2	24.7±1.0
Diabetes (g)	22±2.2	18±1.1*	23±1.2	22±1.3*

*$p<0.05$

TABLE 4.10 Human heart CPTI expression in transgenic mice heart under different metabolic stresses. mRNA level was measured in total RNA extracts from tissues as described in the methods section. AU, arbitrary units normalized to β-actin

TG mice	Control	Diabetes	Insulin	Fasted	High fat diet
mRNA level (AU)	1.0	1.06	1.04	0.95	1.09

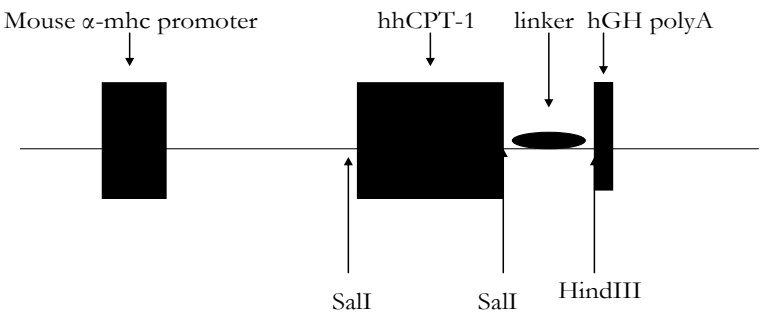

FIG. 4.1. Map of heart specific M-CPTI over-expression plasmid construct.

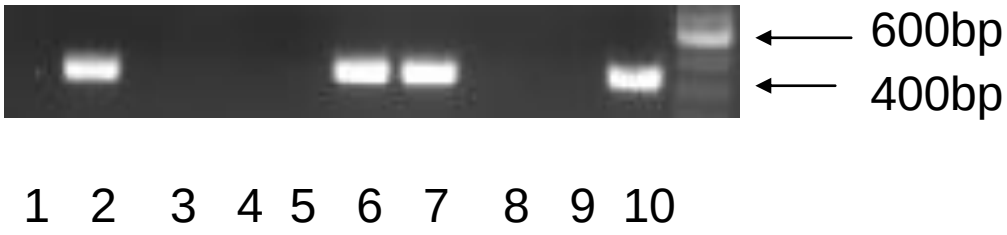

FIG. 4.2. Genotyping mice that over-express mutant M-CPTI. Tail DNA was extracted using Qiagen DNeasy tissue kit and PCR product separated on 1% agrose gel. Mutant F_0 generation. Primer-pair: αMHCFwd-HHCPTR134. Positives: lane 2, 6, 7, 10.

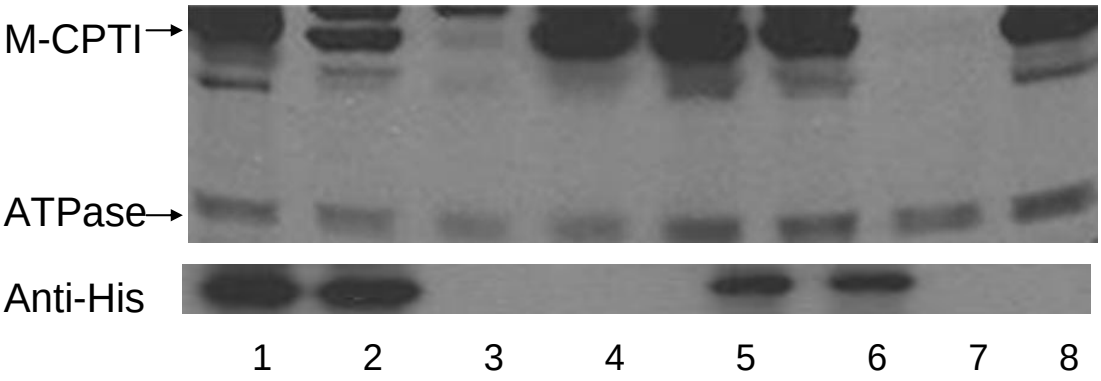

FIG. 4.3. Effect of fasting on M-CPTI expression in the transgenic mice. For Western blot, mitochondia protein was used. Blots were developed using polyclonal antibody for M-CPTI, monoclonal antibodies against β-subunit of F_1-ATPase (ATPase) and monoclonal anti-His antibodies. *Lane1*, TG Female, fed; *lane2*, TG female, fasted; *lane3*, NTG female, fed; *lane4*, NTG female, fasted; *lane5*, TG male, fed; *lane6*, TG male, fasted; *lane7*, NTG male, fed; *lane8*, NTG male, fasted.

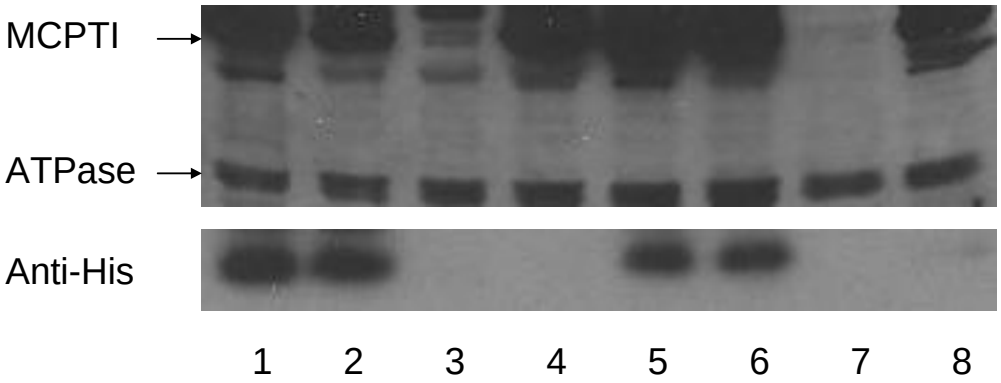

FIG. 4.4. Effect of high-fat diet on M-CPTI expression in the transgenic mice. Blots were developed using polyclonal antibody for M-CPTI, monoclonal antibodies against β-subunit of F_1-ATPase (ATPase) and monoclonal anti-His antibodies. *Lane1*, TG Female, normal fed; *lane2*, TG female, high-fat fed; *lane3*, NTG female, normal fed; *lane4*, NTG female, high-fat fed; *lane5*, TG male, normal fed; *lane6*, TG male, high-fat fed; *lane7*, NTG male, normal fed; *lane8*, NTG male, high-fat fed.

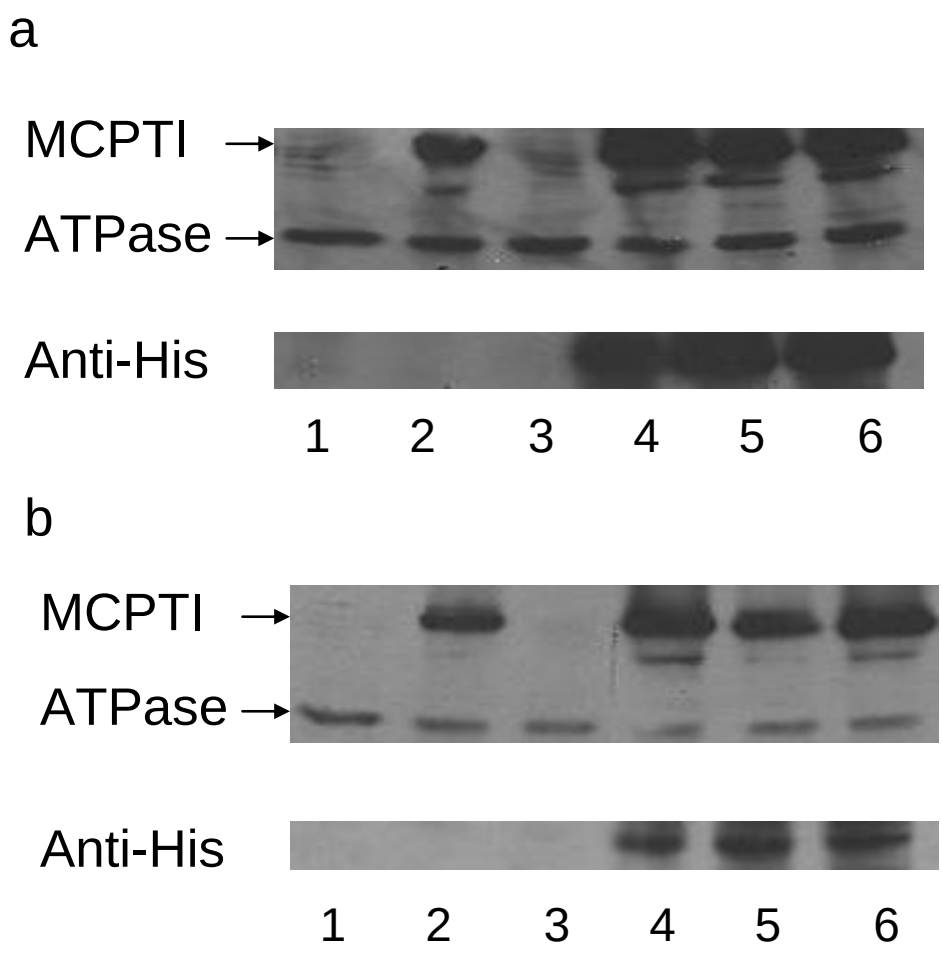

FIG. 4.5. Effect of diabetes and insulin administration on M-CPTI expression in the transgenic mice. Western blot was developed using polyclonal antibody for M-CPTI, monoclonal antibodies against β-subunit of F_1-ATPase (ATPase) and monoclonal anti-His antibodies. a, effect of hormone on female mice. b, effect of hormone on male mice. *Lane1*, NTG control, *lane2*, NTG diabetes, *lane3*, NTG insulin, *lane4*, TG control, *lane5*, TG diabetes, *lane6*, TG insulin.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 SUMMARY OF RESEARCH

5.1.1 The interaction between specific domains in the N-and C-terminal regions of rat liver carnitine palmitoyltransferase I determines malonyl-CoA sensitivity

The mutants that decrease malonyl-CoA sensitivity (Δ18 and M593A) were counteracted by those that increase malonyl-CoA sensitivity (Δ19-30 and E590A) resulting in an increase in inhibitor sensitivity higher than either the Δ18 or M593A mutant. Furthermore, combining the mutants that decrease malonyl-CoA sensitivity (Δ18 + M593A) further decreases the sensitivity of the enzyme to the inhibitor, while combining the mutants that increase malonyl-CoA sensitivity (Δ19-30 + E590A) increased the sensitivity of the enzyme to the inhibitor to a level 100-fold higher than that observed with the wild-type (WT) enzyme. Mutant Δ19-30+M593A and E590A+M593A exhibited malonyl-CoA sensitivity similar to the WT. This data supports the hypothesis that the N-terminal end interacts with a region of the C-terminal end far from the catalytic site. Since the data shows that the mutations are compensatory or additive, we can speculate that malonyl-CoA binding could change the interaction of the N-terminal domain with the C-terminal domain resulting in a conformation with less catalytic activity.

5.1.2 Global knockout of M-CPTI causes embryonic lethality in mice

To address the pathophysiological effect of diminished M-CPTI expression on fatty acids metabolism in vivo, a mouse M-CPTI gene knockout model was generated by a gene target strategy in ES cells that resulted in the deletion of exon 6, 7 and part of exon 5 of M-CPTI. Homozygous deficient mice (M-CPTI -/-) were not viable. However heterozygous (M-CPTI +/-) pups survived. M-CPTI +/- mice became more susceptible to

fasting than wild-type mice. Western blot analysis and CPT activity assay suggested that the heterozygous heart had about half of CPTI protein expression of the wild-type heart, and there were no sex difference on the CPTI activity. The heterozygous mice showed 50% mRNA expression of the wild-type mice in heart and skeletal muscle and were more sensitive to fasting than the wild-type mice. We concluded that targeted deletion of M-CPTI results in embryonic lethality.

5.1.3 Over-expression of human heart malonyl-CoA insensitive carnitine palmitoyltransferase I mutant enhances energy utilization in mice

To gain further understanding of the direct role of M-CPTI in regulating fatty acid metabolism in vivo, we generated a transgenic mice model that specifically over-expresses mutant human heart M-CPTI in heart. An increase in M-CPTI content can upregulate fatty acid metabolism in vivo under basal conditions and stress burden. M-CPTI plays an important role in body weight control, especially for high fat diet mice. Our data set the basis for M-CPTI activation to treat obesity. The over-expression of M-CPTI leads to increased CPTI activity in isolated mitochondria and plays an important role in control of lipid storage in vivo.

We have now developed a novel model to study the M-CPTI regulation of heart metabolism in vivo. However, transgenic mice that over-express the cardiac-specific M-CPTI were found to be more susceptible to diabetes. It is likely that the majority of the metabolic and behavioral changes noted in the CPTI transgenic mice are due to metabolic alterations resulting from the heart specific over-expression of the human heart mutant M-CPTI. This mutant makes the transgenic mice insensitive to malonyl-CoA regulation, and avoids the adaptive counterregulatory responses that would probably be provoked by constitutive transgene expression.

5.2 FUTURE DIRECTIONS

To gain further understanding of the direct role of CPTI in regulating fatty acid metabolism in vivo, we will generate a conditional gene knockout mice model and an

inducible transgenic mice model that specifically overexpresses human heart M-CPTI in different tissues, such as in heart and skeletal muscle.

5.2.1 Construction of conditional knockout M-CPTI using the 2-loxP-2-FRT strategy.

We have a 7.8kb mouse genomic DNA clone for M-CPTI that will be used for the generation of the classical knockout mouse model. The complete sequence of the mouse M-CPTI gene is composed of 19 exons flanked by 18 introns. Our clone is missing the 3'end exons 15-19 and introns 14-18, but is more than adequate for construction of the targeting vector for the conditional knockout mouse model. The targeting vector is ploxPFLPneo plasmid (Transgenic Animal Model Core). Our conditional knockout construct will place our target exon 3 to exon 5 between the two loxP sites, Deletion of these exons will result in an inactive M-CPTI, because the two transmembrane domains are essential for M-CPTI activity and malonyl-CoA sensitivity. PCR will be used to introduce *Swa*I site to the fragment of M-CPTI from exon 6 to exon14, which will then be inserted into *Swa*I site of ploxPFLPneo plasmid. Similarly a *Bam*H I site will be introduced to both ends of the fragment from exon 3 to intron 5 and inserted into *Bam*H I site of ploxPFLPneo plasmid. Additionally, the fragment of exon1 to inton 2 will be placed in the *Xho* I –*Kpn* I site of ploxPFLPneo plasmid. The insertion will be confirmed by sequencing. The final targeting construct will be linearized by *Aat* II and electroporated into embryonic stem cells (ES cells). The neo resistant colony will be selected and used for microinjection into C57BL/6 blastocysts and implantion into pseudopregnant female recipients. The chimeric mice will be crossed for germline transmission, and will be assessed by PCR or Southern blotting. Subsequently FLP-mediated excision will delete the neomycin cassette, leaving behind a single FRT site.

Cre-mediated expression will then delete the targeted exon and leave behind a loxP site and a FRT site.

5.2.2 Inducible human heart CPTI transgene via tet-on system construction

TRE (tetracycline response element)-HHCPT-1 will be constructed into the pTRE2 vector (Clontech). The full-length human heart carnitine palmitoyltransferase (M-

CPTI) cDNA will be amplified using the primer pair pTRE2-F1, 5'-TCTAGAATGGCGGAAGCTCACC-3' and pTRE2-REV, 5'-TCTAGACTAATGATGATGATGATGATGGCTGTA-3' and the 6×His tagged human heart M-CPTI cDNA as the template. An *Xba*I site will be created at the immediate flanking 5'and 3' ends of M-CPTI. The PCR product will be subcloned into the PCR2.1 TA clone vector, then it will be digested by *Xba*I. The target product will be subcloned into the *Xba*I cloning site of plasmid pTRE2 (Clontech) containing the Tet responsive PhCMV-1 promoter. The construct will be checked by PCR and the orientation of the construct examined by digestion with *Hind*III. After confirmation by DNA sequencing, the fragment composed of the TRE and M-CPTI will be linearized by *Xho*I, purified, and will be used for production of transgenic mice by the transgenic mice core facility.

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BIOGRAPHICAL SKETCH

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Publications

Chen Z, Tan Y, Napal L, Woldegiorgis G (in prep). The interaction between specific domains in the N-and C-terminal regions of rat liver carnitine palmitoyltransferase I determines malonyl-CoA sensitivity.

Chen Z, Zheng G, Woldegiorgis G (in prep). Global knockout of M-CPTI causes embryonic lethality in mice.

Chen Z, Woldegiorgis G (in prep). Over-expression of human heart malonyl-CoA insensitive carnitine palmitoyltransferase I mutant enhances energy utilization in mice.