ABSTRACT

LYVERS PEFFER, PASHA A. Unraveling the pathway of lipid oxidation in the young pig: Assessment of hepatic β-oxidation and characterization of carnitine palmitoyltransferase I (CPT I). (Under the direction of Jack Odle).

Hepatic β-oxidation and liver and skeletal muscle carnitine palmitoyltransferase I (CPT I) activity and mRNA expression were evaluated in the pig. In the first study, newborn pigs were allotted to one of four dietary regimens: artificial milk replacer with long chain triglycerides (LCT) as the fat source (Control), the Control diet with 0.5% clofibric acid (CA), the Control diet with medium chain triglycerides replacing LCT as the fat source (MCT), or the Control diet with 40 ppm isoproterenol (ISO). There were no differences between Control and MCT or ISO supplemented groups in total, mitochondrial, or peroxisomal ß-oxidation of [1-^{14}C]-palmitate (1 mM). Total and peroxisomal β-oxidation increased 134 and 186%, respectively, with CA supplementation. Hepatic malonyl-CoA sensitive CPT activity increased ($p < 0.05$) in pigs receiving CA. Changes in relative expression of hepatic LCPT I and skeletal muscle MCPT I mRNA amounts following clofibrate supplementation were not detected, while a modest effect on acyl-CoA oxidase (ACO) relative mRNA amounts was observed (p=0.08). In the second study, hepatic and skeletal muscle CPT I kinetics in pigs during different stages of development were evaluated. Activity of CPT I increased 109 and 67% between birth and 1 wk of age in liver and skeletal muscle, respectively ($p < 0.05$). Realtive expression of hepatic CPT I mRNA in the 24 hr old pig was 7% of the amount detected in the newborn ($p < 0.001$); while hepatic CPT I apparent Km for carnitine decreased 48% from birth to 3 wk of age. The apparent Km for carnitine in skeletal muscle decreased from birth to 1 wk of age, then increased 200% between 1 and 5

wk of age ($p< 0.01$). Plasma and liver free carnitine concentrations increased 200 and 160%, respectively, during the first day of life ($p < 0.05$). High relative expression of γ-butyrobetaine hydroxylase (γBBH) in the kidney indicated high capacity for de novo carnitine synthesis by this tissue. Collectively, the findings from this research are important in understanding how the pig, a species with a low capacity for β-oxidation, utilizes fatty acids.

UNRAVELING THE PATHWAY OF LIPID OXIDATION IN THE YOUNG PIG: ASSESSMENT OF HEPATIC β-OXIDATION AND CHARACTERIZATION OF CARNITINE PALMITOYLTRANSFERASE I (CPT I).

by

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APPROVED BY:

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To my husband, for his endless support

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EDUCATION

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North Carolina State University, Raleigh, NC
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EXPERIENCE

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metabolism at the cellular level with total body function; and practical application of basic principles of nutrient metabolism. Spring 2004.
Research Assistant. Michigan State University, East Lansing, MI
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Vice-President Animal Science Graduate Student Association. Michigan State University. Fall 1998 – Spring 1999.

PUBLICATIONS

Odle, J., P. Lyvers-Peffer, and X. Lin. Hepatic Fatty Acid Oxidation and Ketogenesis in young Pigs. In: *Biology of Metabolism in Growing Animals*. (In Press).

Lyvers-Peffer, P.A., X. Lin, and J. Odle. A molecular and enzymatic evaluation of the pigs' metabolic requirement for L-carnitine, and its supply from de novo synthesis and diet**. (***in preparation***)**

Lyvers-Peffer, P.A., X. Lin, and J. Odle. The effects of dietary clofibrate on gene expression and enzyme activity of hepatic and skeletal muscle CPT-I. (*in preparation*)

Lyvers-Peffer, P.A., X. Lin, L. Averette Gatlin, J. Woodworth, and J. Odle. 2004. Ontogeny of mitochondrial carnitine palmitoyltransferase I in porcine liver and skeletal muscle (Abs). National Animal Science Meeting.

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INTRODUCTION

In the past decade pre-weaning mortality has continued to significantly impact the number of pigs weaned per litter, claiming between 10-15% of total pigs born alive (NAHMS, 2000). Mortality rate is greatest during the initial days postpartum indicating the necessity for early intervention if pre-weaning mortality is to be ameliorated. Data from the National Animal Health Monitoring System (2000) lists starvation as the second leading cause of pre-weaning mortality in pigs, resulting in approximately 17% of the total loss. However, the impact of inadequate nutrition may be even greater, as pigs suffering from starvation become pre-disposed to crushing by the dam. Therefore, additional factors contributing to mortality and morbidity may be secondary to starvation and the impact of nutritional deficiency may be greater than original estimates. Starvation, although a consequence of inadequate nutrient supply, can also result from inadequate nutrient utilization by the neonate.

For the developing fetal pig, maternal glucose serves as the primary energy substrate due to the restrictive permeability of the placenta to fatty acids (Battaglia and Meschia, 1988). During the postnatal period milk lipids replace carbohydrates as the principal substrate for oxidative metabolism with 55-65% of dietary energy originating from milk fat (Girard et al., 1992). As the piglet transitions from a fetal to neonatal energy source there is

a decrease in liver glycogen, which is elevated at birth but precipitously declines until becoming depleted within 48 h (Pégorier et al., 1981). In addition, the piglet does not have an appreciable fat depot from which to mobilize this needed energy source for survival, as adipose stores represent less than 2% of the piglets' total mass at birth (Mannaerts and McCrea, 1963). Therefore, not only must the piglet make the metabolic adaptations necessary for utilizing fat as its main energy source, it must rely on obtaining this energy from the dam's milk. The piglets' ability to quickly up-regulate the machinery required to catabolize dietary fatty acids for energy greatly influences its probability for survival.

Several lines of evidence lend support to the credence that the neonatal piglet is limited in it's capacity to catabolize dietary fatty acids; 1) The capacity of the 1 day old pig to oxidize fatty acids is 32% of the rate of the 24 d old pig (Bieber, et. al., 1973). 2) A large percent of oleate (80 to 95%) taken up by isolated piglet hepatocytes is re-esterified with limited flux through the pathway of β-oxidation (Pergori et. al., , 1983), and 3) Piglets do not show an appreciable accumulation of circulating ketone bodies during the neonatal period (Adams et al., 1997; Pégorier et al., 1981; Bengtsson, et al., 1969).

Carbon flux of long chain fatty acids through β-oxidation spiral is regulated by entrance of the fatty acids into the mitochondrial matrix, and is controlled by the activity of carnitine palmitoyltransferase I (CPT I). Deficiencies in CPT I lead to hypoglycemia and hypoketonemia in the human (Bonnefont et al., 1999). The low β-oxidative capacity of the neonatal pig and the incidence of hypoketonemia may derive from hepatic CPT I regulation. It is apparent that pig CPT I, both liver and skeletal muscle isotypes, are kinetically distinct from their rat and human counterparts. The unique characteristics of CPT I make this enzyme an appealing target for study to tease out the intricacies of lipid oxidation in the

young pig, which appear to deviate from the dogma that has been established using the rodent model.

CENTRAL DOGMA OF FATTY ACID OXIDATION

β-oxidation in the Mitochondria and the Ancillary Pathway of Peroxisomal Oxidation

In-utero the fetus is reliant on carbohydrate energy sources for development, however fatty acids serve as the primary energy source during the neonatal-suckling phase (Girard et al., 1992). As a consequence, the neonate must make an abrupt metabolic switch in fuel source selection. Timely postnatal initiation of β-oxidation, the major route in which fatty acids are oxidized, becomes imperative to the survival of the neonate. The process of β-oxidation is essential in skeletal muscle which relies on the production of acetyl-CoA generated when two carbon units from the acyl chain are sequentially removed. Acetyl-CoA subsequently serves as a substrate for the tricarboxylic acid cycle. The products of β-oxidation are also important to the liver for the synthesis of ketone bodies, an alternative fuel source which serves to spare glucose during physiological states which increase circulating fatty acids.

The enzymes of β-oxidation are confined within the mitochondrial and peroxisomal matrices (Figure 1). Before a fatty acid is accessible to enzymes of either pathway, it must be activated to its CoA thioester, a reaction catalyzed by a family of enzymes classified as fatty acyl-CoA synthetases and localized to the outer mitochondrial membrane, the peroxisomal membrane, or the endoplasmic reticulum. The resulting fatty acyl-CoA is hence primed for β-oxidation. In general, oxidation occurring in the peroxisome is considered ancillary to mitochondrial oxidation. However, the peroxisome is essential in the

oxidation of very long-chain fatty acids which are not oxidized by the mitochondria (Wanders et al., 2001).

The reactions of mitochondrial and peroxisomal β-oxidation are very similar, oxidation proceeds via four consecutive steps involving desaturation, hydration, dehydrogenation, and finally, thiolytic cleavage to produce a fatty acid shortened by two carbon units. Although identical reactions occur in both organelles, they are catalyzed by different enzymes encoded by distinct genes (Wanders et al., 2001). In the mitochondrial β-oxidation scheme the hydration and dehydration enzyme activities reside within a bi-functional protein, in contrast these activities along with the final thiolysis reside in a tri-functional protein within the peroxisomal β-oxidation pathway.

Although the enzyme activities of the β-oxidation pathways are comparable, key differences are observed in the initial desaturation reactions. The desaturation of acyl-CoA is catalyzed by a family of acyl-CoA dehydrogenases in the mitochondria versus acyl-CoA oxidase within the peroxisome, and both are coupled to the reduction of a flavoprotein (FAD). In the mitochondria FAD reduction is coupled to the electron transport chain. However, the peroxisome lacks an electron transport chain, therefore electrons are transferred directly to molecular oxygen (forming H_2O_2) to regenerate the oxidized form of the flavoprotein, which is essential to keep the cycle engaged (Mannaerts, et al., 1979). The uncoupling of the desaturation step from the electron transport chain results in the generation of heat for the initial step of β-oxidation within the peroxisome, while this step in the mitochondria is coupled to ATP production (Hashimoto, 1995). In addition, acyl-CoA oxidase activity is greatest with longer carbon chain lengths, and in humans is inactive toward fatty acyls with carbon chain lengths of four or less (van Hove, et al., 1993).

Consequently, fatty acyls are only chain shortened, yielding the end products acetyl-CoA and short chain fatty acyls within the peroxisome. Consequently, peroxisomal β-oxidation of palmitate produces approximately 30% less energy when compared to mitochondrial β-oxidation (Reddy and Mannaerts, 1994). It is unknown exactly how many cycles of β-oxidation a fatty acyl chain will undergo in the peroxisome, or what determines the extent to which a fatty acyl chain is shortened. In the mitochondria, activated fatty acids are committed to β-oxidation, the process will continue until the initiating fatty acyl-CoA has been completely combusted to multiple acetyl-CoA units.

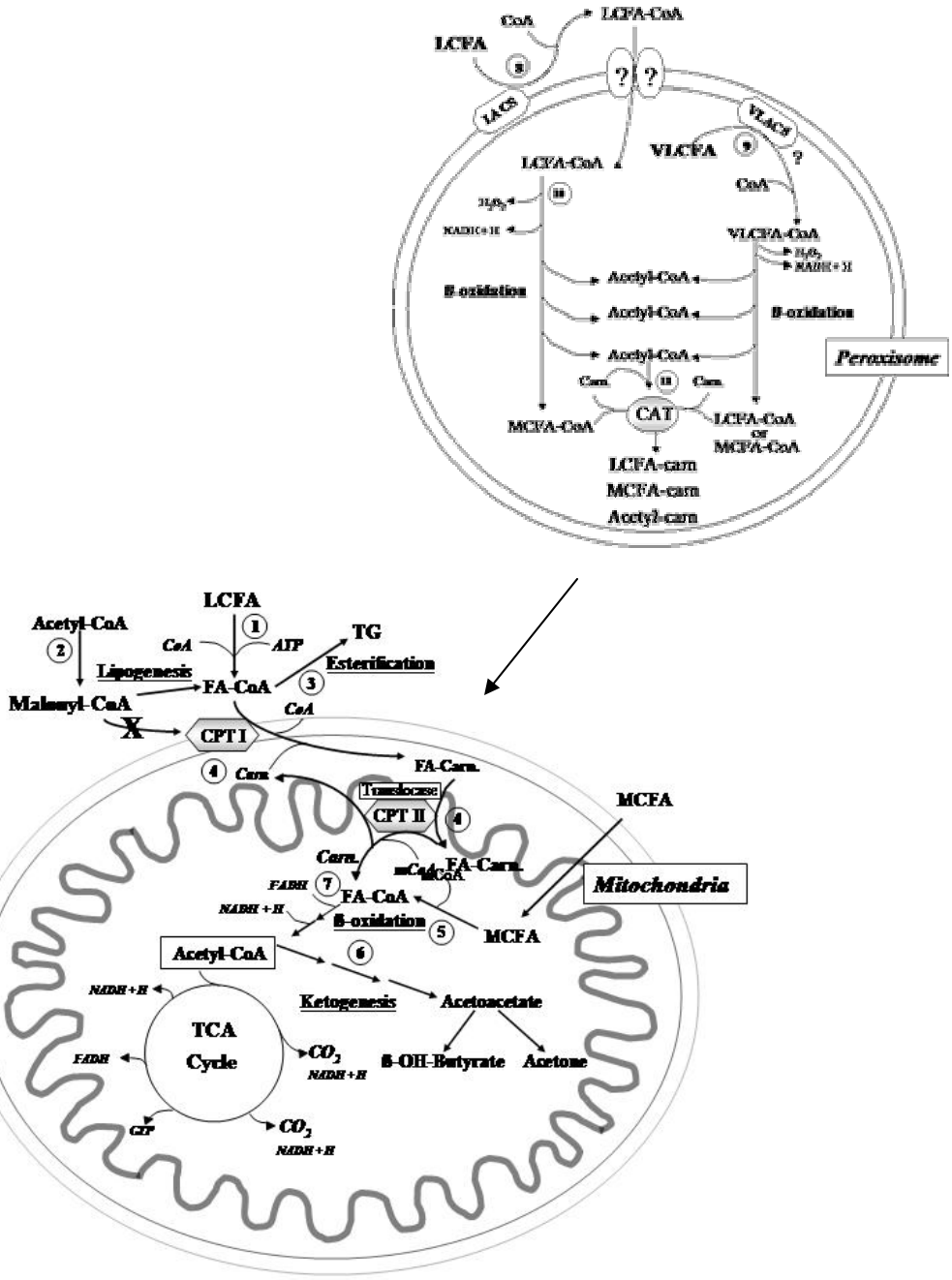


Figure 1.1
Pathways of hepatic lipid metabolism with emphasis on oxidative metabolism. Enzymes/pathways are numbered as follows: 1) long-chain acyl-CoA synthetase, 2) acetyl-CoA carboxylase, 3) various acyl-CoA transferases (ie. monoglycerol-, diglycerol- glycerol phosphate acyl transferase) 4) carnitine shuttle consisting of CPT 1, translocase, and CPT II, 5) medium-chain acyl-CoA synthetase, 6) mitochondrial hydroxymethylglutaryl-CoA synthase, 7) acyl-CoA dehydrogenase, 8) long chain acyl-CoA synthetase, 9) very long chain acyl-CoA synthetase, 10) acyl-CoA oxidase, 11) carnitine octanoyltransferase.

Peroxisome Proliferator Activated Receptors

In the fed state, peroxisomal oxidation represents approximately 9% of total fatty acid oxidation (Grum, et al., 1994). Under conditions of peroxisomal proliferation, this value may rise to 25% in the rat (Veerkamp, et al., 1986). The proliferation of peroxisomes involves the up-regulation of peroxisomal genes (mainly those involved with β-oxidation), an increase in peroxisomal size, and the synthesis of new peroxisomes (Purdue and Lazarow, 2001). In mammals, peroxisomal proliferation is induced by both physiological stimuli (long-chain fatty acids) and a variety of xenobiotics through their direct binding with the peroxisome proliferator activated receptor (PPAR) (Kliewer, et al., 1997; Totland et al., 2000). Although multiple PPARs have been identified (PPAR β, PPARγ_1, and PPARγ_2), only PPARα induces peroxisome proliferation. In mice and rats PPARα is highly expressed in cells which have high fatty acid catabolic rates including the liver, kidney, heart, and skeletal muscle (Braissant, et al., 1996). However, only peroxisomes of the liver and, to a lesser degree, the kidney undergo significant proliferation (Schoonjans, et al., 1996). Proliferation is also species dependent, while the rat is quite responsive to peroxisome proliferation the human remains unresponsive (Vamecq and Draye, 1989). Furthermore, the response in males is greater than that observed in females (Yamada et al., 1991).

In the rat, prolonged activation of PPARα results in hepatomegaly and ultimately hepatic tumor development. The exact cause of tumorogenesis is unknown since peroxisome proliferators are nongenotoxic (Chu et al., 1995). Ligands for the PPARs show little structural similarity, however all can be classified as carboxylic acids, or metabolized to a carboxylic acid derivative (Kliewer, et al., 2001). Several hypolipidemic agents, many belonging to the family of fibrates, are potent activators of PPARα. Although less

efficacious, long chain fatty acids also function as PPARα ligands. Stimulated during fasting (Sterchele et al., 1996) and with increased fatty acid intake (Ouali et al., 2000), PPARα results in increases in acyl-CoA oxidase (Neschen et al., 2001). In addition, peroxisome proliferators increase expression and activity of key enzymes required by the β-oxidative pathway of the mitochondria (Mascaró et al., 1998). Notable enhancement of both mitochondrial and peroxisomal enzymes reveals a complex interplay between the two organelles in regulating fatty acid metabolism.

The Carnitine Shuttle and Regulation of Fatty Acid Oxidation

The activation of a fatty acid to its CoA thioester prevents its translocation to the mitochondrial matrix, therefore the acyl-CoA substrate is not accessible to the enzymes of β-oxidation. The exclusion of the fatty acyl-CoA from the mitochondrial matrix is a consequence of the site of fatty acid activation, and the nature of the mitochondrial membrane. The mitochondrion is composed of two lipid bilayers: an outer membrane and a highly invaginated inner membrane. The outer membrane is imbedded with porins that allow the passage of molecules less than 5000 Da (Hancock et al., 1979). The inner membrane, however, is highly impermeable due to the absence of such channels. The activation of a fatty acid to its CoA thioester prevents its translocation across the inner mitochondrial membrane due to size exclusion as a result of the CoA moiety. In addition, although the porins of the outer membrane create channels large enough for passage of the fatty acyl-CoA, this is most likely not the case. The acyl-CoA associates with an acyl-CoA binding protein, resulting in a complex of 11000 Da. Therefore, its passage across the outer mitochondrial membrane is restricted as well (Abo-Hashema et al., 2001; Zammit, 1999). The impervious

nature of the mitochondrial membrane toward activated fatty acids is overcome by presence of the carnitine acyltransferase system.

The carnitine acyltransferase system first came to light in the early 1960's due to the work conducted by Fritz and Yue (1963). It was concluded that acyl-CoA esters are able to overcome the impermeability of the mitochondrial membrane by the exchange of the CoA moiety for carnitine in a reaction catalyzed by carnitine acyltransferase. The newly formed acylcarnitine permeates the inner mitochondrial membrane where the carnitine is released and the acyl group is re-esterified to CoA residing within the matrix. The re-generated acyl-CoA is thus committed to β-oxidation within the matrix. Two decades after the pioneering work by Fritz and Yue (1963) the currently accepted model of the mitochondrial carnitine shuttle system was established. The shuttle is comprised of three fundamental proteins: 1) carnitine acyltransferase I, 2) carnitine/acylcarnitine translocase, discovered by Pande (1975), and 3) carnitine acyltransferase II (Figure2).

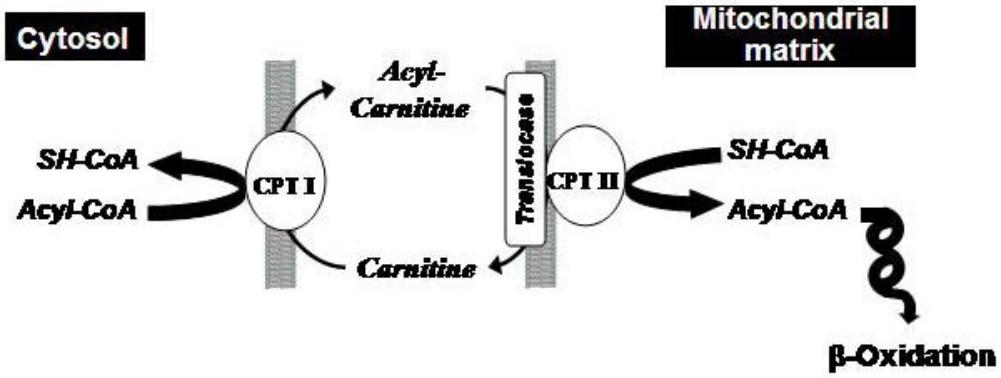


Figure 1.2
The mitochondrial CPT system.

Carnitine acyltransferase I resides as an integral protein in the outer mitochondrial membrane. It initiates the translocation of fatty acids across the outer lipid bi-layer by first exchanging the CoA molecule for cytosolic carnitine. The generated acylcarnitine is subsequently transported across the inter-mitochondrial space driven by the affinity of the carnitine/acylcarnitine translocase (CACT) for cytosolic long chain acylcarnitine. Carnitine/acylcarnitine translocase catalyzes the unidirectional translocation of the acylcarnitine across the inner mitochondrial membrane in exchange for matrix carnitine (Pande and Parvin, 1980). The transfer proceeds by a mole to mole exchange, and it is the greater affinity for long chain acylcarnitines that governs the direction of substrate translocation. Rat liver translocase demonstrates a greater affinity for long chain acylcarnitine than that for either cytosolic carnitine or short chain acylcarnitines, and as a result, long chain acylcarnitine produced in the cytosol is transported into the matrix and translocation in the reverse direction is not favored (Madiraju, et al., 1984). Once the acylcarnitine is transported across the inner mitochondrial membrane, carnitine acyltransferase II, located on the inner membrane and oriented toward the mitochondrial matrix, exchanges carnitine for mitochondrial CoA to regenerate acyl-CoA within the mitochondrial matrix (McGarry and Brown, 1997), thereby completing the catalytic cycle.

The activity of carnitine acyltransferase I is represented by a family of enzymes with optimum activities toward specific fatty acid chain lengths (C2, C8, and C16). In contrast, carnitine acyltransferase II shows optimal activity over a broader range of fatty acids (Feike at al., 2000). The most characterized of the acyltransferases are carnitine palmitoyltransferase I (CPT I) and II (CPT II). Although it was originally hypothesized that CPT I and CPT II were the same protein, it was later shown that the two are distinct proteins

arising from different genes. One of the first distinguishable attributes of CPT I in comparison to CPT II is the loss of activity following treatment of mitochondria with detergents (McGarry et al., 1978). The loss of activity is a result of CPT I losing its catalytic capacity when removed from its natural environment (de Vries et al., 1997); however, CPT II remains catalytically active despite membrane disruption. Furthermore, CPT I and CPT II are distinctly regulated by the inhibitory substrate malonyl-CoA. Malonyl-CoA, the first committed intermediate in lipogenesis, is generated from acetyl-CoA in a reaction catalyzed by acetyl-CoA carboxylase (Kim, 1997). Only CPT I is inhibited by malonyl-CoA. In tissues that possess both pathways of fatty acid synthesis and degradation, inhibition of CPT I prevents the futile cycling of fatty acids that would occur if they were committed to both pathways concurrently. Due to the requirement for fatty acid oxidation, and its regulation by malonyl-CoA, CPT I is considered rate limiting for the oxidation of fatty acids. The notion of a single rate limiting step within a pathway is often questioned, and control is believed to be shared between several steps (Fell and Thomas, 1995). According to new mathematical approaches designed to determine rate limiting steps within a pathway, CPT I is found to exert significant control over β-oxidation (New at al., 1999; Krauss et al., 1996).

Topology of CPT I Determines Malonyl-CoA Sensitivity

Carnitine palmitoyltransferase I, originally thought to reside on the inner mitochondrial membrane, was later assigned to the outer mitochondrial membrane (McGarry and Brown, 1997). The protein is targeted to the mitochondria by an amino-terminus leader sequence. The enzyme retains its amino terminus upon translocation to the mitochondria resulting in a mature protein of 773 amino acids (88 kDa) in the rat (McGarry and Brown,

1997). The topology of CPT I in the outer mitochondrial membrane is essential to its activity in situ, and its elucidation has proven invaluable to the study of the mature enzyme. Prior to cloning and sequencing of rat CPT, it was suggested that the active amino-terminus (palmitoyl-CoA binding site) and regulatory carboxyl-terminus (malonyl-CoA binding site) regions were spatially separated with the regulatory domain exposed to the cytosol, and the catalytic domain confined to the inter-membrane space, however it was determined that both the active and regulatory domains reside in the cytosol (Fraser et al., 1997) and the protein contains two highly conserved hydrophobic domains (residues 48-75 and 103-122) (Fraser et al., 1997).

Following the acceptance of CPT I topology research ensued to determine the effect of the amino-terminus on malonyl-CoA inhibition. Carnitine palmitoyltransferase I constructs of various amino-terminus deletions revealed that the extreme amino-terminus (residues 1-83) was not essential to maintaining a catalytically active protein, however, deletion of residues 1-83 increased concentration of malonyl-CoA required for 50% inhibition (IC_{50}) from 2.0 uM to 170-380 uM, when constructs were expressed in Pichia pastoris, a strain of yeast with no endogenous CPT I activity (Shi et al., 1998). The increase in malonyl-CoA required for inhibition was a result of both loss in sensitivity and decreased malonyl-CoA binding.

The effect of deletion mutants on the regulatory influence of malonyl-CoA led to the identification of specific residues involved in malonyl-CoA binding and inhibition (Shi et al., 1999). A point mutation of the third residue, replacing glutamate with alanine, resulted in complete loss of sensitivity to malonyl-CoA, coinciding with a loss of malonyl-CoA binding. While the third glutamate residue appears to act as a negative determinant of malonyl-CoA

sensitivity, there is evidence that positive determinants of sensitivity are also present in the amino-terminus. The positive determinants reside between residues 19-30. Deletion of these residues results in considerably lower IC_{50} values for malonyl-CoA (1.1 uM versus 38 uM for control) (Jackson et al., 2000).

The findings that deletions within the amino-terminus negatively impacted malonyl-CoA binding without subsequent negative effects on CPT I activity provided further evidence that malonyl-CoA and palmitoyl-CoA binding occurred at separate locations on the enzyme. The original inference that malonyl-CoA and palmitoyl-CoA interacted with CPT I at the same site implied competitive inhibition. However, identification of a high affinity malonyl-CoA binding site in the regulatory domain of the protein distinct from the palmitoyl-CoA binding site that is assigned to the catalytic domain, is indicative of true allosteric inhibition. The idea of competitive inhibition has not been abandoned. There is evidence of an additional low affinity malonyl-CoA binding site that partially overlaps the active binding site of palmitoyl-CoA (Shi et al., 1999; Cook et al., 1993). The existence of a second low affinity binding site in the active domain infers that malonyl-CoA behaves as a both competitive and allosteric inhibitor of CPT I.

The highly invaginated nature of the inner mitochondrial membrane, creates areas where the two membranes come within 4 nm of each other. These regions are identified as contact sites and represent 5 to 10% of the outer membrane surface area. It is estimated that up to 40% of both the CPT I and CPT II proteins are confined to contact sites (Fraser and Zammit, 1998). A comparison between CPT I within contact sites and the enzyme confined to the outer regions of the membrane reveal differences in substrate binding and malonyl-CoA inhibition. Within the contact sites, the affinity for palmitoyl-CoA is 2.4-fold greater,

however, the greater affinity for palmitoyl-CoA is greatly diminished by the presence of malonyl-CoA. In contrast, malonyl-CoA has no apparent effect on the Km for palmitoyl-CoA in the outer regions of the membrane. Conversely, malonyl-CoA reduces the maximal velocity of CPT I by 70% in regions on the outer membrane, while having little effect on the rate of enzyme activity confined to the contact sites (Fraser et al., 2001). The increased Km for palmitoyl-CoA within contact sites following exposure to malonyl-CoA is indicative of competitive inhibition, whereas the decrease in Vmax observed in CPT I confined to the outer regions of the membrane is indicative of non-competitive inhibition.

Discovery of Distinct CPT I Isotypes

Over the past three decades research to resolve the structure and function of CPT I has revealed a complex protein, whose complexity is even further enhanced by the discovery that CPT I exists in at least two isotypes. Designated as either L-CPT I (liver isotype) or M-CPT I (muscle isotype), based upon the tissues from which they were first isolated, the isotypes have been identified in a variety of tissues. The liver isotype (also referred to as CPTIα) is expressed in liver, kidney, lung, spleen, intestine, pancreas, and ovary. Muscle isotype (also referred to as CPTIβ) expression occurs predominantly in skeletal muscle, brown and white adipose, and testis (McGarry and Brown, 1997). The heart expresses the liver isotype at birth, and undergoes isotype switching during development to express primarily the muscle isotype in adult hood (Brown et al., 1995). The analysis of CPT II in a variety of rat tissues indicates the same protein is expressed in all tissues (Woeltje et al., 1990).

Both the liver and muscle isotypes are approximately 88 kDa, but diverge in their catalytic and regulatory properties. The two isotypes are kinetically distinct in respect to their Km for carnitine and their inhibition by malonyl-CoA. In the rat, the Km for carnitine of the liver isotype is ≈ 30 uM, while in skeletal muscle ≈ 500 uM of carnitine is required for the enzyme to operate at half its maximal velocity. The concentration of malonyl-CoA required to inhibit CPT I in liver compared to skeletal muscle tissues is reciprocal to the concentration of carnitine required to stimulate enzyme activity, with less malonyl-CoA required for skeletal muscle enzyme inhibition. For the rat, the IC_{50} of malonyl-CoA in the liver is 2.7 uM, while in skeletal muscle it is a mere 34 nM (McGarry et al., 1983). As stated previously, malonyl-CoA is a precursor for fatty acid synthesis and is associated with lipogenic tissues. However, in non-lipogenic tissues, such as skeletal muscle, malonyl-CoA has been measured, although reported concentrations are less than those measured in liver, a lipogenic tissue in the rat. The concentration of malonyl-CoA required to inhibit the isotypes (2.7 uM for LCPT I and 34 nM for MCPT I) is in accordance with the levels of tissue malonyl-CoA observed between lipogenic and non-lipogenic tissues. What remains puzzling, however, is that malonyl-CoA concentrations measured in skeletal muscle are significantly greater than the IC_{50} of malonyl-CoA, indicating skeletal muscle CPT I should be inhibited at all times. It is speculated that not all malonyl-CoA measured in the tissue is accessible to CPT I, possibly as a result of protein binding or mitochondrial compartmentalization (Ruderman et al., 1999).

The ability of the two isotypes to respond differently with respect to inhibiting malonyl-CoA concentrations resides within the proteins structure. In liver, malonyl-CoA inhibition of CPT I is dictated by the presence of both positive and negative determinants of

malonyl-CoA sensitivity, the presence of negative determinants has not been established for the skeletal muscle isotype. Deletion of residues 19-30 which increase malonyl-CoA sensitivity in the liver isotype, has no effect on the already high sensitivity to the inhibitor in the muscle isotype (Jackson et al., 2000). Although the motifs responsible for malonyl-CoA inhibition in the liver do not operate the same in muscle, they are not quiescent. These residues influence the affinity of the muscle isotype for carnitine (Jackson et al., 2000). The actions of these regions on malonyl-CoA inhibition in liver and carnitine affinity in muscle may account for the reciprocal relationship for the affinities of malonyl-CoA and carnitine within the two tissues.

It is important to note that the amino-terminus alone does not dictate the overall nature of the isotype. Generation of a chimeric protein with the amino-terminus of liver and carboxy-terminus of skeletal muscle, or the amino-terminus of skeletal muscle and carboxy-terminus of liver did not exhibit sensitivity to malonyl-CoA based on the origin of the amino-terminus. The chimera with the liver amino-terminus exhibited sensitivity toward malonyl-CoA comparable to the native skeletal muscle CPT I isotype, the reverse held true with the chimera constructed from the muscle amino-terminus (Swanson et al., 1998). These findings provided evidence of the importance of the interaction between the amino and carboxy terminus for determining CPT I's overall sensitivity to malonyl-CoA.

CPT I Activity and Sensitivity to Inhibition in Response to Physiological Stimuli

The inhibition of hepatic CPT I by malonyl-CoA is responsive to physiological changes. For instance, in the rat CPT I is less sensitive to inhibition by malonyl-CoA under various physiological changes which elevate circulating fatty acids such as the suckling

period (Krauss and Quant, 1996), induction of diabetes (Cook et al., 1984; Bremer, 1981), fasting (Cook et al., 1980), and high-fat diets (Pégorier, et al., 1988; Power and Newsholme, 1997). This decrease in sensitivity is paralleled by a decline in tissue content of malonyl-CoA (Chien et al., 2000; McGarry et al., 1983). In skeletal muscle, high-fat diets have no effect on the sensitivity of CPT I to inhibition by malonyl-CoA during the initial four weeks of consumption. After 10 weeks of consuming a high-fat diet, CPT I becomes less sensitive to inhibition, an effect observed only with unsaturated fatty acids and not saturated fatty acids (Power and Newsholme, 1997). Membrane fluidity impacts CPT I inhibition, with increased fluidity decreasing sensitivity (Zammit et al., 1998), and may account for the different effects of unsaturated versus saturated fatty acids on CPT I inhibition in skeletal muscle.

In addition, fasting results in an acute up-regulation of hepatic CPT I mRNA (Kersten et al., 1999), and increased activity (Bremer, 1981). Similar results have been observed in the diabetic state (Park et al., 1995) and with hyperthyroidism (Mynatt et al., 1994). Physiological perturbations which affect hepatic CPT I are also important modifiers of the muscle isotype. Starvation (McGarry et al., 1983), exercise (Berthin et al., 1998), and diets high in unsaturated fatty acids (Power and Newsholme, 1997) increase CPT I activity concomitant with decreased skeletal muscle malonyl-CoA content (Dean et al., 2000; Rasmussen et al, 1997). In contrast, hyperglycemia/hyperinsulinemia increase malonyl-CoA content and decrease the oxidation of oleate compared to octanoate (Rasmussen et al., 2002). The comparative oxidation of octanoate versus oleate is used as an indirect measure of CPT I inhibition. The oxidation of octanoate, a medium chain fatty acid, is not dependent on the carnitine shuttle system for entrance into the mitochondria. The reason behind this

phenomenon is that medium chain acyl-CoA synthetases are confined to the mitochondria (Fujino et al., 2001), therefore medium chain fatty acids can diffuse across the mitochondrial membrane prior to CoA esterification, thereby by-passing the carnitine shuttle.

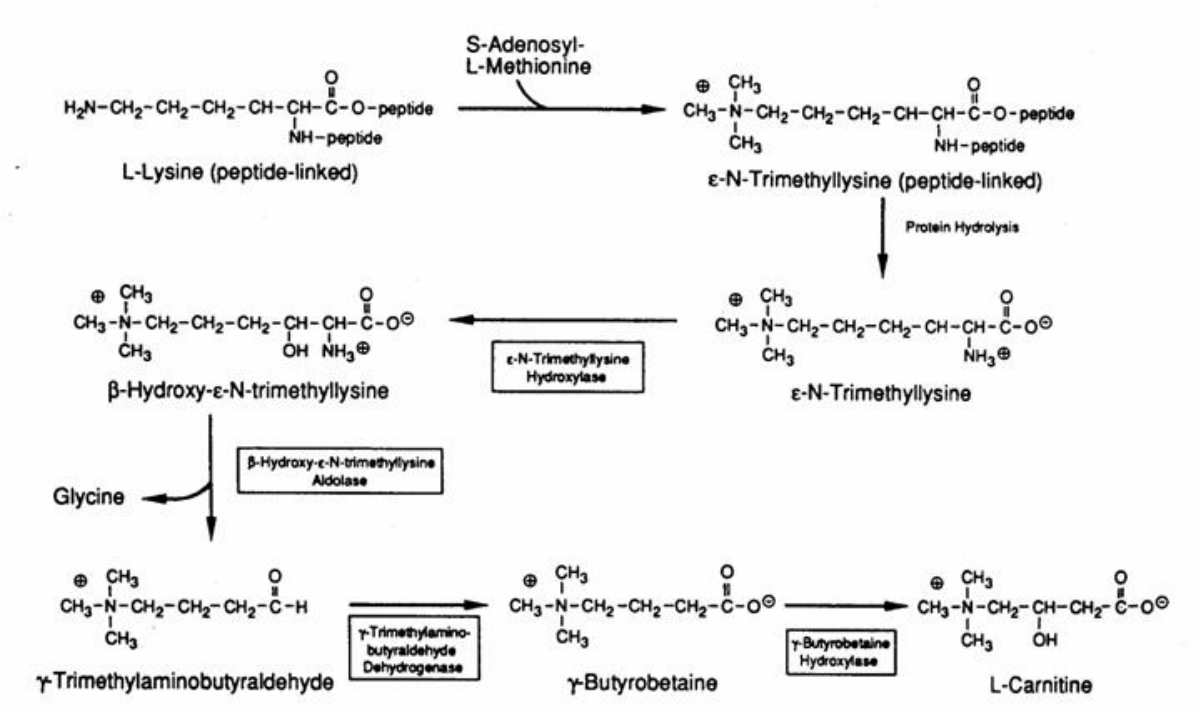
Another salient feature of the CPT I protein is the developmental regulation that has been characterized in the suckling rat. In fetal rats, hepatic CPT I activity and mRNA expression are low. Within 24 hr after birth, there is a 5-fold increase in CPT I activity and expression (Thumelin et al., 1994). Maximal hepatic CPT I mRNA levels are observed at 3 days of age and remain elevated until weaning (Asins et al., 1995). After weaning CPT I activity and expression decrease precipitously and values measured mimic those observed in the adult. Carnitine palmitoyltransferase I remains refractory to the weaning induced decline in activity when rats are maintained on a high fat diet after the suckling period. In contrast to the changes noted for the CPT I protein, CPT II levels are high in the fetal rat and do not change during development (The dramatic changes in CPT activity coincide with the increase in glucagon and decrease in insulin that occurs at the onset of the suckling period. While the proximal promoter of CPT I contains CREB-binding sites, these sites are absent in CPT II (reviewed in Louet et al., 2001). The CREB-binding site, which is responsive to cAMP, could account for the differences in developmental regulation of CPT I and II.

Carnitine: An Essential Co-factor for the Carnitine Shuttle

In the majority of documented human cases, CPT I deficiency is highly correlated with elevated plasma carnitine (reviewed in Bonnefonet at al., 1999). There is an absolute requirement of carnitine for the function of the CPT system, which renders carnitine a physiological necessity in fuel homeostasis. In mammals, adequate tissue carnitine status is

acquired through dietary carnitine and de novo synthesis of carnitine. Dietary carnitine is obtained primarily through the consumption of meat and dairy products. The majority of cereals, fruits, and vegetables serve as inadequate sources as they contain little or no carnitine (Mitchell, 1978). For the neonate, colostrum and milk are the major sources of carnitine in the diet (Rebouche, 1992).

De novo synthesis of carnitine requires two essential amino acids and several co-factors (reviewed by Rebouche, 1991; Figure 3). The carbon backbone and amino group of carnitine are derived from lysine. The three methyl groups are donated from methionine via *S*-adenosylmethionine. In mammals, the post-translational methylation of lysine only occurs for protein bound lysine (Rebouche, 1982). Following protein hydrolysis, ε-N-trimethyllysine is hydroxylated by the enzyme ε-N-trimethyllysine hydroxylase resulting in the formation of β-hydroxy-ε-N-trimethyllysine. β-hydroxy-ε-N-trimethyllysine is subsequently cleaved to glycine and γ-trimethylaminobutyraldehyde, a reaction catalyzed by β-hydroxy-ε-N-trimethyllysine aldolase (also known as serine hydroxymethyltransferase). The formation of γ-butyrobetaine follows with the oxidation of γ-trimethylaminobutyraldehyde catalyzed by γ-trimethylaminobutyraldehyde dehydrogenase. The final reaction is the hydroxylation of γ-butyrobetaine catalyzed by γ-butyrobetaine hydroxylase (Figure 3). The two hydroxylation reactions are catalyzed by α-ketoglutarate dependent dioxygenases. The decarboxylation of α-ketoglutarate to succinate is coupled to the hydroxylation of the substrate. The enzymes require ascorbic acid as a reducing agent and iron in its reduced state (Lindstedt and Lindstedt, 1970).



Rebouche, 1991

Figure 1.3.
Pathway of carnitine biosynthesis in mammals (Rebouche, 1991).

The first four enzymes in the pathway are ubiquitous in rat and human tissues, however, γ-butyrobetaine hydroxylase activity is confined to the liver and/or kidney of all species studied to date (reviewed by Rebouche, 1982). In the rat and guinea pig, γ-butyrobetaine hydroxylase activity was located in the liver, however, none was detected in kidney of either species, or rat muscle. In humans, rabbit, and cat, significant activity has been detected in the liver and kidney (Englard et al., 1978; Englard and Carnicero, 1978). In cat and humans the activity present in the kidney exceeds that measured in the liver. Work by Galland and co-workers (1999) validated the tissue distribution of γ-butyrobetaine hydroxylase mRNA in the rat. Northern blot analysis of γ-butyrobetaine hydroxylase mRNA expression of various tissues concluded that expression was confined primarily to the liver. Earlier research in humans noted significant mRNA expression in both the kidney and liver,

with kidney expression being greater than liver, congruent with the earlier reports on enzyme activity (Vaz et al., 1998).

While the expression of γ-butyrobetaine hydroxylase is specific to the liver and kidney, a variety of tissues are capable of converting ε-N-trimethyllysine to γ-butyrobetaine. However, the kidney is the only organ studied that shows an appreciable rate of absorption for circulating ε-N-trimethyllysine (Henderson et. al., 1982; Zaspel, 1980). Due to transport across many tissues being low, and the ubiquitous expression of the intermediate enzymes in the pathway, conversion of ε-N-trimethyllysine ultimately to γ-butyrobetaine most likely occurs in the tissue which ε-N-trimethyllysine originates. Per milligram of protein, skeletal muscle contains the highest amount of ε-N-trimethyllysine, followed by heart, kidney, and liver (Davis and Hoppel, 1981). Consequentially, muscle may be the greatest supplier of γ-butyrobetaine for carnitine synthesis. The γ-butyrobetaine formed in skeletal muscle must be exported to liver (or kidney) for hydroxylation to form carnitine, after which, carnitine must be transported back to skeletal muscle.

In rats, dietary ε-N-trimethyllysine increases the rate of carnitine biosynthesis (Rebouche et al., 1986), however, the same effect is not as pronounced in humans (Rebouche et al., 1989). Although ε-N-trimethyllysine is efficiently absorbed across the intestine, the low absorption of circulating ε-N-trimethyllysine by many tissues may exclude exogenous sources from entering the carnitine biosynthetic pathway. Dietary lysine also has been examined as an exogenous substrate for stimulating carnitine synthesis. Because significant methylation of the amino group occurs only with protein bound lysine, circulating lysine is not a direct precursor for carnitine synthesis. While adequate lysine intake is important,

lysine deficiency in rats does not coincide with symptoms of carnitine deficiency (Rebouche, 1992).

γ-Butyrobetaine Hydroxylase: Age and Tissue Specific Expression

Despite the requirement of carnitine for fatty acid metabolism, it is not classified as an essential dietary component as de novo synthesis in the adult is adequate in supplying carnitine needed for metabolic homeostasis. Indeed, adult vegetarians, who receive little dietary carnitine, do not display symptoms of carnitine deficiency (Feller and Rudman, 1988). The capacity for de novo carnitine synthesis is both tissue and age specific. The intermediate γ-butyrobetaine is synthesized at all ages of development, in all animals studied (Borum, 1983). However, hepatic carnitine synthesis does not occur in the rat until 8 days of life (Hahn, 1981). Furthermore, expression of γ-butyrobetaine hydroxylase mRNA remained undetectable by Northern blot analysis in rat liver until after weaning, at which time detected levels remained lower than reported adult values (Galland et al., 1999). Similar results were observed for human γ-butyrobetaine hydroxylase. Hepatic activity in infants was 12% of the adult mean. This value rose to 30% by 2.5 years of age, and did not reach the adult mean until 15 years of age (Rebouche and Engel, 1980). However, in humans kidney γ-butyrobetaine hydroxylase is not developmentally regulated (Olson and Rebouche, 1987).

Importance of Adequate Tissue Carnitine Concentrations

In the neonate, research strongly suggests dietary L-carnitine serves as the main source of tissue carnitine (Florez et al., 1996; Coffey et al., 1987). In rats, up to 50% of tissue carnitine is derived from milk carnitine (Davis, 1989). A comparison of fetal and

neonatal rats indicates that carnitine levels in plasma, heart, liver, and skeletal muscle increase during the first day of life (Borum, 1983; Robles-Valdez et al., 1976). The accumulation of maternal carnitine in the neonate is postulated to result from the movement of carnitine from the mothers' liver → milk → neonatal liver (Robles-Valdez et al., 1976). Even in the human infant, where kidney γ-butyrobetaine hydroxylase activity is not age dependent, plasma carnitine levels are low when soy-based infant formulas devoid of carnitine are fed (reviewed by Hahn and Novak, 1985).

Plasma carnitine levels are used as an indice of carnitine status, however they are not a reliable indicator of tissue carnitine. In the past decade a family of organic cation transporters has been described. One in particular (OCTN2) has been identified in a variety of tissues including kidney, heart, skeletal muscle, and liver; has a high affinity for carnitine; and mutation of the protein results in systemic carnitine deficiency. Both free carnitine and short chain acylcarnitine are transported across tissues by this sodium dependent active transporter (reviewed by Lahjouji et al., 2001; Bererdi et al., 2000).

The concentration of free carnitine within tissues may serve as a predictor of the capacity of CPT I for β-oxidation when circulating fatty acids are elevated. The apparent Km for carnitine approximates the carnitine concentration necessary for CPT I to catalyze the conversion of fatty acyl-CoA to acylcarnitine at a rate which is half of its maximal velocity. For many key enzymes, when the substrate concentration is in the vicinity of the Km, the rate at which the reaction proceeds is proportional to the substrate concentration. Prevailing tissue carnitine concentrations at or below CPT I's apparent Km for carnitine could limit flux of acyl groups into the mitochondria for β-oxidation. In the dog, hepatic carnitine concentrations are 50% greater than the apparent carnitine Km of CPT during the initial 24

hours after birth. After 24 hours, carnitine concentrations begin to decline and are below the apparent carnitine Km by 3 weeks of age, suggesting that the rate of CPT may be limited by tissue carnitine levels at this age (Lin and Odle, 2003).

Another central function of carnitine is maintenance of the acetyl-CoA/CoA pool (Rebouche and Seim, 1998). Carnitine acetyltransferase (CAT) located within the mitochondrial matrix catalyzes the conversion of acetyl-CoA to acetylcarnitine, thereby liberating free CoA. The Km of CAT for acetyl-CoA is responsive to changes in substrate concentration, such that increasing amounts of acetyl-CoA are used for acetylcarnitine synthesis through mass action effect (Zammit et al., 1999; Beiber et al., 1982). In liver, acetylcarnitine concentrations are increased following high rates of fatty acid oxidation (Hancock et al., 1979). The conversion of acetyl-CoA to acetylcarnitine is also important in regulating the metabolic branchpoint through which glucose and fatty acid oxidation are reciprocally regulated. In the perfused rat heart L-carnitine is considered essential for enhancing glucose oxidation and decreasing fatty acid oxidation by decreasing the acetyl-CoA/CoA ratio (Zammit et al., 1999).

LIPID METABOLISM IN THE NEONATAL PIG: REDEFINING CURRENT VIEWS

Fatty Acid Oxidation in the Neonatal Pig

Like other mammalian neonates the pig must rapidly adapt from a carbohydrate diet in utero to a high fat diet upon suckling. These adaptations for many neonates include an up-regulation of the machinery of gluconeogenesis, fatty acid oxidation, and ketogenesis, all of which serve to ensure an adequate energy supply for hepatic and extra-hepatic tissues.

Within the initial 24 hr after birth, liver glycogen in both fed and starved piglets decreases precipitously, and unless suckling ensues will become depleted within 48 hr postnatal (Pégorier et al., 1981). This renders the neonatal pig highly susceptible to hypoglycemia.

The susceptibility of the neonatal pig to hypoglycemia necessitates prompt ingestion of colostrum and milk. As suckling is initiated there is an increase in circulating fatty acids, which must be met with increased oxidative capacity of the cell to ensure an adequate supply of energy to tissues for growth and development. Between birth and 2 days of age there is an increase in oxidative capacity of isolated hepatocytes. This increase in oxidative rate is concomitant with an increase in mitochondrial number which occurs 6 to 12 hr postnatal (Mersmann et al., 1972). As mitochondria are the primary site for oxidation in most neonates, early proliferation is important for increased rates of fatty acid oxidation. The rate of oxidation of palmitate increases approximately fourfold between birth and 7d of age in liver homogenates (Mersmann and Phinney, 1973), however, oxidation rates do not differ between 1 and 7 days of age (Miller et al., 1971). It can be inferred that the increase in fatty acid oxidation occurs prior to 24 hr of age and is a primary consequence of mitochondrial proliferation.

Despite the increase in oxidation rates during the initial 24 hour of life, hepatic fatty acid oxidation in the neonatal pig is considered low in comparison to other mammals. Oxidation of palmitate (represented by summation of CO_2 and acid-soluble products or ASP) in fasted neonatal pigs compared to fasted adult rats indicates considerable less flux of carbon through these pathways for the pig (Adams et al., 1997; Duée et al., 1994). Increased oxygen consumption rates, indicative of increased basal metabolic rate, for the adult rat could partially account for decreased β-oxidation by neonatal pig liver homogenates (Adams et al.,

1997). Thus, although total β-oxidative capacity of the neonatal pig liver is low in comparison to the rat, it may be sufficient in meeting cellular energy needs due to lower tissue metabolic rate (Odle et al., 1991). However, in many mammalian neonates a significant portion of metabolites derived from β-oxidation of fatty acids are found as ketone bodies (Duée et al., 1985; Ferré et al., 1983; Girard et al., 1981; Robles-Valdes et al., 1976). This is not the case in neonatal pigs.

Lack of Ketogenesis During the Suckling Phase

While plasma ketone body concentrations (acetoacetate and β-hydroxybutyrate) may exceed 2mM in the rat (Robles-Valdes et al., 1976), the concentration of these metabolites in the plasma of pigs is less than 0.25 mM in fed and fasted newborns (Pégorier et al., 1981; Bengtsson et al., 1969). Furthermore, ketone bodies represent 20-40% of acid soluble products formed in the oxidation of palmitate for the rat, but only 3-7% for the young pig (Adams et al., 1997). At physiological concentrations β-hydroxybutyrate contributes to less than 5% of the piglets' energy requirement (Tetrick et al., 1995). The attenuation in circulating ketones is attributed to decreased synthesis and not increased utilization. Ketone bodies represent a major fuel source for extra-hepatic tissues when glucose is limited. Because of the lack of ketogenesis in the pig, it has been proposed that carbon sources other than those derived from ketone bodies may be more important to energy homeostasis in the pig. In isolated hepatocytes, up to 40% of the carbon recovered in acid soluble products (ASP) following the oxidation of palmitate was in free acetate (Odle et al., 1995), however, subsequent research indicated that acetate turnover was independent of fatty acid oxidation (Adams and Odle, 1998).

In other mammalian neonates the initiation of ketogenesis is concomitant with a fall in the insulin:glucagon ratio and an increase in circulating fatty acids (McGarry and Foster, 1980). Although the pig displays these physiological changes within 48 hr postpartum, ketone body production is unaffected (Pégorier et al., 1981; Bengtsson et al., 1969). Using isolated primary hepatocytes, Pegorier and co-workers (1983) observed cellular uptake of oleate by the neonatal pig comparable to the fed and starved adult rat. In addition, 98-99 % of the initial radioactivity of oleate was recovered, with the greatest activity (80–95%) in esterified lipids. The incorporation of oleate into esterified fats occurred regardless of nutritional status (fed versus fasted) or age (newborn versus 15 d).

Does CPT I play a Role in the Reduced Oxidative Capacity of the Neonatal Pig

The shuttling of dietary fatty acids toward esterification and away from oxidation has led to the implication of CPT I in the low oxidative capacity of the newborn pig liver. It has long been established that the activity of CPT develops rapidly in the neonatal pig. In fed and fasted pigs, there is a precipitous increase in hepatic CPT activity between birth and 24 h, with activity in fed animals developing faster than their fasted counterparts, indicating that the initiation of suckling is important for maximal CPT activity following birth (Beiber, 1973).

In skeletal muscle, CPT I activity is increased at 5 days of age compared to birth, an effect that is dependent on the specific mitochondrial population and muscle type. A comparison of subsarcolemmal versus intermyofibrillar mitochondria revealed a 91 and 62% increase, respectively, of CPT I activity from birth to 5 days of age. Furthermore, the effect in subsarcolemmal mitochondria was also impacted by muscle type, where CPT I activity

increased 7 fold for longissimus dorsi muscle and 3 fold for rhomboideus muscle during the first 5 days of life. While the change in CPT I activity was more pronounced in longissimus dorsi (fast glycolytic muscle), the rhomboideus (slow oxidative muscle) had greater overall CPT I activity (Schmidt and Herpin, 1998).

The inhibition of CPT I by malonyl-CoA is a ubiquitous feature in that all membrane bound CPT studied to date is subject to inhibition. However, in the pig a unique pattern of inhibition for the liver and muscle isotypes has recently come to light, revealing distinct species differences in sensitivity of CPT I activity to malonyl-CoA inhibition . The IC_{50} for malonyl-CoA of hepatic CPT I is significantly less in the pig than that of liver isotype in many mammals, while skeletal muscle CPT I appears to be more resistant to inhibition in the pig compared to other species. In fact, the IC_{50} for inhibition of CPT I by malonyl-CoA is reported to be greater in pig muscle (5.48–6.34 uM) than in pig liver (0.103 uM) (Schmidt and Herpin, 1998). Furthermore, this high degree of sensitivity for hepatic CPT I is observed in both fed and fasted neonatal pigs (Schmidt and Herpin, 1998; Duée et al., 1994), while skeletal muscle CPT I inhibition by malonyl-CoA is attenuated following birth. In the rat, inhibition of skeletal muscle CPT I is less responsive to physiological factors, while hepatic CPT I sensitivity to malonyl-CoA is enhanced or diminished abruptly in response to the changing milieu. The increase in IC_{50} that occurs between birth and 5 days of age is paralleled by a decrease in tissue malonyl-CoA content (Schmidt and Herpin, 1998).

The finding that hepatic CPT I was 20 times more sensitive to inhibition by malonyl-CoA ultimately led to the characterization of the enzyme. Nicot and co-workers (2001) were the first to suggest that pig CPT I behaves as a natural chimera of rat liver and skeletal muscle isotypes. Pig hepatic CPTI exhibits saturation kinetics similar to the rat for

carnitine (126.34 uM) and palmitoyl-CoA (35.44 uM). However, the IC_{50} for malonyl-CoA was 141 nM, comparable to human skeletal muscle CPT I. Although the liver is not a lipogenic organ for the pig (Mersmann et al., 1973), which in part could account for a higher sensitivity to malonyl-CoA, it is unknown what the direct consequences of this increased sensitivity are toward fuel homeostasis in the neonatal pig. Indeed, the use of TDGA, an irreversible inhibitor of CPT I, reduces the flux of carbon from palmitate through the oxidative pathway and reciprocally increases its rate of esterification. The addition of carnitine redirected carbon flux from palmitate through the oxidative pathway concomitant with a reduction in the rate of esterification (Odle et al., 1995).

Carnitine Status

At birth, blood and liver contain low levels of carnitine, whereas, heart and skeletal muscle contain much greater amounts of total carnitine, the majority of which is non-esterified. Within 2 days of life, levels of carnitine increase in heart, skeletal muscle, and liver. The liver reflects the greatest change with an approximately 3 fold increase (Kerner et al., 1984). Carnitine levels within liver and skeletal muscle reach a plateau by 4 days of age, however, in the heart levels continue to increase (Borum, 1991). In the rat switching from the liver isotype to the skeletal muscle isotype that occurs in the heart dictates the need for higher carnitine levels. This occurs as the contribution of the liver isotype decreases and the skeletal muscle isotype reciprocally increases with age (Brown et al., 1995).

Sow colostrum and milk contains considerable amounts of carnitine, however, it is unknown whether this serves as the primary source of carnitine for the neonatal pig as the majority of milk carnitine is found as short chain acylcarnitines, while piglet tissues are high

in free carnitine. Research has not yet determined when γ-butyrobetaine hydroxylase levels reach adult levels in the pig, or whether the activity of the enzyme is confined to the liver and/or kidney, or is age dependent. Therefore, it is unknown when de novo synthesis of carnitine begins to sufficiently meet the carnitine needs of the pig and if this could contribute to the different carnitine profiles observed between sow milk and neonatal piglet tissues. It has been hypothesized that when pigs are transitioned from a mixed diet containing animal products at 7-8 wk of age, to a primarily plant based diet, de novo synthesis is not adequate to compensate for the decrease in dietary carnitine (Heo et al., 2000). Research to examine the role of supplemental L-carnitine in weanling pigs allowed ad-libitum access to diets has been variable (Rincker et al., 2003; Owen et al., 1996; Hoffman et al., 1993) making it difficult to discern when de novo synthesis compensates for decreased dietary intake.

Peroxisomal Oxidation in the Neonatal Pig

Research indicates peroxisomal β-oxidation in the young piglet represents a greater percentage of total β-oxidation independent of the administration of peroxisome proliferators, contributing 40–50% of total β-oxidation (Yu et al., 1997a,b). This increased contribution of peroxisomal oxidation to total oxidation is observed not only in hepatic tissue, but also skeletal muscle. Depending on muscle type, rhomboideus or longissimus lumborm, peroxisomal oxidation represents 42 to 32% of total oxidation, respectively. Furthermore, peroxisomal oxidation as a percent of total oxidation remains constant between birth and 5 days of age, consequently, peroxisomal oxidation increases by 170% during this time as total oxidation also increases with age (Herpin et al., 2003). The induction of peroxisomal oxidation occurs immediately post-partum, is greater in the suckled versus fasted piglet (Yu

et al., 1997b,c), and is reliant on the initiation of suckling (Yu et al., 1997c). The greater contribution of peroxisomal β-oxidation to total oxidation in this species may provide an ancillary route in which milk lipids can be metabolized, bypassing the control over oxidation exerted by the mitochondrial pathways. Furthermore, expression of hepatic CPT I is regulated through the use of peroxisome proliferators. Yu and co-workers (2001) were able to show a greater than 2 fold increase in total CPT activity when pigs were fed clofibrate for 2 wks, and a 3 fold increase in peroxisomal β-oxidation of the liver.

CONCLUSION

For over 30 years scientists have attempted to ascertain the answers to why the neonatal pig does not follow the conventional dogma of lipid catabolism displayed by other mammalian neonates. In review of the research conducted to date, the pig continues to remain an enigma when attempting to delineate the mechanisms it employs to maintain energy homeostasis during the initial days of postnatal development. What is certain is that the initiation of suckling is extremely important to the survival of the neonatal pig as it is born with limited fat stores, and liver glycogen is depleted within 48 hr. Furthermore, the lack of ketogenesis limits the supply of glucose sparing carbon for extra-hepatic tissues. Recent research in the neonatal pig indicates that peroxisomal oxidation provides an alternate route for the oxidation of dietary lipids, and may play a substantial role in the pigs' ability to adapt to changing fuel sources at birth. This is further highlighted by the fact that peroxisome proliferators increase mitochondrial CPT I activity within the liver. As CPT I exerts significant control over the oxidation of fatty acids it remains a target of study in elucidating the mechanisms of fatty acid oxidation in the pig.

Rapid induction of CPT I activity in the newborn pig indicates that essential metabolic adaptations required for oxidizing fatty acids do occur, yet the rate of fatty acid oxidation remains considerably less than other mammals and may be attributed to the high senstitivity of hepatic CPT I to inhibition by malonyl-CoA. The research outline herein was conducted to determine if control at the level of CPT I may be alleviated through enhancement of peroxisomal β-oxidation. Furthermore, to gain a better understanding of the role of CPT I in the oxidative capacity of the piglet liver, gaps in the research base must be addressed. Despite various research aimed at CPT I, no studies have been conducted on determining when carnitine is synthesized adequately by the pig, this information becomes essential in determining if this co-factor could impart be limiting in vivo fatty acid utilization. In addition, a complete assessment of the development of CPT I activity throughout the life of the pig has not been conducted. Knowledge in the developmental pattern of CPT I, coupled with information on the synthesis and status of its co-factor carnitine, is imperative to the understanding of piglet lipid metabolism.

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Clofibrate Increases Hepatic β-Oxidation and Malonyl-CoA Sensitive Carnitine Palmitoyltransferase I (CPT I) activity Without Induction of Hepatic CPT I Gene Expression in Pigs∗.

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Running head: Clofibrate supplementation in young pigs

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ABSTRACT

Newborn pigs were collected 48 h after birth and used to characterize hepatic β-oxidation after suckling or allotted to one of four dietary regimens: artificial milk replacer with long chain triglycerides (LCT) as the fat source (Control), the Control diet with 0.5% clofibric acid, the Control diet with medium chain triglycerides replacing LCT as the fat source (MCT), or the Control diet with 40 ppm isoproterenol. Dietary treatments were applied for 12 days (± 2 d) and an increase in liver weight was measured ($P < 0.05$) following clofibrate supplementation. There were no differences between Control and Newborn, MCT or isoproterenol supplemented groups in total, mitochondrial, or peroxisomal ß-oxidation of [1-^{14}C]-palmitate (1 mM). Total and peroxisomal β-oxidation increased 134 and 186%, respectively, with clofibrate supplementation. Malonyl-CoA sensitive CPT I activity increased ($P < 0.05$) in pigs receiving clofibrate, while MCT and isoproterenol supplementation had no effect on CPT activity when compared to Control animals. Changes in relative expression of hepatic LCPT I and skeletal muscle MCPT I mRNA amounts following clofibrate supplementation were not detected, while a modest effect on acyl-CoA oxidase (ACO) relative mRNA amounts was observed (p=0.08). Collectively, these findings indicate that the use of a peroxisome proliferator induced CPT activity, concomitant with increased fatty acid oxidation.

Key Words: Clofibrate, medium chain triglycerides, isoproterenol, β-oxidation, carnitine palmitoyltransferase I (CPT I), acyl-CoA oxidase, peroxisome, pigs

INTRODUCTION

In mammals, β-oxidation systems reside in the mitochondria and peroxisome (Wanders et al., 2001). Estimates of peroxisomal β-oxidation in rats range from 10 to 30%, and are dependent on postnatal age and physiological state (Grum, et al., 1994; Veerkamp, et al., 1986; Krahling et al., 1979). Research indicates peroxisomal β-oxidation in young pigs contributes to a greater percentage of total β-oxidation, representing 40–50% of the total (Yu et al., 1997a,b). This increased contribution of peroxisomal oxidation to total oxidation is observed not only in hepatic tissue, but also skeletal muscle where 32 to 42% of total oxidation occurs within the peroxisome (Herpin et al., 2003). In both tissues, the induction of peroxisomal oxidation occurs immediately post-partum, is reliant on the initiation of suckling (Yu et al., 1997c), and is greater in the suckled versus fasted piglet (Yu et al., 1997b,c).

During the suckling period milk lipids are the principle substrate for oxidative metabolism with 55-65% of dietary energy originating from milk fat. This is in stark contrast to the situation which occurs in utero, where maternal carbohydrate sources are essential for fetal development (Girard et al., 1992). During the peri- to postnatal transition in the rat there is a marked increase in β-oxidation followed by a hyperketonemic state (Ferre et al., 1983; Foster and Bailey, 1976). However, several lines of evidence suggest that the neonatal piglet is limited in its capacity to catabolize dietary fatty acids supplied in colostrum and milk (Adams et al., 1997; Odle et al., 1995; Pergori et. al., 1983; Pégorier et al., 1981; Bieber, et. al., 1973; Bengtsson et al., 1969). The greater contribution of peroxisomal β-oxidation to total oxidation in this species may provide an ancillary route in which milk lipids are metabolized.

An important feature of the peroxisomal β-oxidation system is the induction by physiological stimuli (fatty acids) and a variety of xenobiotics (including the fibrate family of hypolipidemic agents) through their direct binding with peroxisome proliferator activated receptor (PPAR)α (Kliewer, et al., 1997; Totland et al., 2000). The proliferation of peroxisomes involves up-regulation of peroxisomal genes (mainly those involved with β-oxidation), an increase in peroxisomal size, and the production of more peroxisomes (Purdue and Lazarow, 2001). In rodents PPARα is highly expressed in cells which have high fatty acid catabolic rates including liver, kidney, heart, and skeletal muscle (Braissant, et al., 1996). However, only the liver and to a lesser degree, the kidney, undergo peroxisome proliferation (Schoonjans, et al., 1996). Proliferation is also species dependent, while there is a dramatic induction of peroxisome proliferation, the human is refractory (Vamecq and Draye, 1989), and the pig is intermediate (Yu et al., 2001). Furthermore, activation of PPARα also enhances mitochondrial β-oxidation as evidenced by increased carnitine palmitoyltransferase I (CPT I) expression and activity (Lawrence et al., 2001; Chatelain et al., 1996).

Carnitine palmitoyltransferase I, an integral outer mitochondrial membrane protein, is essential to the translocation of acyl-CoA into the mitochondrial matrix for subsequent β-oxidation, as such, it is often considered rate limiting for mitochondrial oxidation of fatty acids (McGarry and Brown, 1997). The reaction that is catalyzed by CPT I, conversion of acyl-CoA to acylcarnitine, is regulated by the enzymes physiological inhibitor, malonyl-CoA. Two catalytically active isotypes, liver (CPT Iα) and skeletal muscle (CPT Iβ), have been characterized, each differs in its sensitivity to inhibition. For the rat, the IC_{50} of malonyl-CoA in the liver is 2.7 uM, while in skeletal muscle it is a mere 34 nM (McGarry et

al., 1983). Furthermore, it has recently been suggested that CPT I is subject to covalent modification through phosphorylation, with phosphorylation increasing activity while decreasing sensitivity to malonyl-CoA inhibition (Kerner, et al., 2004). A different picture has emerged for CPT I expressed in pig liver. While activity of CPT develops rapidly in the pig following birth (Beiber et al., 1973), sensitivity to inhibition by malonyl-CoA is considerably greater than that measured in the rat, with an IC_{50} of 141 nM for the pig isotype (Nicot et al., 2001). The greater sensitivity to inhibition by malonyl-CoA for the pig isotype may contribute to the lower oxidative capacities noted in this species.

While CPT I is often considered rate limiting for the oxidation of fatty acids, it should be noted that the oxidation of octanoate, a medium chain fatty acid, is not dependent on CPT I. The reason behind this phenomenon is that MCFA are activated to their CoA esters within the mitochondria (Fujino et al., 2001), therefore MCFA can diffuse across the mitochondrial membrane prior to CoA esterification. However, greater than 95% of porcine milk fatty acids are long- or very-long chain fatty acids (Lucas and Lodge, 1961), rendering CPT I essential for lipid oxidation in the suckling pig.

Rapid induction of CPT I activity in the newborn pig (Beiber et al., 1973) indicates that essential metabolic adaptations required for oxidizing fatty acids do occur, yet the rate of fatty acid oxidation remains considerably less than other mammals. The goals of the study outlined herein were to determine if β-oxidation and CPT activity could be enhanced through dietary supplementation. Due to the inducability of peroxisomes in pigs, clofibrate was chosen for supplementation. It was hypothesized that β-oxidation and CPT activity would increase based on previous research (Yu et al., 2001), however, to provide more complete knowledge of the effects of clofibrate in pigs malonyl-CoA sensitive CPT activity and

relative abundance of mRNA were assessed. In addition, medium chain triglyceride and isoproterenol were also supplemented to pig diets. The choice of MCT for supplementation was due to CPT I independent β-oxidation. Isoproterenol, a β_1/β_2 mixed agonist for the β-adrenergic receptor elicits its effects by stimulation of a cAMP mediated phosphorylation cascade. Isoproterenol was an intriguing target for study due to known effects of the β-adrenergic system including decreased lipogenesis and increased lipolysis, coupled with the paucity of research of β-agonist in neonatal pigs.

MATERIALS AND METHODS

Animals and diets.

Procedures for this study were approved by the North Carolina State University Animal Care and Use committee. Piglets were obtained from North Carolina State University Swine Education Unit within 24-48 hr after birth (suckled). Piglets were individually housed in an environmentally controlled (30° C) facility and allotted by sex and weight to one of four dietary treatments: 1) control milk replacer, 2) control milk replacer supplemented with 0.5% (wt/wt) clofibrate, 3) control milk replacer with medium chain triglyceride (MCT) source replacing 95% of soybean oil, and 4) control milk replacer supplemented with 40 ppm isoproterenol (Table 1). Milk replacer was reconstituted with water to 15% (wt/vol) dry matter and fed via gravity flow through a nipple (McLead et. al., 1990). Milk replacer was mixed and replaced twice daily, and dietary treatments were offered an average of 12 d (± 2 d).

Reagents and Chemicals.

L-[N-Methyl-^{3}H]carnitine (2.5 GBq/mol), [1-^{14}C]-Palmitate (2.0 GBq/mmol) and [1-^{14}C]-glucose (2.0 GBq/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Palmitoyl-CoA, antimycin A, rotenone and other chemicals were purchased from Sigma Chemicals (St. Louis, MO). Scintillation fluid (Scintisafe) was purchased from Fisher Scientific (St. Louis, MO).

Collection of tissue samples.

Pigs were euthanized via American Veterinary Medical Association approved electrocution. Liver and a portion of muscle (semitendonosis) tissue were excised immediately. Liver was weighed and a portion was placed in ice-cold isolation buffer containing 220 mmol/L mannitol, 70 mmol//L sucrose, 2 mmol/L HEPES, and 0.1 mmol/L EDTA (pH 7.4). Liver was blotted, minced, and manually homogenized in 10 volumes of the isolation buffer using a Potter-Elvejhem homogenizer. Homogenate and mitochondrial fractions were obtained by differential centrifugation (Mersmann, 1972). Respiratory control ratios were determined on the mitochondrial fraction to assess the integrity of the mitochondrial membrane (Aprille and Asimakis, 1980). Protein was determined by the biuret method (Gornall et al., 1949) in both the homogenate and mitochondrial fractions. Additional samples of liver and muscle were frozen immediately in liquid N and stored at -80 °C.

Analysis of carnitine palmitoyltransferase-I activity.

Liver mitochondria were used to assess CPT activity as previously described by Bremer (1981). The assay measured the rate of formation of palmitoylcarnitine from palmitoyl-CoA and carnitine. The reaction medium contained 75 mmol/L KCL, 50 mmol/L Hepes, 0.2 mmol/L EGTA, 1% fatty acid free bovine albumin, and 80 umol/L of palmitoyl-CoA for determination of total CPT activity. Malonyl-CoA insensitive activity was determined in parallel reactions by the addition of 250 umol/L of malonyl-CoA to the reaction medium. Malonyl-CoA sensitive CPT activity was determined by difference. Reactions were initiated by the addition of 1 mmol/L of L-[N-methyl-^{3}H]-carnitine (.05 uCi/reaction), and terminated by the addition of 6% $HClO_4$ after 6 min. The labeled palmitoyl-carnitine was extracted with butanol and quantified by liquid scintillation spectrometry.

Assays of mitochondrial and peroxisomal β-oxidation.

Rates of first-cycle total, mitochondrial, and peroxisomal β-oxidation of [1-^{14}C] palmitate or [1-^{14}C] glucose were measured in liver homogenates by a modification of the procedures as described by Yu and co-workers (1997a). In duplicate reactions, aliquots of homogenate (300 uL) were added to Erlenmyer flasks containing 1.7 mL of a reaction medium (50 mmol/L sucrose, 150 mmol/L Tris-HCl, 20 mmol/L KH_2PO_4, 10 mmol/L $MgCl_2 \cdot 6H_20$, 2 mmol/L EDTA, 1 mmol/L L-carnitine, 0.2 mmol/L CoA, 2 mmol/L NAD, 0.1 mmol/L malate, 10 mmol/L ATP; pH 7.4). The reaction medium contained either: 1) [1-^{14}C]-palmitate, 2) [1-^{14}C]-glucose, 3) [1-^{14}C]-palmitate plus antimycin/rotenone, 4) [1-^{14}C]-palmitate plus glucose, or 5) [1-^{14}C]-glucose plus palmitate. Each reaction contained 0.5 uCi

of either [1-^{14}C]-palmitate or [1-^{14}C]-glucose. Palmitate was added to the reaction medium as Na-palmitate bound to fatty acid free albumin (complexed in a 5:1 molar ratio) to a final concentration of 1 mmol/L. Glucose was added to the reaction medium to achieve a final concentration of 5 mmol/L. Antimycin (50 umol/L) and rotenone (10 umol/L) were added to inhibit mitochondrial oxidation for indirect determination of peroxisomal oxidation.

Reactions were initiated by the addition of the homogenate and allowed to proceed for 30 min. in a shaking water bath at 37 °C. Incubations were terminated by the addition of 1 mL of 3 mol/L $HClO_4$ to the reaction medium. After termination of the reaction, flasks were incubated at room temperature (2 hr) for collection of $^{14}CO_2$ into a suspended well containing 500 uL of ethanolamine. When [1-^{14}C]-palmitate was contained in the reaction medium, acid soluble products (ASP) were collected following centrifugation of flask contents to precipitate protein and non-metabolized palmitate. Blanks were prepared by immediate acidification of the reaction medium following addition of the homogenate. Radioactivity in CO_2 and ASP was quantified by liquid scintillation spectrometry.

The rate of total β-oxidation was measured as the rate of accumulation of ^{14}C in $^{14}CO_2$ plus [^{14}C-]ASP. The rate of accumulation of ^{14}C into [^{14}C-] ASP following the addition of the inhibitors antimycin and rotenone was reported as peroxisomal β-oxidation. Negligible $^{14}CO_2$ production was measured under the presence of the inhibitors (data not shown). Mitochondrial β-oxidation was calculated to be the difference between total β-oxidation and peroxisomal β-oxidation.

Extraction of total RNA.

Total RNA was extracted from liver and muscle tissue using guanidine isothiocyanate and phenol (TRI™ Reagent solution; Sigma-Aldrich, Inc.). Briefly, tissue was homogenized .1g tissue/mL TRI Reagent), and .2 mL of chloroform (per 1 mL TRI Reagent) was added to the homogenate. Following centrifugation (12,000 x g, 15 min.) the RNA was precipitated from the aqueous phase by the addition of isopropanol (.5 mL/mL TRI Reagent) followed by centrifugation (12,000 x g, 10 min.). The resulting pellet was washed with 75% ethanol and resuspended in RNase free water. Gel electrophoresis with ethidium bromide staining was used to asses RNA integrity. The RNA was quantified and purity assessed by UV spectrophotometric analysis of $_{A}260$ and A_{260}/A_{280} ratio (> 1.7), respectively.

Reverse Transcription.

The RNA (10 ug/50uL) was treated with 4 units of TurboDNase™ (Ambion, Inc.) according to the manufacturers instructions for removal of genomic DNA. The RNA was recovered following phenol:chloroform extraction and 2 ug was reverse transcribed using Omniscript™ reverse transcription kit according to the manufacturer's instructions (Qiagen, Inc). First strand cDNA was synthesized at 42 ºC for 60 min by priming with oligo-dT (Roche Diagnostics, Inc). A negative control (using pooled RNA) containing all of the reverse transcription reagents in the absence of RT enzyme was included to verify absence of genomic DNA during PCR (no RT control).

Primer Design.

The primer design program Lasergene Primer Select (DNAstar, Inc.) was used to design pig specific primers for hypoxanthine phosphoribosyltransferase (HPRT), CPT I (liver and muscle isotypes), and acyl-CoA oxidase (ACO) (Table 2). Primer pairs were selected for optimum annealing temperatures and negligible secondary structure. Primers were purchased through Sigma-Genosys, Inc. and resuspended in 1x Tris/EDTA.

Real-time RT-PCR with SYBR Green.

Real-time RT-PCR was performed using the DNA Engine Opticon® (MJ Research, Inc.). Real-time fluorescence detection was performed in 96 well plates using Quantitect™ SYBR Green buffer (Qiagen, Inc). Samples were assayed in triplicate. Each 20 uL reaction contained cDNA (equivalent to 100 ng RNA) and .3 uM each of the forward and reverse primers. Following initial 15 min. incubation at 95 °C for activation of the Hot Start Taq DNA polymerase, all templates were amplified for 40 cycles using the following protocol: denaturation for 15 sec at 94 °C, primer annealing for 30 sec at 60 °C, and elongation for 30 sec at 72 °C. Fluorescence data were acquired at the end of each elongation phase. Following the amplification program, melting curves were generated by measuring the fluorescence as the temperature was raised from 50 °C to 95 °C in .2 °C increments to verify specificity of fluorescence detection. Specificity of amplification products was further verified by gel electrophoresis and ethidium bromide staining. Background fluorescence was set as the average fluorescence between cycles 3 and 15 and threshold (Ct) was determined based on 10 standard deviations above background fluorescence (Giulietti et al., 2001).

Positive (no template reaction) and negative (no reverse transcriptase) controls were run in triplicate and included in each PCR assay.

Analysis of Real Time RT-PCR Data.

Standard curves for the reference gene HPRT and the target genes of interest for liver (LCPT I and ACO) and muscle (MCPT I) were generated from pooled cDNA (equivalent to 200, 100, 50, 25, and 12.5 ng of total RNA). The Ct values were regressed on the log of template cDNA concentration. The quality of the standard curves were assessed according to the correlation coefficient ($R^2 > .95$) and the slope of the lines were used to determine efficiency of amplification according to the equation $E = 10^{-1/slope}$ (Pfaffl, 2001; Figure 1). Due to unequal amplification efficiencies between the reference gene and target genes, a relative expression ratio indicating fold change was determined according to the method by Pfaffl (2001) for assessment of treatment differences:

$$\text{Expression Ratio} = \frac{E^{\Delta Ct_{target}}}{E^{\Delta Ct_{reference}}}$$

Where ΔCt = sample – control for the target gene and reference gene. Differences in Ct values for pigs fed clofibrate (sample) were assessed against Ct values obtained from control pigs for HPRT and the target genes of interest (LCPT I, MCPT I, or ACO) to determine a ΔCt values. The ΔCt was used to determine abundance of mRNA using the efficiency of amplification for target genes and reference gene. The target gene was corrected for differences in reference gene amplification to obtain the expression ratio.

Variance of the expression ratio was determined according to Marino and co-workers (2003):

$$\text{Variance}_{\text{lnR}} = \left(\frac{sCt_1}{\beta}\right)^2 + \left(\frac{sCt_2}{\beta}\right)^2 + \left(\frac{(Ct_2\text{-}Ct_1)\, s_\beta}{\beta^2}\right)^2$$

where Ct_1 = sample, Ct_2 = control and sCt_1 and sCt_2 = estimate of standard error (average of triplicate readings) for the sample and control (respectively), β = slope of the gene being evaluated and s_β = error of the slope. Log variance was determined for the target genes and reference gene. Target gene variance was corrected for differences in reference gene using the following equation:

$$\text{corrected variance}_{\text{lnR}} = \text{variance}_{\text{lnRTarget}} + \text{variance}_{\text{lnRreference}}$$

The corrected variance was linearized and the standard error determined.

Statistical Analysis:

Carnitine palmitoyltransferase activity and β-oxidation data were subjected to ANOVA using the general linear models procedure of SAS (SAS Inc., Cary, NC) appropriate for a completely randomized design (CRD). Effects of substrate (palmitate or glucose) oxidation were analyzed as a sub-plot within the CRD. A one-sided students t-test was used for determining significance in fold change of hepatic ACO and LCPT I, and

skeletal muscle MCPT I expression for clofibrate supplementation relative to control. Data are reported significant when $p < 0.05$.

RESULTS

Average daily gain was greater in control pigs than pigs supplemented with clofibrate, MCT, or isoproterenol (290 g ± 8 g, 239 g ± 11 g, 227 g ± 12 g, 255 g ± 12 g, respectively; $p < 0.05$). Liver weights were 165g (± 7), 228g (± 10), 179g (± 10), and 142g (± 10) for control, clofibrate, MCT, and isoproterenol supplemented pigs, respectively. Clofibrate supplementation increased liver weight by 138% ($p < 0.0001$). There was no difference in liver weight following MCT or isoproterenol supplementation.

Using [1-^{14}C]-palmitate as substrate, total, mitochondrial, and peroxisomal first-cycle β-oxidation rates were similar between suckled newborn pigs, control pigs, and pigs supplemented with MCT or isoproterenol (Figure 2). Total and mitochondrial oxidative capacities were increased 134 and 186%, respectively, in liver homogenates from clofibrate supplemented pigs in comparison to similar age control pigs ($p < 0.05$; Figure 2). Peroxisomal β-oxidation accounted for 41, 28, 39, and 43% of total β-oxidative capacity for control, clofibrate, MCT, or isoproterenol supplemented pigs, respectively. Peroxisomal oxidation was 37% greater for clofibrate supplemented pigs compared to control pigs ($p = 0.06$); however, it represented less of the total oxidation due to increased mitochondrial oxidative capacity. Production of $^{14}CO_2$ measured in liver homogenates from clofibrate, MCT, or isoproterenol supplemented pigs did not differ from control pigs, but the rate of ^{14}C accumulation into ASP of mitochondrial oxidation increased 197% following clofibrate acid supplementation ($p < 0.001$; Figure 2).

Liver homogenates from control, clofibrate, MCT, or isoproterenol supplemented pigs exhibited no preference toward metabolic fuel substrate as evidenced by similar rates of CO_2 or ASP production with the addition of glucose to [1-^{14}C]-palmitate or palmitate to [1-^{14}C]-glucose (Table 3). However, in newborn pigs there was a 30% reduction in CO_2 derived from [1-^{14}C]-glucose by the addition of palmitate and a 127% reduction in ASP when glucose was added to [1-^{14}C]-palmitate. Production of CO_2 from glucose increased 28 and 23% for MCT and isoproterenol supplemented pigs, respectively, in comparison to similar age control pigs.

Mean respiratory control ratios for isolated mitochondria exceeded 3 for all dietary treatments. Activity of CPT increased 105% in control compared to suckled newborn pigs ($p < 0.05$; Figure 3). An additional 80% increase in CPT activity was measured in pigs supplemented with clofibrate. ($p < 0.05$). Supplementation with clofibrate increased both malonyl-CoA sensitive and insensitive CPT activity by 64 and 105%, respectively. Supplementation with MCT or isoproterenol had no effect on total, malonyl-CoA sensitive, or insensitive CPT activity.

For pigs receiving clofibrate supplementation, the relative abundance of LCPT I, MCPT I, and ACO mRNA was also determined. As assessed by real time RT-PCR, clofibrate had no detectable effect on relative transcript amount of ACO or LCPT I in liver, or MCPT I in skeletal muscle (Figure 4).

DISCUSSION

Effect of age on hepatic β-oxidation.

In the prenatal to postnatal transition the neonate must make the metabolic adaptations necessary for the oxidation of fatty acids, which serve as the primary energy substrate during the suckling period (Girard et al., 1992). Previous research evaluating the β-oxidative capacity of pig liver homogenates has shown that the rate of oxidation of palmitate increases approximately fourfold between birth and 7 d of age (Mersmann and Phinney, 1973); however, oxidation rates do not differ between 1 and 7 d of age (Miller et al., 1971), as the postnatal increase in β-oxidative capacity of the liver occurs within the first 24 h of life (Yu et al., 1997b). It can be inferred that maximal oxidative capacity was measured in suckled newborn pigs as total oxidative products, represented by the summation of CO_2 and ASP, of [1-^{14}C]-palmitate did not differ between suckled newborns and 12 d old control pigs in the current study (Table 3, Figure 2).

At birth, approximately 50% of the total β-oxidative capacity can be ascribed to peroxisomal oxidation. Yu and co-workers (1997a,b) showed that by 24 hr of age, peroxisomal oxidation increases 66% in suckled newborn pigs, however, continues to represent 50% of total oxidation due to increased rates of mitochondrial oxidation. By 10 d of age, peroxisomal oxidation decreased to represent 40% of total oxidation (Yu et al., 1997b). This is in agreement with the current study, where peroxisomal oxidation contributed 40% of total oxidation in control pigs. However, Yu and co-workers (1997b) reported a decrease in peroxisomal oxidation between 24 h of age and 10 d of age in piglets allowed to suckle the sow, while peroxisomal oxidation in pigs reared artificially was equivalent between suckled newborn and control pigs.

In the pig, the early postnatal rise in β-oxidation coincides with hepatic mitochondrial proliferation which occurs within 6 to 12 hr after birth (Mersmann et al., 1972); furthermore, the increase in peroxisomal oxidation rates that occur in early life may also be attributable to an increase in peroxisomal number. Indeed, peroxisome proliferation has been shown to occur between birth and 28 d of life in the pig (Laging et al., 1990). In rats, hepatic peroxisomes number increases rapidly after birth, reaching adult levels by 6 d of age. Similarly, palmitoyl-CoA oxidase activity increases 2.5-fold within 1 d of life, and then progressively decreases to adult values by 7 d of age (Krahling et al., 1979). However, in the young animal peroxisomal oxidation represents only 10% of total oxidation, a value that increases to 30% for adult rats (Krahling et al., 1979). In the neonatal rat, increased hepatic β-oxidative capacity is marked by increased rates of ketogenesis (Girard et al., 1992). In the neonatal pig, which has limited capacity for ketogenesis (Odle et al., 1995; Pegorier et al., 1981; Bengtsson et al., 1969), the greater contribution of peroxisomal oxidation to total oxidation may provide an alternative path for catabolism of milk lipids, due to limited mitochondrial oxidation as a result of low ketogenic capacity.

Early postnatal changes in oxidative capacity are congruent with changes in CPT activity in the pig (Beiber et al., 1973) and rat (Saggerson and Carpenter, 1982). Beiber and co-workers (1973) reported maximal CPT activities in 24 h old fed piglets that were not further increased by fasting at 24 d of age. Furthermore, while fasting delayed the induction of CPT activity in the 24 h old pig, in the fed pig CPT activity at 12 h was similar to that measured in the 24 h fed animal. In the current study, CPT activity increased 105% between suckled newborn and 12 d old control pigs, indicating that CPT activity continues to increase after 24 h of age while β-oxidative rates remain constant.

Oxidative capacity of the liver following clofibrate supplementation.

To enhance β-oxidative capacity, clofibrate was supplemented in piglet diets for an average of 12 days. In general, clofibrate results in enlargement of the rodent liver after 10-14 d of treatment (Hess et al., 1965). The marked increase in liver size is attributed to an increase in peroxisomal number and size (Lazarow et al., 1976). Coincident with increased peroxisome proliferation are greater peroxisomal oxidation rates and increased expression and activity of acyl-CoA oxidase (Schoonjans, et al., 1996). Limited research has been conducted in pigs regarding the putative effects of clofibrate supplementation. In the current study, clofibrate supplementation increased liver size by 78% and tended to increase acyl-CoA oxidase expression 3.7-fold, however, there was only a weak effect (37% increase; p=0.06) on the rate of peroxisomal oxidation. This is in contrast to a previous study conducted in pigs (Yu et al., 2001) where clofibrate supplementation resulted in a 2.7 fold increase in peroxisomal oxidation and >3-fold increase in acyl-CoA oxidase activity. In the current study peroxisomal oxidation was assessed by rotenone- and antimycin-insensitive β-oxidation of [1-^{14}C]-palmitate, which measures only the initial round of β-oxidation. Yu and co-workers (2001) used the palmitoyl-CoA-dependent, KCN-insensitive reduction of NAD+ to quantitate all cycles of peroxisomal β oxidation.

Despite differences between the current study and that conducted by Yu and co-workers (2001), the effects of clofibrate supplementation are attenuated in the pig in comparison to the effects observed in the rat, where hypolipidemic drugs induce a 20-fold increase in peroxisomal oxidation (Lazarow et al., 1976) and a 6-fold increase in acyl-CoA oxidase activity (Lawrence et al., 2001). The lesser effect seen in pigs may be ascribed to the already high rates of peroxisomal oxidation in this species compared to the rodent.

Furthermore, the effect of clofibrate is mediated via the PPARα isotype (Kliewer et al., 2001). In humans lower expression of the PPARα isotype in the liver (Palmer et al., 1998) has been attributed to lower transcript amount of acyl-CoA oxidase following peroxisome proliferation (Ammerschlaeger et al., 2004). In mature pigs the PPARα isotype is highly expressed in the liver, however, expression in the neonatal pig is breed dependent, with Landrace pigs showing lower expression compared to the Duroc breed (Sunvold et al., 2001).

While peroxisomal oxidation was enhanced only mildly, total oxidation increased approximately 2-fold as a result of greater mitochondrial oxidative rates. In rats, clofibrate treatment increases hepatic mitochondrial number (Kurup, et al., 1979) and mitochondrial oxidation (Mannaerts, et al., 1978). While mitochondrial mass was not determined in the current study, increased mitochondrial number is correlated with increased rates of mitochondrial oxidation (Mersmann et al., 1972) and could account for the increased rate of oxidation for the current study. Another explanation for the increased rates of mitochondrial oxidation can be attributed to the effects of clofibrate on CPT activity. In rodents clofibrate treatment increases both CPT I and CPT II mRNA expression (Chatelain et al., 1996) and CPT I activity (Lawrence et al., 2001). Indeed, malonyl-CoA sensitive CPT activity was increased, in agreement with observations made in the rat. However, we failed to detect a difference in hepatic mRNA expression for CPT I with clofibrate supplementation. Similarly, an effect of clofibrate supplementation on expression of skeletal muscle CPT I was not detected. In humans, PPARα is expressed in skeletal muscle (Auboeuf, et al., 1997) and MCPT I expression is regulated through PPARα activation (Mascaro, et al., 1998). In pigs, however, PPARα has not been detected in skeletal muscle (Sundvold et al., 2001).

Oxidation of palmitate and glucose following MCT feeding.

The use of MCT as an alternative metabolic fuel for the neonatal pig has been researched extensively in light of the pigs' ability to digest, absorb, and utilize medium chain fatty acids released from this energy source (Odle, 1997). The rate (mmol ATP·kg$^{-.75}$·min^{-1}) and extent (mmol ATP·kg$^{-.75}$) of MCT utilization is 3- and 4-fold greater, respectively, than long chain triglyceride (LCT) utilization (Heo et al., 2002). Pegorier and co-workers (1983) suggested that the piglets' inability to oxidize long chain fatty acids was due to a high rate of re-esterification and a low rate of oxidation. Because acyl-transferases have a low affinity for MCFA and the re-esterification and deposition of MCFA in adipose is limited (Odle, 1997; Bach and Babayan, 1983) MCFA are considered an obligatory fuel.

While direct feeding of MCFA results in a greater oxidative flux of carbon (Odle et al.,1991) prolonged feeding of MCT in the current study had no effect on subsequent oxidation of [1-^{14}C]-palmitate to CO_2 or accumulation of ^{14}C into ASP. Furthermore, MCT supplementation had no effect on malonyl-CoA sensitive CPT activity. These findings are in agreement with research conducted in rats where oxidation of oleate and CPT I activity was not effected by prolonged MCT feeding, however, hepatic lipogenesis was increased 6-fold (Pegorier et al., 1988). Although not determined in this study, MCT feeding would not be expected to increase hepatic lipogenesis as the capacity for lipogenesis in neonatal pig hepatocytes is extremely low (Mersmann, 1973).

In contrast to oxidative rates for [1-^{14}C]-palmitate, oxidation of [1-^{14}C]-glucose by MCT supplemented pigs was increased over control pigs. Furthermore, [1-^{14}C]-glucose oxidation remained high when measured in the presence of palmitate. The increased rate of glucose oxidation (77% greater for MCT supplemented pigs compared to controls) may be

required to meet hepatic energy requirements, as the liver weight:body weight ratio was 80% greater for MCT supplemented pigs (data not shown).

It is interesting to note that MCT supplementation had no deleterious effects on peroxisomal oxidation. Medium chain fatty acids are poor substrates for oxidation by the peroxisome, due to the lower affinity of acyl-CoA oxidase toward acyl substrates of eight or fewer carbons (Hashimoto, 1997). Furthermore, peroxisomal oxidative capacity is responsive to high-fat feeding of long chain fatty acids (Neat et al., 1980). While suckling is important for establishment of optimal peroxisomal β-oxidative capacities (Yu et al., 1997b, Yu et al., 1997c), it appears that maintaining high rates of peroxisomal β-oxidation is not dependent on a continual source of long chain fatty acids in the young pig.

Effects of isoproterenol on palmitate oxidation.

Effects of β-adrenergic ligands on lipid oxidation during the suckling period of the pig are not known. In vitro studies in older pigs have shown a decrease in lipogenesis and an increase in lipolysis when porcine adipose tissue explants were incubated with isoproterenol (a β1/β2 agonist) (Pterela and Scanes, 1990). The effect on lipolysis also has been observed in vivo following isoproterenol infusion (Mersmann, 1987). Limited research in the young pig has shown that isoproterenol infusion increases blood glucose, concomitant with increased liver glycogen phosphorylase activity, an effect that is more pronounced in the 7 d old pig compared to the newborn (Stanton and Mueller, 1973). Furthermore, in the fed newborn isoproterenol had no effect on plasma NEFA, but increased plasma NEFA values 2-fold by 7 d of age (Stanton and Mueller, 1973).

In the current study, rates of first-cycle oxidation of [1-^{14}C]-palmitate and hepatic CPT activity were unaffected by isoproterenol. Phosphorylation sites on CPT I have recently been identified, and are thought to play a role in sensitivity to malonyl-CoA inhibition (Kerner, 2004). As effects of β agonists, such as isoproterenol, are mediated by cAMP phosphorylation cascades (Levitzki, 1988), regulation of CPT I via phosphorylation could be a putative role of β agonist in hepatic lipid metabolism. However, in the pig CPT I is acutely sensitive to inhibition by malonyl-CoA (Nicot et al., 2001) and malonyl-CoA concentrations are markedly lower than those measured in the rat, owing to the fact that the liver is not a lipogenic organ for the pig (Mersmann, 1973).

It has been suggested that first-pass metabolism by the liver may limit the bioavailability of isoproterenol when fed. In rats, the effects of isoproterenol on lipolysis are lost when the β agonist is fed at .5mg/kg body weight (Fotovati et al., 2000). Furthermore, desensitization and/or down-regulation of the β-adrenergic receptor following continual exposure of the ligand are a known mode of regulation (Levitzki, 1988). While the fetal liver is resistant to regulation (Slotkin et al., 2003), continual exposure to β agonist following birth have resulted in hepatic desensitization of the receptor (Auman et al., 2002).

In summary, this study provides evidence that the pig is less responsive to supplementation with clofibrate, an effect which may be due to the already high capacity for peroxisomal oxidation in this species. Despite only a modest increase in peroxisomal oxidative rates, mitochondrial oxidative capacity was dramatically induced by clofibrate, coincident with increased malonyl-CoA sensitive CPT activity.

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CHAPTER 2 TABLES

Table 2.1
Diet Composition

	Diet[a]			
Item	Control	Clofibrate	MCT[b]	Isoproterenol
Ingredients, %				
Non-fat skim milk	39.7	39.7	39.7	39.7
Whey protein concentrate	7.92	7.92	7.92	7.92
Soybean oil	20	20	1	20
MCT	-	-	19	-
Lactose	13.5	13.0	13.5	13.5
Sodium Casein	11.88	11.88	11.88	11.88
Arginine	0.5	0.5	0.5	0.5
Histidine	0.17	0.17	0.17	0.17
$CaCO_3$	0.5	0.5	0.5	0.5
Dicalcium phosphate	2.7	2.7	2.7	2.7
Mineral Premix	0.5	0.5	0.5	0.5
Vitamin Premix	0.13	0.13	0.13	0.13
KCl	0.5	0.5	0.5	0.5
Xanthum gum	1	1	1	1
Lecithin	1	1	1	1
Clofibrate	-	0.5	-	-
Isoproterenol	-	-	-	.004

[a] Expressed on a dry weight basis, 150 g of dry diet was reconstituted with 1 L of water.
[b] Contained C_6 (5%) and a mixture of C_8 and C_{10} (95%). Provided as a gift from Lonza, Inc. (Fairlawn, NJ 07410).

Table 2.2
Primers used for amplification of hypoxanthine phosphoribosyltransferase (HPRT), liver and skeletal muscle isotypes of carnitine palmitoyltransferase I (L CPT I and M CPT I), and acyl-CoA oxidase (ACO) and expected amplicon size following SYBR Green RT-PCR. Accession numbers listed reference published Genbank sequences for pigs which were used to design forward and reverse primers.

Gene	Primer sequence (5' → 3')	Amplicon Size (bp)	Accession
HPRT	F: CCATCACATCGTAGCCCTCTG R: TACTTTTATATCGCCCGTTGACTG	172	AF143818
L CPT I	F: GGACCGCCACCTGTTCTGCCTCTA R: GCCCCCTCCGCTCGACACATAC	175	AF288789
M CPT I	F: GCACGCCAGGCCTTCTTCAGC R: TGGCCTCGTCTTCCGGGTCATAGT	121	AY181062
ACO	F: ACCACGGTGAAGAAGATAAGG R: TCCAGGCGGGCATGAAGAAGC	181	AF185048

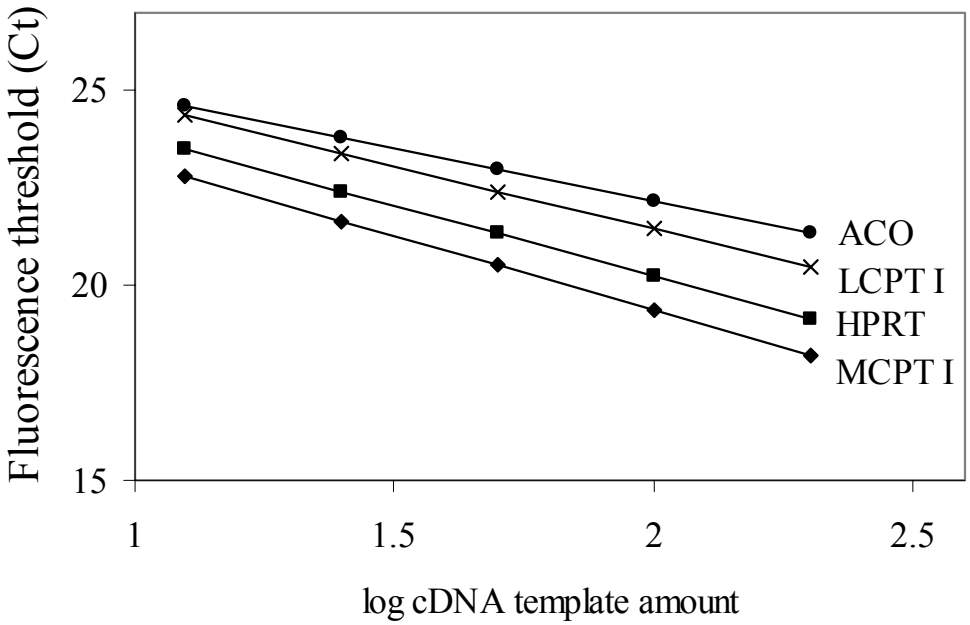
Table 2.3
Carbon accumulation in CO_2 and ASP from incubations of pig liver homogenates with [1-^{14}C]-palmitate or [1-^{14}C]-glucose with or without the addition of unlabeled glucose or palmitate, respectively (see MATERIALS and METHODS for details)[1].

CO_2 (nmol/h*mg protein)	**n**	**[1-^{14}C]-Palmitate**	**[1-^{14}C]-Palmitate + Glucose**	**[1-^{14}C]- Glucose**	**[1-^{14}C]-Glucose + Palmitate**	**SEM**
Newborn	4	1.62^{x}	1.04^{x}	$27.78^{b,y}$	$21.34^{abc,z}$	2.23
Control	9	0.97^{x}	0.92^{x}	$21.59^{a,y}$	$21.72^{a,y}$	1.49
Clofibrate	6	1.58^{x}	1.52^{x}	$17.95^{a,y}$	$17.04^{b,y}$	1.82
MCT	5	0.75^{x}	0.72^{x}	$27.74^{b,y}$	$26.96^{c,y}$	1.99
Isoproternol	6	0.71^{x}	0.65^{x}	$26.53^{b,y}$	$22.9^{ac,y}$	1.82
ASP (nmol/h*mg protein)						
Newborn	4	$22.27^{ac,x}$	$9.79^{a,y}$	-	-	2.5
Control	9	19.07^{ac}	19.35^{b}	-	-	1.67
Clofibrate	6	45.29^{b}	43.8^{c}	-	-	2.04
MCT	5	23.37^{a}	20.41^{b}	-	-	2.24
Isoproternol	6	16^{c}	15.11^{ab}	-	-	2.04

[1] Substrates for oxidation were included in incubations as 1 mmol/L of [1-^{14}C]-palmitate or palmitate and/or 5 mmol/L of [1-^{14}C]-glucose or glucose.
[abc] Means within a column lacking a common superscript differ ($p < 0.05$).
[xyz] Means within a row lacking a common superscript differ ($p< 0.05$).

CHAPTER 2 FIGURES

(a)



(b)

Gene	Slope	r^2	Efficiency	Average Ct
HPRT	-3.60	0.99	1.89	22.4 ± 2.00
LCPT I	-3.21	1.00	2.04	23.5 ± 2.43
MCPT I	-3.8	0.99	1.82	21.5 ± 1.33
ACO	-2.68	0.99	2.34	24.3 ± 1.73

Figure 2.1
Relationship between cDNA template concentration and fluorescene threshold Ct. (a) Linear regression of log concentration of template versus C_T. Template concentrations were equivalent to 200, 100, 50, 25, and 12.5 ng RNA. Values are means of triplicate readings for the genes acyl-CoA oxidase (ACO; ●), hypoxanthine phosphoribosyltransferase (HPRT; ■), skeletal muscle carnitine palmitoyltransferase I (MCPT I; ♦), and liver carnitine palmitoyltransferase I (LCPT I; ×). (b) Efficiency of amplification for each gene. Efficiency calculated as $E = 10^{-1/slope}$. Average Ct values are representative of starting template concentration equivalent to 100 ng RNA for control pigs.

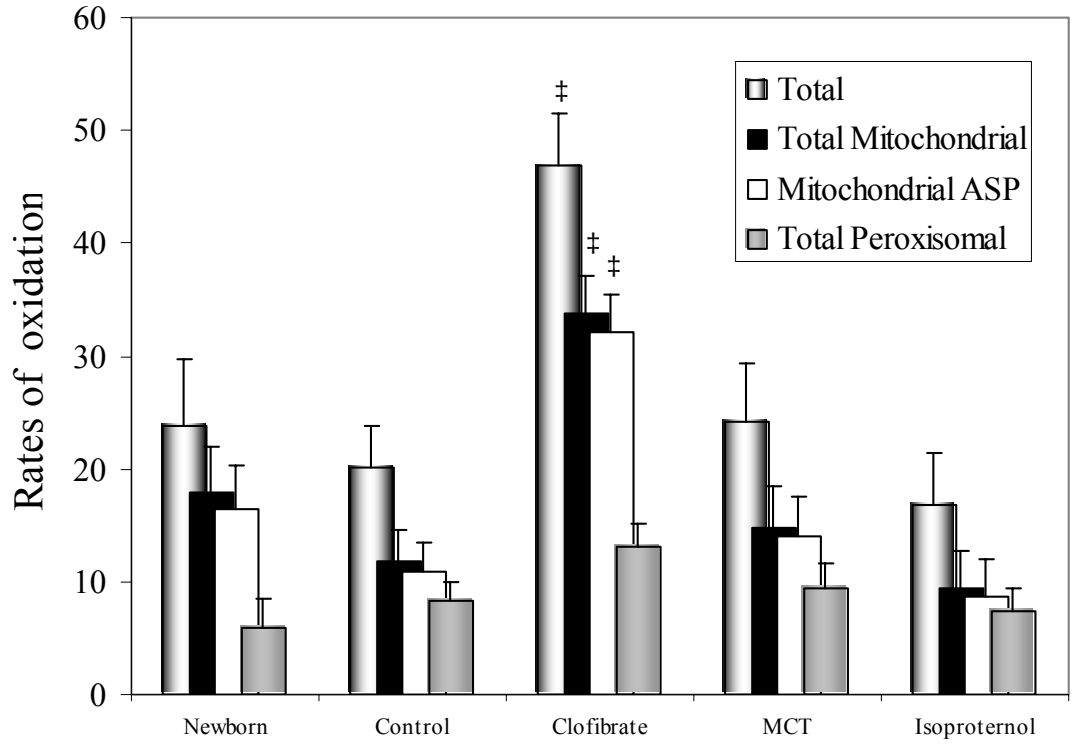


Figure 2.2
Rates of total, mitochondrial, and peroxisomal β-oxidation ($^{14}CO_2$ and [^{14}C]-ASP accumulation); and mitochondrial [^{14}C]-ASP production (nmol/h·mg protein). Bars represent means (n=5-9) ± SE. ‡ Differ from control ($p < 0.01$) comparison made between similar coded bars.

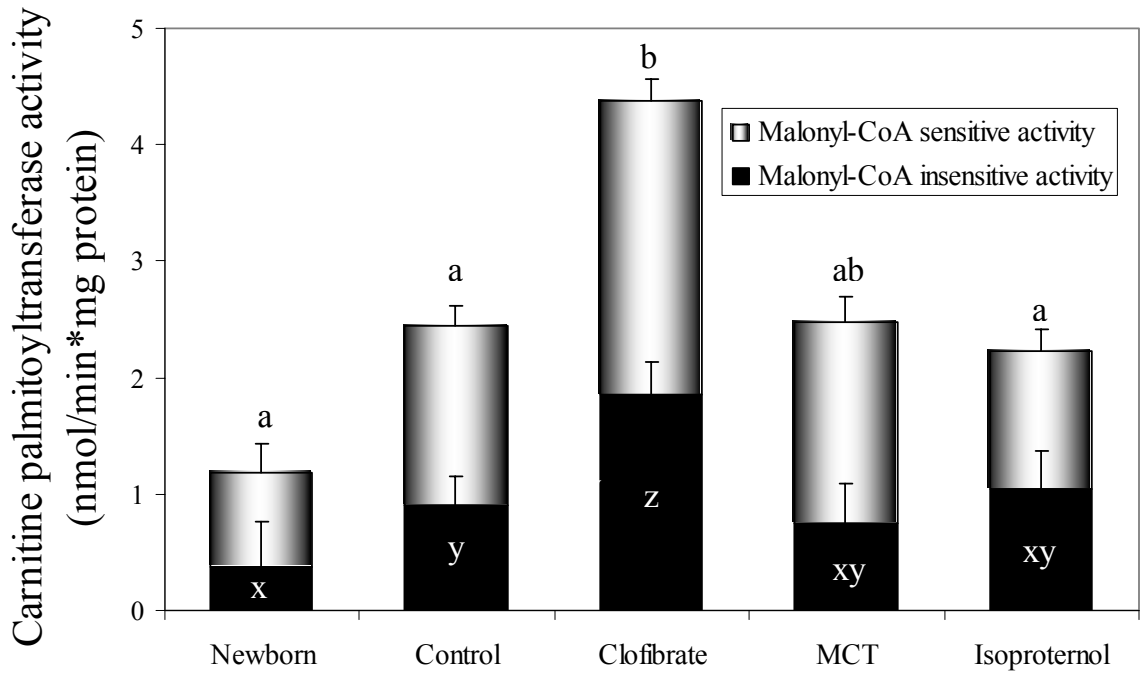


Figure 2.3
Malonyl-CoA sensitive and malonyl-CoA insensitive hepatic carnitine palmitoyltransferase activity (CPT). Total activity is represented by summation of malonyl-CoA sensitive and insensitive activities. Bars represent means ± SE. Different letters (a,b,c) denote significant difference between dietary treatment for malonyl-CoA sensitive CPT activity ($p < 0.05$). Different letters (x,y) denote significant difference between dietary treatment for malonyl-CoA insensitive CPT activity ($p < 0.05$).

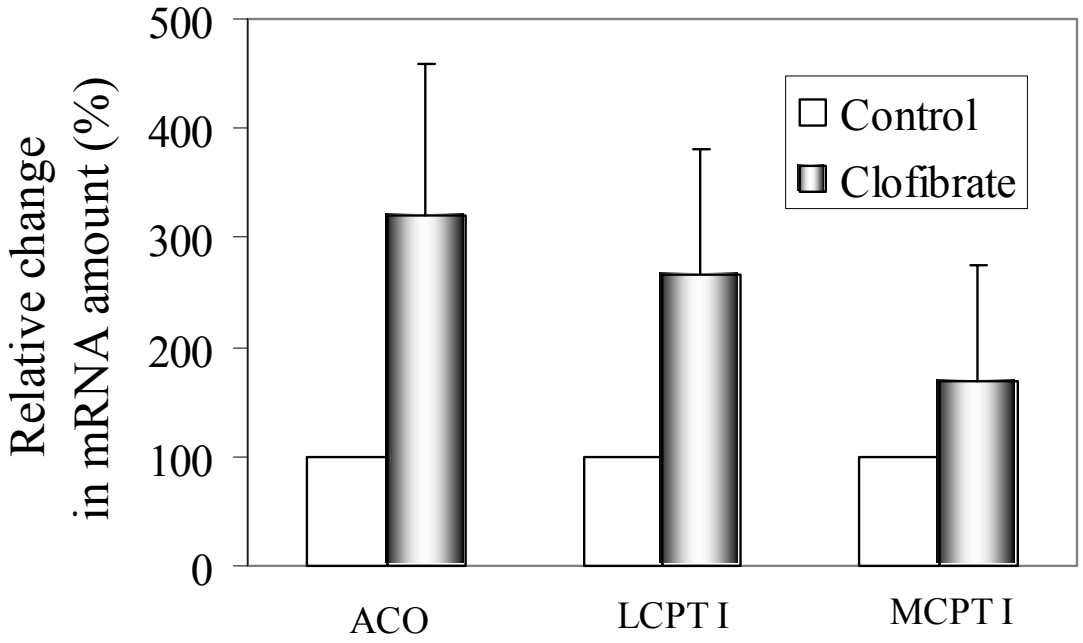


Figure 2.4
Relative hepatic acyl-CoA oxidase (ACO) and liver isotype carnitine palmitoyltransferase I (LCPT I), and skeletal muscle isotype carnitine palmitoyltransferase I (MCPT I) mRNA amounts. Amounts of mRNA for control pigs are set as 100% and data are reported as % change for clofibrate supplemented pigs relative to control pigs. Data were corrected for changes in reference gene expression according (Pfaffl, 2001). Bars represent mean (n=4-5) ± SE; p = 0.08; 0.16; and 0.3 for ACO, LCPT I, and MCPT I, respectively, for clofibrate supplemented versus control pigs.

Ontogeny of Hepatic and Skeletal Muscle Carnitine Palmitoyltransferase I (CPT I) activity: Evidence of decreased Gene Expression for Hepatic CPT I After Birth and Capacity for De Novo Carnitine Synthesis in the Kidney.

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Running Head: Ontogeny of carnitine palmitoyltransferase I in pigs

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ABSTRACT

Hepatic and skeletal muscle CPT I kinetics, along with relative expression of transcript amounts for both isotypes of CPT I, were assessed in pigs during different stages of development. Pigs were selected at birth (non-suckled), 24 hr (suckled), 1, 3, 5, 8 wk, and 7 mo of age (fed). Activity of CPT I increased 109 and 67% between birth and 1 wk of age in liver and skeletal muscle, respectively ($p < 0.05$). Hepatic CPT I mRNA relative expression decreased 13 fold during the initial 24 hr of life and remained lower than amounts expressed at birth, until adulthood ($p < 0.05$). Hepatic CPT I apparent Km for carnitine decreased by 48% from birth to 3 wk of age. The apparent Km for carnitine in skeletal muscle decreased from birth to 1 wk of age, then increased 200% between 1 and 5 wk of age ($p< 0.01$). Plasma and liver free carnitine concentrations increased 200 and 160%, respectively, during the first day of life ($p < 0.05$). Free carnitine concentrations measured in skeletal muscle were elevated at birth and were not affected by age. In liver, there was a 3.8- to 5.9-fold increase in relative γbutyrobetaine hydroxylase (γBBH) expression at 1 and 3 wk of age, respectively ($p < 0.05$). Despite no detectable differences in γBBH mRNA expression in the kidney throughout development, transcript amounts were on the order of 50- to 300-fold greater than those determined for the liver. To our knowledge, this study is the first to provide a complete molecular and kinetic assessment of the ontogeny of CPT I, and provide evidence for high capacity of de novo carnitine synthesis in the kidney of neonatal pigs.

Key words: carnitine palmitoyltransferase I (CPT I), γbutyrobetaine hydroxylase, carnitine development, pigs

INTRODUCTION

The rate of β-oxidation is regulated through the entry of fatty acids into the mitochondria, catalyzed by carnitine palmitoyltransferase I (CPT I). Located on the outer mitochondrial membrane, three isotypes have been identified: liver isotype (CPT Iα), skeletal muscle isotype (CPT Iβ) (McGarry and Brown, 1997), and a recently discovered isotype within the brain (CPT Iγ) (Price et al., 2002). The translocation of fatty acids by CPT I is often considered rate limiting, in part due to activity being regulated through inhibition by malonyl-CoA. In general, hepatic CPT I activity exhibits decreased sensitivity to inhibition by malonyl-CoA compared to skeletal muscle (IC_{50} 2.7 uM versus 34 nM, respectively, in rats) (McGarry et al., 1983). Additionally, hepatic CPT I sensitivity to malonyl-CoA is decreased under various physiological conditions which elevate circulating fatty acids (Krauss and Quant, 1996; Power and Newsholme, 1997; Pégorier, et al., 1988; Cook et al., 1984).

In recent years, research has shown that the nature of the protein is not conserved across species, as distinct differences exist for both liver and skeletal muscle isotypes expressed in the pig in comparison to the rat. Indeed hepatic CPT I expressed in the pig is a natural chimera of rat liver and muscle isotypes, and is 20 times more sensitive to inhibition by malonyl-CoA than the rat liver isotoype (Nicot et al., 2001). Furthermore, the skeletal muscle isotype of pig CPT I is less sensitive to inhibition than the liver isotype (Schmidt and Herpin, 1988). These differences in CPT I activity have led to the implication of CPT I in the low β-oxidative capacity of neonatal pigs (Odle et al., 1995).

In suckling rats, increased ketogenesis (Girard et al., 1992) is concomitant with increased activity of hepatic CPT I (Thumelin et al., 1994; Saggerson and Carpenter, 1982). At weaning, hepatic CPT I activity decreases as rats are transitioned onto a high carbohydrate diet (Thumelin et al., 1994). The changes in CPT I activity are paralleled by changes in mRNA expression (Asins et al., 1995). Research indicates that hepatic CPT I activity doubles during the first 24 hr of life and remains elevated through the suckling period, with the rate of change prior to 24 h being dependent on the initiation of suckling (Beiber et al., 1973). Furthermore, there is a 61 to 92% increase in skeletal muscle CPT I following birth (Schmidt and Herpin, 1998). Developmental changes in hepatic and skeletal muscle CPT I mRNA expression in the pig, however, remain unknown.

Activity of CPT I is dependent on adequate tissue carnitine concentrations. Within 2 days of life, levels of carnitine increase in heart, skeletal muscle, and liver of newborn pigs. The liver reflects the greatest change with an approximately 3 fold increase (Kerner et al., 1984). Carnitine levels continue to increase within liver and skeletal muscle until 4 d of age (Borum, 1991). Changes in tissue carnitine reflect changes in milk carnitine for the newborn rat (Robles-Valdez et al., 1976). Sow colostrum and milk contain considerable amounts of carnitine, however, it is unknown whether this serves as the primary source of carnitine for the neonatal pig as the majority of milk carnitine is found as short chain acylcarnitines, while piglet tissues are high in free carnitine.

Different carnitine profiles observed between sow milk and neonatal pig tissues could be a result of de novo carnitine synthesis. In the rat, hepatic carnitine synthesis is negligible until 8 d of age due to limited expression of the enzyme γ-butyrobetaine hydroxylase (γBBH) (Hahn, 1981). In humans the liver is also unable to synthesize carnitine at birth, however,

γBBH is expressed in the kidney also, and expression is not dependent on age (Olson and Rebouche, 1987).

The study outlined herein was conducted to provide a kinetic and molecular characterization of CPT I ontogeny. Piglets were chosen at different ages from birth until adulthood to determine developmental changes in CPT I activity and hepatic and skeletal muscle carnitine concentrations. Furthermore, exogenous carnitine supply from the diet and capacity for de novo synthesis of carnitine by measurement of γBBH mRNA expression in liver and kidney was assessed. The results presented provide a comprehensive evaluation of the supply of carnitine through exogenous and endogenous sources and the demand for carnitine by CPT I in the developing pig.

MATERIALS AND METHODS

Animals.

Procedures for this study were approved by the North Carolina State University Animal Care and Use committee. Commercial cross-bred pigs of normal body weight were obtained from NCSU Swine Education and Research Facility. Male and female pigs (n=5 per age group) were randomly selected at birth (non-suckled; 1.2 kg ± 0.23), 24 hr (1.52 kg ± 0.18), 1 wk (3.2 kg ± 0.24), 3 wk (5.45 kg ± 1.17), 5 wk (14.12 kg ± 2.60), and 8 wk of age (19.57 kg ± 5.32). The 3 wk old pigs were removed from the sow and represented the end of the suckling period. Data between 3 and 5 wk old pigs characterize the suckling-weaning transition. In addition, female pigs (7 ± 1 mo of age) were selected to represent mature animals (n=4). At the time of selection, milk or diet samples were collected for each age of pig.

Reagents.

[Methyl-^{3}H]carnitine (2.5 GBq/mol) and [1-^{14}C]acetyl-CoA (148 MBq/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Palmitoyl-CoA, acetyl-CoA, acetyltransferase and other chemicals were purchased from Sigma Chemicals (St. Louis, MO). Ion-exchange resin (AG 1x8, 100-200, Cl form)was purchased from Bio-Rad Laboratories (Richmond, CA).

Isolation of Liver and Muscle Mitochondria.

Pigs less than 8 weeks of age were euthanized via American Veterinary Medical Association approved electrocution. Adult pigs were euthanized by captive bolt. Liver, kidneys, and a portion of skeletal muscle (semitendonosis) were excised immediately. Liver and kidneys were weighed. Approximately 4 g of fresh liver and muscle were chilled on ice. Liver mitochondria were isolated by differential centrifugation as described by Mersmann et al., (1972). Muscle mitochondria were isolated following the procedure of Power and Newshome (1997) using isolation medium described by Saggerson and Carpenter (1981). Mitochondrial protein was determined by the biuret method (Gornall et al., 1949). In addition to fresh tissue, samples of liver, kidney, and skeletal muscle were rapidly frozen in liquid N and stored at -80 °C.

Analysis of carnitine palmitoyltransferase-I activity and measurement of L-carnitine.

Liver and muscle mitochondria were used to assess CPT-I activity as previously described by Bremer (1981). The assay measured the rate of formation of palmitoylcarnitine from palmitoyl-CoA and carnitine. Activity of CPT I was determined over a range of

carnitine concentrations from 0 to 2.5 mmol/L, while palmitoyl-CoA was fixed at 80 umol/L to generate Michaelis-Menten kinetic curves and determine the Km for carnitine. The reaction was initiated by the addition of [methyl-^{3}H]-carnitine, and terminated by the addition of 6% $HClO_4$ after 6 min. The labeled palmitoyl-carnitine was extracted with butanol and quantified by liquid scintillation spectrometry.

Levels of free L-carnitine were assayed in liver, muscle, plasma, and diet samples. Liver and muscle were prepared using the procedure described by Bhuiyan and co-workers (1992). Approximately 200 mg of tissue were homogenized in 400 uL ice-cold $HClO_4$ (1 mol/L) using a PowGen polytron homogenizer (Fisher Scientific, Pittsburgh, PA). The homogenate was centrifuged (10,000 x g, 5 min.) and the supernatant retained. The pellet was washed with 200 uL $HClO_4$ (.1 mol/L), recentrifuged, and the supernatants combined. The supernatant was neutralized with 400 uL KOH (1 mol/L) and the resulting $KClO_4$ was precipitated by centrifugation. The supernatant was subsequently used for analysis of free carnitine. For plasma, the above procedure was followed with the following modifications: 200 uL of ice-cold $HClO_4$ (1 mol/L) and 200 uL of plasma were vortexed and centrifuged as above, the pellet was washed with 100 uL $HClO_4$ (.1 mol/L), and the combined supernatant was neutralized with 200 uL KOH (1 mol/L). Free carnitine was extracted from diet samples following the procedure outlined for tissues samples. For milk samples, 200 uL was used for the extraction procedure. For dry diet samples, 200 uL of water was added to 100 mg of diet prior to homogenization in ice-cold $HClO_4$. Extracted free carnitine was assayed by the enzymatic radioisotope method described by McGarry and Foster (1976). Carnitine extracted samples were incubated with [1-^{14}C]acetyl-CoA and carnitine acetyltransferase I.

The formation of [1-^{14}C]acetyl-carnitine was determined by liquid scintillation spectrometry following elution of [1-^{14}C]acetyl-carnitine from an anionic (Cl-) column.

Extraction of total RNA.

Total RNA was extracted from kidney using RNeasy kit (Qiagen, Inc). Liver and muscle total RNA was isolated using the guanidine isothiocyanate and phenol based TRI Reagent solution (Sigma-Aldrich, Inc.). Briefly, tissue was homogenized in TRI reagent (.1g tissue/mL TRI Reagent), and .2mL of chloroform (per 1 mL TRI Reagent) was added to the homogenate. Following centrifugation (12,000 x g, 15 min.) the RNA was precipitated from the aqueous phase by the addition of isopropanol (.5 mL/mL TRI Reagent) followed by centrifugation (12,000 x g, 10 min.). The resulting pellet was washed with 75% ethanol and resuspended in RNase free water. Gel electrophoresis with ethidium bromide staining was used to asses RNA integrity. The RNA was quantified and purity assessed by UV spectrophotometric analysis of A_{260} and A_{260}/A_{280} ratio (> 1.7), respectively.

Reverse Transcription.

The RNA (10 ug/50uL) was treated with 4 units of TurboDNase™ (Ambion, Inc.) according to the manufacturers instructions for removal of genomic DNA. The RNA was recovered following phenol:chloroform extraction and 2 ug was reverse transcribed using Omniscript™ reverse transcription kit according to the manufacturer's instructions (Qiagen, Inc.). First strand cDNA was synthesized at 42ºC for 60 min by priming with oligo-dT (Roche Diagnostics, Inc). A negative control (using pooled RNA) containing all of the reverse transcription reagents in the absence of RT enzyme was included (no RT control).

Primer Design.

The primer design program Lasergene Primer Select (DNAstar, Inc.) was used to design pig specific primers for cyclophilin, β-actin, hypoxanthine phosphoribosyltransferase (HPRT), and CPT I specific for liver and muscle (Table 1). For γ-butyrobetaine hydroxylase (γBBH), rat, human, and mouse γBBH partial cds were aligned (Megalign, DNAstar, Inc.), highly conserved regions were identified, and primers selected from these regions due to lack of information regarding pig sequence data. A BLAST search (Nucleotide database for BLAST; NCBI) was conducted on all primer pairs for assessment of primer specificity. Primers were obtained through Sigma-Genosys, Inc. and were resuspended in 1x Tris/EDTA.

Real-time RT-PCR with SYBR Green.

Real-time RT-PCR was performed using the DNA Engine Opticon® (MJ Research, Inc.). Real-time fluorescence detection was performed in 96 well plates using Quantitect™ SYBR Green buffer (Qiagen, Inc). Samples were assayed in triplicate. Each 20 uL reaction contained cDNA (equivalent to 100 ng RNA) and .3 uM each of the forward and reverse primers. Following an initial 15 min. incubation at 95 °C for activation of the Hot Start Taq DNA polymerase, all templates were amplified for 40 cycles using the following protocol: denaturation for 15 sec at 94 °C, primer annealing for 30 sec at 60 °C, and elongation for 30 sec at 72 °C. Fluorescence data was acquired at the end of each elongation phase. A melting curve was generated by measuring the fluorescence as the temperature was raised from 50 °C to 95 °C in .2 °C increments to verify specificity of fluorescence detection. Specificity of amplification products was further verified by gel electrophoresis and ethidium bromide staining (Figure 1). Background fluorescence was set as the average fluorescence between

cycles 3 and 15 and threshold (Ct) was determined based on 10 standard deviations above background fluorescence (Giulietti et al., 2001). Positive (no template reaction) and negative (no reverse transcriptase) controls were run in triplicate and included in each PCR assay.

Analysis of Real Time RT-PCR Data.

Cyclophilin, HPRT, and β-Actin were evaluated as potential reference genes for this experiment. Expression was assessed over time and across tissues. Based on preliminary data showing constitutive expression of hypoxanthine phosphoribosyltransferase, HPRT was selected and standard curves for the reference gene and the target genes of interest for liver (LCPT I and γBBH), kidney (γBBH), and muscle (MCPT I) were generated from pooled cDNA (equivalent to 200, 100, 50, 25, and 12.5 ng of total RNA). The Ct values were recorded and a plot of the linear regression of the log of template cDNA concentration against the Ct was made. The quality of the standard curve was assessed according to the correlation coefficient ($R^2 > .95$) and the slope of the line was used to determine efficiency of amplification according to the equation $E = 10^{-1/slope}$ (Pfaffl, 2001; Figure 2). Due to unequal amplification efficiencies between the reference gene and target genes, a relative expression ratio indicating fold change was determined according to the method by Pfaffl (2001) for assessment of treatment differences using the following equation:

$$\text{Expression Ratio} = \frac{E^{\Delta Ct}{}_{\text{target}}}{E^{\Delta Ct}{}_{\text{reference}}}$$

Where ΔCt = sample – control for the target gene and reference gene. Within each tissue, differences in Ct values for all ages (sample) were assessed against Ct values obtained for newborn pigs (control). In addition, a comparison of γBBH expression between liver (control) and kidney (sample) was made, for this comparison Ct values for each age in kidney was assessed against the same age in liver tissue.

Variance of the expression ratio was determined according to Marino and co-workers (2003),

$$\text{Variance}_{\text{lnR}} = \left(\frac{sCt_1}{\beta}\right)^2 + \left(\frac{sCt_2}{\beta}\right)^2 + \left(\frac{(Ct_2\text{-}Ct_1)\, s_\beta}{\beta^2}\right)^2$$

where Ct_1 = sample, Ct_2 = control and sCt_1 and sCt_2 = estimate of standard error (average of triplicate readings) for the sample and control (respectively), β = slope of the gene being evaluated and s_β = error of the slope. Log variance was determined for the target genes and reference gene. Target gene variance was corrected for differences in reference gene using the following equation:

$$\text{corrected variance}_{\text{lnR}} = \text{variance}_{\text{lnRTarget}} + \text{variance}_{\text{lnRreference}}$$

The corrected variance was linearized and the standard error determined.

Statistical Analysis:

Carnitine palmitoyltransferase I activity data were fit to Michaelis-Menten kinetic curves using the non-linear (NLIN) model of SAS (SAS Inc., Cary, NC) for determination of

Vmax and Km. The apparent Vmax, Km, and levels of L-carnitine were subjected to ANOVA using the general linear model procedure of SAS. A one-sided students t-test was used for determining significance in fold change of CPT I and γBBH expression relative to the newborn and differences in γBBH expression in the kidney relative to the liver. Data were reported significant when $p < 0.05$.

RESULTS

Carnitine palmitoyltransferase I kinetics in liver and skeletal muscle.

Hepatic CPT I activity (Figure 3a) increased from birth to 1 wk of age. By 1 wk of age hepatic CPT I activity was 2 fold greater than that measured in the newborn ($p < 0.05$). There was no difference in hepatic CPT I activity between 1 and 8 wk of age (Table 2). By 7 mo of age, activity had declined to values comparable to those measured in the 24 h old pig. In skeletal muscle, the pattern of CPT I activity followed a similar trend to that observed in the liver (Figure 2b). Activity was low in the newborn, increased 1.7-fold by 1 wk of age ($p <0.05$) and remained elevated until 8 wk of age. Between 8 wk and 7 mo of age, skeletal muscle CPT I activity declined to levels similar to that measured in the newborn and 24 h old pig (Table 2). There were no differences in CPT I activity between liver and skeletal muscle at the various ages ($p > 0.05$).

Hepatic CPT I apparent Km for carnitine progressively decreased by 48% from birth to 3 wk of age (Table 3). By 5 wk of age the Km for carnitine increased to levels comparable to those measured in the newborn, before decreasing 28% by 7 mo of age. The apparent Km for carnitine in skeletal muscle decreased from birth to 1 wk of age. The apparent Km increased 200% from 1 wk of age to 5 wk of age in skeletal muscle ($p< 0.01$), and then

decreased 60% from 5 wk to 7 mo of age ($p < .05$). At 3 wk and 5 wk of age the apparent Km of CPT I for carnitine was 2- and 1.6-fold greater, respectively, in skeletal muscle compared to liver ($p < 0.05$).

Carnitine Concentrations.

Plasma free carnitine concentrations doubled within the first 24 h of life for the suckled pig compared to the non-suckled newborn ($p < 0.05$). Concentrations in the liver increased 160% during the first day of life ($p < 0.05$) and remained elevated during the suckling period (Table 4). The suckling-weaning transition coincided with a 37% decrease in hepatic free carnitine concentrations ($p < .01$). Free carnitine concentrations in 7 mo old pigs did not differ from those measured in the newborn ($p > 0.1$). Plasma values decreased 37% between 24 h and 1 wk of age, and were not further affected by development. Free carnitine concentrations measured in skeletal muscle were elevated at birth and were not affected by age.

Validation of a suitable reference gene.

Hypoxanthine phosophoribosyltransferase was evaluated at the various ages and across tissues to verify constitutive expression for use of the gene as reference. Standard errors of readings within each age and across all ages were similar, indicating that HPRT did not differ significantly with age. Similar calculations of standard error for each tissue and across all tissues also indicted HPRT expression was not affected by tissue type. Furthermore, the efficiency of amplification between tissues was similar (Figure 4), validating the use of HPRT as a suitable internal reference.

Assay variability of RT-PCR.

Intra-assay variability was assessed from the coefficient of variance (CV) from samples run in triplicate. The average intra-assay variation ranged from 0.89 to 1.66% for $HPRT_{Liver}$, 1.48 to 2.09% for LCPT I, 0.84 to 1.25% for MCPT I, and 0.94 to 1.34% for γBBH_{Kidney}. Overall CV's for triplicate readings of samples for $HPRT_{Liver}$ (n=34), L CPT I (n=34), M CPT I (n=33), and γBBH_{Kidney} (n=34) were 1.20%, 1.78%, 1.05%, and 1.08%, respectively.

Expression of CPT I and γBBH.

Hepatic CPT I mRNA relative expression decreased 13 fold during the initial 24 hr of life ($p < 0.001$), and continued to remain lower than levels expressed at birth until 7 mo of age (Figure 5a). In skeletal muscle expression of CPT I mRNA was reciprocal to that observed in liver during the initial 3 wk of life. Transcript amounts of skeletal muscle CPT I increased until 3 wk of age relative to that measured at birth, and decreased precipitously from an 11 fold difference measured between 3 wk and birth to a 2.4 fold difference measured between 5 wk of age and birth (Figure 5b).

In liver, there was a 3.8- to 5.9-fold increase in relative γBBH expression at 1 and 3 wk of age, respectively ($p < 0.05$; Figure 6a). Relative expression of hepatic γBBH at 5 wk of age decreased 50% compared to relative expression measured at 3 wk of age. By 8 wk of age γBBH expression had increased and was similar to that observed in the 1 wk old pig. Unlike hepatic γBBH mRNA expression, expression in the kidney did not appear to be subject to developmental regulation. Despite no detectable differences in γBBH mRNA expression in the kidney throughout development, transcript amounts were on the order of 50- to 300-fold greater than those determined for the liver (Figure 6); with average combined

kidney weight being 24, 26, 11, 22, 16, 18, and 21% of average liver weight for the newborn, 24 hr, 1 wk, 3 wk, 5 wk, 8 wk, and 7 mo old pig, respectivley.

DISCUSSION

The research outlined herein is the first to provide a developmental assessment of post-natal CPT I kinetics and mRNA expression, contributing to the growing body of research highlighting the unique properties of pig CPT I.

Developmental changes in CPT I activity.

Previous research in pigs (Beiber et al., 1972) and rats (Saggerson and Carpenter, 1982; Thumelin et al., 1994) indicates that CPT activity increases rapidly to a maximal rate during the first day of life, and remains high throughout the suckling period. The current research provides evidence that CPT I activity continues to increase until 1 wk of age in pigs and remains elevated until 7 mo of age. In rats, the suckling to weaning transition is coincident with a 50% decrease in hepatic CPT I activity. Furthermore, CPT I specific activity determined post weaning is equivalent to activity determined for 7 mo old pigs (Thumelin et al., 1994). In contrast, activity measured 2 wk after weaning in the pig was 22% greater than activity measured at 24 hr of age and similar to activity determined at 1 wk of age. The decrease in CPT I activity that occurs at weaning is attenuated when the rat pup is weaned to a high fat diet. Activity of CPT I for rat pups weaned onto a carbohydrate rich diet is 70% of the activity determined for pups maintained on a high fat diet at weaning (Thumelin et al., 1994). In the dog, it has also been inferred that CPT activity is reflected by changes in fat content of the diet (Lin and Odle, 2003). The decrease in lipid, and reciprocal

increase in carbohydrate energy sources that occurs at weaning in the pig, however, had no affect on hepatic CPT I activity.

There is strong evidence that long chain fatty acids have a role in the transcription of various genes, including CPT I (Louet, et al., 2001), however, the effect of dietary fat on modulation of CPT I activity is less convincing. While Thumelin and co-workers observed an increase in CPT I activity with feeding a high fat diet (72% fat content) compared to a carbohydrate rich diet, Power and co-workers (1994) only observed an effect of safflower oil on CPT I specific activity, despite also feeding diets containing 20% coconut oil, olive oil, or fish oil, inferring that elevated fatty acids in the diet are not solely responsible for modulating CPT I activity. Instead, dietary fatty acids influence inhibition of CPT I by malonyl-CoA. For instance, in the rat CPT I is less sensitive to inhibition by malonyl-CoA under conditions which elevate circulating fatty acids such as the suckling period (Krauss and Quant, 1996), induction of diabetes (Cook et al., 1984), fasting (Cook et al., 1980), and high-fat diets (Pégorier, et al., 1988; Power and Newsholme, 1997). This decrease in sensitivity is paralleled by a decline in tissue content of malonyl-CoA (McGarry et al., 1983; Chien et al., 2000). In skeletal muscle, the effects of a high fat diet on CPT I inhibition are less pronounced, and only apparent after prolonged consumption of a high fat diet (Power and Newsholme, 1997). In the pig, hepatic CPT I is 20 times more sensitive to inhibition by malonyl-CoA, and behaves as a natural chimera of rat liver and muscle isotypes (Nicot et al., 2001). Consequentially, malonyl-CoA inhibition of hepatic CPT I is not altered by changes in physiological state (ie starvation) (Schmidt and Herpin, 1998; Duée et al., 1994).

In skeletal muscle CPT I activity followed a similar pattern of development as did hepatic CPT I activity (Figure 2 a and b). Specific activity increased 164% during the initial

wk of life, in agreement with early studies which evaluated the change in CPT I expression between birth and 5 d of age (Schmidt and Herpin, 1998). Less information is available regarding the activity of CPT I in skeletal muscle of pigs. Schmidt and Herpin (1998) have provided evidence of enhanced CPT I in slow oxidative (rhomboideous) versus fast glycolytic (longissimus dorsi) muscles, a consequence of the subpopulation (intermyofibrillar subsarcolemmal) of mitochondria present in these muscle types (Cogswell et al., 1993).

Changes in CPT I mRNA expression from birth to adulthood.

Early post-natal hepatic CPT I activity in the rat parallels both CPT I protein and CPT I mRNA expression (Thumelin et al., 1994, Asins et al., 1995). Expression of CPT I mRNA is undetectable in the rat fetus, increases 7-fold at birth, and continues to increase until 3 d of age. Expression remains elevated during suckling, but progressively decreases from 3 d of age until weaning. At weaning, CPT I mRNA expression is equivalent to that measured in the adult. In contrast, hepatic CPT I activity in the pig does not coincide with mRNA expression (Figure 8). A 93% reduction in mRNA expression was measured between birth and 24 h of age. Relative mRNA amount increased slightly at 3 and 8 wk of age (80% of the transcript amount present at birth) and rebounded to values equivalent to the newborn by 7 mo of age.

Hepatic CPT I activity and expression in the rat mimics the developmental changes that occur with 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase (Asins et al., 1995), where expression rises rapidly after birth, and falls with the suckling-weaning transition (Thumelin et al., 1994). Although not measured in this study, research suggests that HMG-CoA synthase transcript amounts are minimal during the first week of life for the young pig

and increase during the second and third postnatal week (Adams et al., 1997b). Low hepatic CPT I mRNA expression for the young pig determined in the current study coincides with the developmental pattern established for HMG-CoA synthase. Concomitant regulation of CPT I and HMG-CoA synthase indicate that both genes are targets of the same transcriptional activator.

The elevated CPT I activity that persists until adulthood despite decreased mRNA expression marks the importance of post-transcriptional events in regulation of hepatic CPT I. While malonyl-CoA has been shown to impart significant control over CPT I activity in the pig (Nicot et al., 2001), the method of mitochondrial isolation for the current study should provide a malonyl-CoA free system. However, changes in protein half-life or phosphorylation state are unknown. Recently, covalent modification of CPT I by phosphorylation has been suggested, with phosphorylation increasing CPT I velocity 20-25% (Kerner et al., 2004).

Age related changes in tissue and circulating carnitine concentration.

In accord with early research examining the neonatal profile of tissue carnitine concentrations (Borum, 1983), hepatic carnitine concentrations increased 164% during the initial day of life, remained elevated through 1 wk of age, before decreasing 16% at 3 wk of age (Table 4). During the suckling-weaning transition carnitine concentrations dropped dramatically and were 67% of the value measured prior to weaning. Hepatic and skeletal muscle carnitine concentrations in the suckling rat and rabbit are directly correlated to dietary carnitine levels. Failure to suckle in the rabbit results in diminished carnitine concentrations in the liver and skeletal muscle (Penn and Schmidt-Sommerfeld, 1988). In rats, hepatic and

skeletal muscle carnitine concentrations increase 5- and 3-fold, respectively, in starved versus fed neonatal animals (Flores et al., 1996). Three lines of evidence support the relationship between dietary carnitine and tissue carnitine: 1) Carnitine concentration is highest in milk immediately preceding parturition, after which, the concentration steadily decreases until weaning, 2) The relationship between carnitine concentration in milk through development parallels the change in free and total liver carnitine concentration measured in rat pups during the suckling period, and 3) [^{14}C]-butyrobetaine administered to rat mothers accumulates as labeled carnitine in maternal liver, milk, and subsequently the neonatal liver over time (Robles-Valdez, 1976).

Sows milk is also high in carnitine. The greatest concentrations are measured immediately post-partum, gradually decreasing until weaning (Figure 9). It is unknown whether this serves as the primary source of neonatal carnitine as the majority of carnitine in sow's milk is isolated as acyl-carnitines, while piglet tissues are high in free carnitine (Kerner, 1984). However, the dramatic decrease in hepatic carnitine concentrations during the suckling-weaning transition parallels the decrease in dietary carnitine that occurs after weaning, and would support the role of dietary carnitine in maintaining tissue carnitine in the young pig. In skeletal muscle, the already high carnitine concentrations present at birth were unaffected by development. While pig hepatic carnitine concentrations respond to dietary carnitine, research suggests that skeletal muscle carnitine concentrations do not emulate dietary changes (Coffey et al., 1991) and may contribute to the lack of developmental effect observed in the pig compared to the rat.

Expression of γBBH and the potential for de novo carnitine synthesis.

The elevated concentrations of carnitine in skeletal muscle from the non-suckled newborn pig reflects the placental transfer of carnitine to fetal tissues (Schmidt-Sommerfeld et al. 1981). However, the maintenance of high skeletal muscle carnitine concentrations in a tissue which does not respond to dietary carnitine (Coffey et al., 1991) may be a reflection of the capacity of the young pig for de novo carnitine synthesis. Research indicates that the rat is unable to adequately synthesize carnitine as a neonate due to lack of γBBH activity in the kidney and limited expression in the neonatal liver (Englard et al., 1978). In other species, including dog, cat, and human, activity of γBBH is found in the kidney and liver. Furthermore, activity within the kidney exceeded that of the liver in cats and humans (Rebouche, 1982). It has been suggested, that humans are capable of de novo synthesis at all stages of life due to kidney γBBH activity (Olson and Rebouche, 1987). To this end, γBBH mRNA expression was assessed in liver and kidney of developing pigs. While kidney showed no developmental regulation, liver expression increased 4- and 6-fold by 1 wk and 3 wk of age when compared to newborn pigs (Figure 6). However, when liver expression was compared with kidney, there was a 50 to 300-fold greater amount of γBBH confined to the liver. When amounts of mRNA expressed were adjusted for differences in tissue size, γBBH expression confined to the kidney was 10- to 70-fold greater than liver, providing evidence that the neonatal pig has the potential for de novo carnitine synthesis and based on transcript amount, the kidney is the major site of de novo carnitine synthesis for this species.

Plasma carnitine status.

Carnitine originating from de novo synthesis or dietary sources must be transported across the tissue from the plasma. Although uptake of carnitine by tissues involves active transport (Lahjouji, et al., 2001), making plasma carnitine not a reliable index of tissue carnitine, changes in plasma carnitine are indicative of overall changes in whole animal carnitine status. Circulating plasma carnitine concentrations increased 2-fold between birth an 24 h of age, similar to the change observed for hepatic carnitine concentrations. This rapid change in circulating carnitine may be attributed to the onset of suckling and the supply of carnitine from the dams' milk, which is similar to the situation that occurs in the rat (Robles-Valdez et al., 1976). It is interesting to note, that plasma carnitine concentrations did not change from 1 wk through 7 mo of age (Table 4); however, at 5 wk of age liver carnitine concentrations were at their lowest values while maximal concentrations in skeletal muscle carnitine were measured. The increased skeletal muscle carnitine, coupled with decreased dietary carnitine and decreased liver carnitine, without changes in plasma carnitine infers that carnitine status is being maintained through increased de novo synthesis, ensuring adequate tissue carnitine in skeletal muscle for CPT I activity.

Relationship between tissue carnitine concentrations and CPT I Km for carnitine.

The concentration of free carnitine within tissues may serve as a predictor of the capacity of CPT I for β-oxidation when circulating fatty acids are elevated. The apparent Km for carnitine approximates the carnitine concentration necessary for CPT I to catalyze the conversion of fatty acyl-CoA to acylcarnitine at a rate which is half of its maximal velocity. For many key enzymes, when the substrate concentration is in the vicinity of the Km, the rate

at which the reaction proceeds is proportional to substrate concentration. Prevailing tissue carnitine concentrations at or below CPT I apparent Km for carnitine could limit flux of acyl groups into the mitochondria for β-oxidation. At all developmental ages, free carnitine measured in skeletal muscle met or exceeded the apparent Km for carnitine of CPT I (Figure 10). In dogs, skeletal muscle carnitine concentrations are 10-fold their apparent Km in skeletal muscle (Lin and Odle, 2003), and humans have skeletal muscle carnitine concentrations considerably greater than their apparent Km (approximately 6-fold) (McGarry, 1995; Cederblad et.al.,1974). In pigs, skeletal muscle carnitine concentrations exceeded the Km by a maximal of 2-fold. As increased carnitine can increase CPT activity and flux of fatty acids across the mitochondrial membrane for subsequent oxidation (Pande and Parvin, 1980), significantly higher carnitine concentrations may be important for ensuring increased capacity for fatty acid oxidation.

In the liver, apparent Km for carnitine progressively decreased from birth until weaning at 3 wk age (Table 3). While, hepatic carnitine was below the Km at birth, levels exceeded the Km for carnitine in suckled 1 d old pigs. Between 3 and 5 wk of age the Km doubled, and exceeded the concentration of free carnitine in the liver through 7 mo of age (Figure 9). The change in hepatic carnitine paralleled the change in hepatic γBBH transcript amounts (Figure 6a) and suggests that the rate of CPT may be limited by tissue carnitine levels at this age due to decreased dietary supply and decreased hepatic de novo synthesis.

The current study supports earlier suggestions that dietary sources of carnitine are inadequate during the suckling-weaning transition carnitine (Owens et al., 1996) and biosynthesis of carnitine in the young pig is limited (Kerner et al., 1984), thereby indicating a need for carnitine supplementation. However, research examining the role of supplemental

L-carnitine on growth parameters in young pigs has been variable (Rincker et al., 2003., Heo et al., 2000a; Owen et al., 1996; Hoffman, et al., 1993). In rats (Clouet et al., 1996), humans (Novak et al., 1983), and pigs (Coffey et al., 1991) plasma carnitine concentrations are increased following carnitine supplementation. Furthermore, in rats (Clouet et al., 1996) and pigs (Heo, et al., 2000b) free carnitine concentrations of liver and skeletal muscle are also increased with supplementation. Increased hepatic carnitine coincided with decreased sensitivity of CPT I to malonyl-CoA and increased CPT I activity with increasing concentrations of palmitoyl-CoA in the rat (Clouet et al., 1996). In preterm infants, carnitine supplementation has been reported to increase circulating ketone bodies (Schmidt-Sommerfeld et al., 1988) and enhance lipid oxidation (Sulkers et al., 1990). In the pig, artificial diets high in carnitine during the suckling period do not alter lipid oxidation or β-hydroxybutyrate production, when compared to piglets allowed to suckle the dam (Coffey et al., 1991). The majority of research concerned with the effects of L-carnitine supplementation on parameters of lipid metabolism in the pig have focused on early postnatal development, in part due to the transition to a high carbohydrate diet at weaning, therefore, the metabolic effects of L-carnitine supplementation in the weaned pig remains unknown.

In summary, this study demonstrates that post-natal development of hepatic and skeletal muscle CPT I activity follows a pattern similar to that observed in the rat (Thumelin et al., 1994; Saggersen and Carpenter, 1982). Activity is lowest in the newborn, increases dramatically during the first week of life, and remains elevated during the suckling period. Carnitine palmitoyltransferase I activity during the suckling period is supported by tissue carnitine concentrations, that are most likely a direct result of the high levels of carnitine supplied by the milk. However, the decrease in hepatic carnitine coinciding with an increase

in CPT I's Km for carnitine during the suckling to weaning transition may be indicative of inadequate carnitine status during this transition. In contrast, skeletal muscle carnitine is constitutive, and does not appear to reflect changes in dietary carnitine. The maintenance of skeletal muscle carnitine appears to be due to the kidney supplying carnitine though de novo synthesis. In liver, minimal γBBH mRNA expression implies limited capacity of this tissue for carnitine synthesis, as a result, the liver is unable to maintain adequate carnitine levels when dietary supply is decreased.

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CHAPTER 3 TABLES

Table 3.1
Primers used for amplification of Cyclophilin, hypoxanthine phosphoribosyltransferase (HPRT), β-Actin, liver and skeletal muscle isotypes of carnitine palmitoyltransferase I (LCPT I and MCPT I), and γbutyrobetain hydroxylase (γBBH) cDNA and expected amplicon size following SYBR Green RT-PCR. Accession numbers listed reference published Genbank sequences which were used to design forward and reverse primers.

Gene	Primer sequence (5' → 3')	Amplicon Size (bp)	Accession
Cyclophilin	F: CCCTTGGGCCGCGTCTCCTT R: CACCCTGGCACATAAATCCTG	148	AY008846
HPRT	F: CCATCACATCGTAGCCCTCTG R: TACTTTTATATCGCCCGTTGACTG	172	AF143818
β-Actin	F: AGCGGGAGATCGTGCGGGACAT R: AGGAAGGAGGGCTGGAAGAG	187	SSU07786
L-CPT I	F: GGACCGCCACCTGTTCTGCCTCTA R: GCCCCCTCCGCTCGACACATAC	175	AF288789
M-CPT I	F: GCACGCCAGGCCTTCTTCAGC R: TGGCCTCGTCTTCCGGGTCATAGT	121	AY181062
γBBH	F: GATGATAAAGGCCAAGTGGTTCGC R: CTCATCACCTGGATTCATCTTG	171	$AY033514_{mouse}$ $AF160958_{rat}$ $AF082868_{human}$

Table 3.2
Maximal activity of carnitine palmitoyltransferase I in liver and skeletal muscle of developing pigs[1]

Age		Liver	Skeletal Muscle
	(n)	*V_{max} (umol/h·g mitochondrial protein)*	
Newborn	5	17.0 ± 5.5 [a]	24.6 ± 4.8 [ac]
24 h	5	25.1 ± 5.5 [a]	25.8 ± 4.8 [ac]
1 wk	5	35.6 ± 5.5 [b]	41.2 ± 4.8 [b]
3 wk	5	33.5 ± 5.5 [b]	38.9 ± 4.8 [bc]
5 wk	5	32.3 ± 5.5 [b]	44.4 ± 4.8 [b]
8 wk	5	39.0 ± 5.5 [b]	38.0 ± 4.8 [bc]
7 mo	5[2]	16.2 ± 5.5 [a]	17.3 ± 5.4 [a]

[1] Values are means ± SEM
[2] For skeletal muscle n=2
[abc] Means within a column lacking a common superscript differ ($p < 0.05$).

Table 3.3
Apparent Km of carnitine palmitoyltransferase for carnitine in liver and skeletal muscle of developing pigs[1]

Age		Liver	Skeletal Muscle
	(n)	*Apparent K_M for L-Carnitine (uM)*	
Newborn	5	278 ± 29 ^{ab}	381 ± 52 ^{ac}
24 h	5	254 ± 29 ^{ac}	245 ± 52 ^{bc}
1 wk	5	179 ± 29 ^{c}	228 ± 52 ^{bd}
3 wk	5	147 ± 29 $^{c;x}$	314 ± 52 $^{acd;y}$
5 wk	5	280 ± 29 $^{b;x}$	460 ± 52 $^{a;y}$
8 wk	5	244 ± 29 ^{ac}	326 ± 52 ^{acd}
7 mo	5[2]	218 ± 29 ^{ac}	280 ± 58 ^{bc}

[1] Values are means ± SEM
[2] For skeletal muscle n=2
^{abc} Means within a column lacking a common superscript differ ($p < 0.05$).
^{xy} Means within a row lacking a common superscript differ ($p < 0,05$).

Table 3.4
Concentration of free carnitine in plasma, liver, and skeletal muscle of developing pigs[1]

		Free carnitine (nmol/mL or g wet tissue)		
Age	(n)	Plasma	Liver	Muscle
Newborn	5	62 + 17	189 + 33[ac]	397 + 19
24 h	5	121 + 17[a]	311 + 33[b]	396 + 19
1 wk	5	83 + 17	329 + 33[b]	408 + 19
3 wk	5	73 + 17	277 + 33[bc]	386 + 19
5 wk	5	84 + 17	104 + 33[a]	415 + 19
8 wk	5	75 + 17	183 + 33[ac]	369 + 19
7 mo	4[2]	86 + 19	130 + 37[a]	390 + 19

[1] Values are means ± SEM
[2] For skeletal muscle n=5
[abc] Means within a column lacking a common superscript differ ($p < 0.05$).

CHAPTER 3 FIGURES

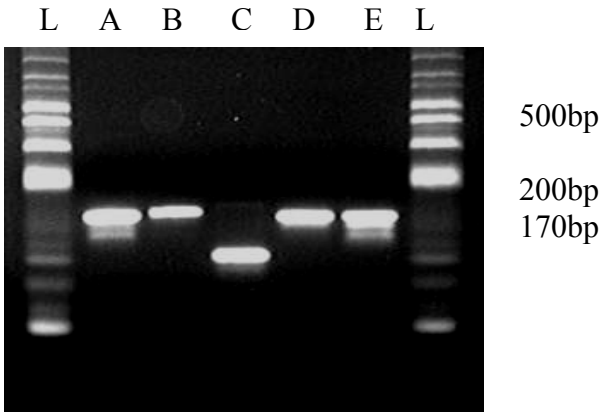
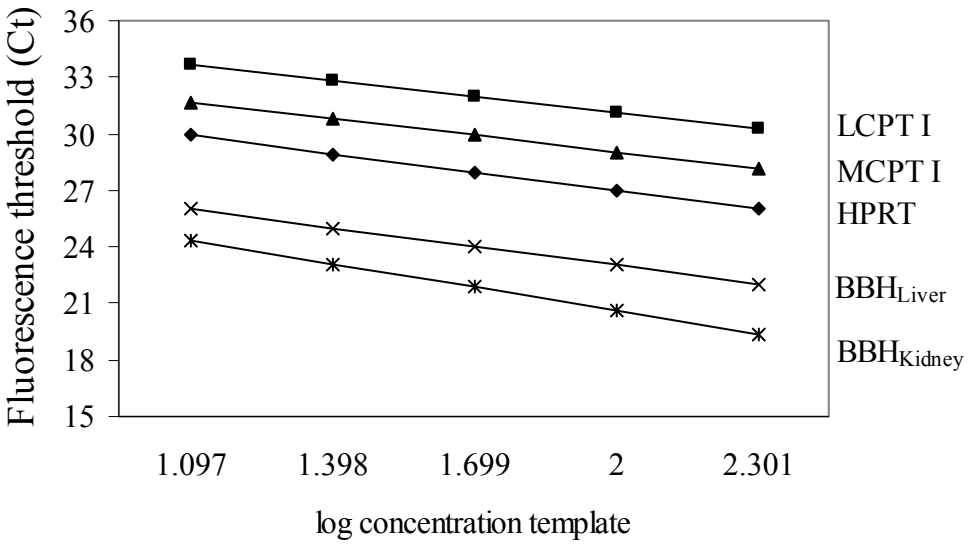


Figure 3.1
Amplification specificity by real time PCR of hypoxanthine phosphoribosyltransferase (A), carnitine palmitoyltransferase I, liver isotype (B), carnitine palmitoyltransferase I, skeletal muscle isotype (C), γ-butyrobetaine hydroxylase expressed in liver (D), and γbutyrobetaine hydroxylase expressed in kidney (E). Visualization of PCR products in agarose gel. Products size was identified by running samples alongside a 500 bp DNA ladder (L).

(a)

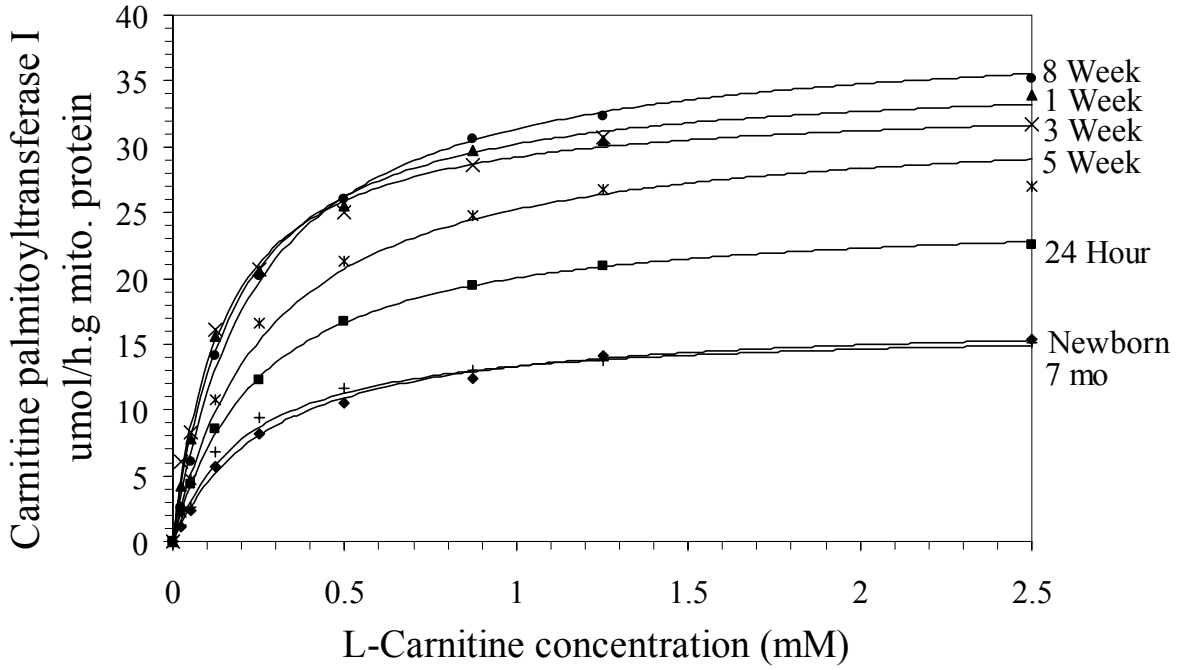


(b)

Gene	Slope	r^2	Efficiency	Average Ct
HPRT	-3.35	0.97	1.99	24.1 ± 2.58
LCPT I	-2.83	0.99	2.23	30.3 ± 3.85
M CPT I	-2.94	0.97	2.19	28.4 ± 2.66
BBH_{Liver}	-3.30	1	2.01	27.9 ± 2.26
BBH_{Kidney}	-4.12	0.98	1.75	22.0 ± 1.83

Figure 3.2
Relationship between cDNA template concentration and Ct. (a) Linear regression of log concentration of template versus C_t. Template concentrations were equivalent to 200, 100, 50, 25, and 12.5 ng RNA. Values are means of triplicate readings for the genes hypoxanthine phosphoribosyltransferase (HPRT; ♦), liver isotype carnitine palmitoyltransferase I (LCPT I; ■), skeletal muscle isotype carnitine palmitoyltransferase I (MCPT I; ▲), γbutyrobetaine hydroxylase expressed in liver(γBBH_{Liver}; ×), γbutyrobetaine hydroxylase expressed in kidney (γBBH_{Kidney}; +). (b) Efficiency of amplification for each gene. Efficiency calculated as $E = 10^{-1/slope}$. Average Ct values ± SE are representative of starting template concentration equivalent to 100 ng and averaged across all ages

(a)



(b)

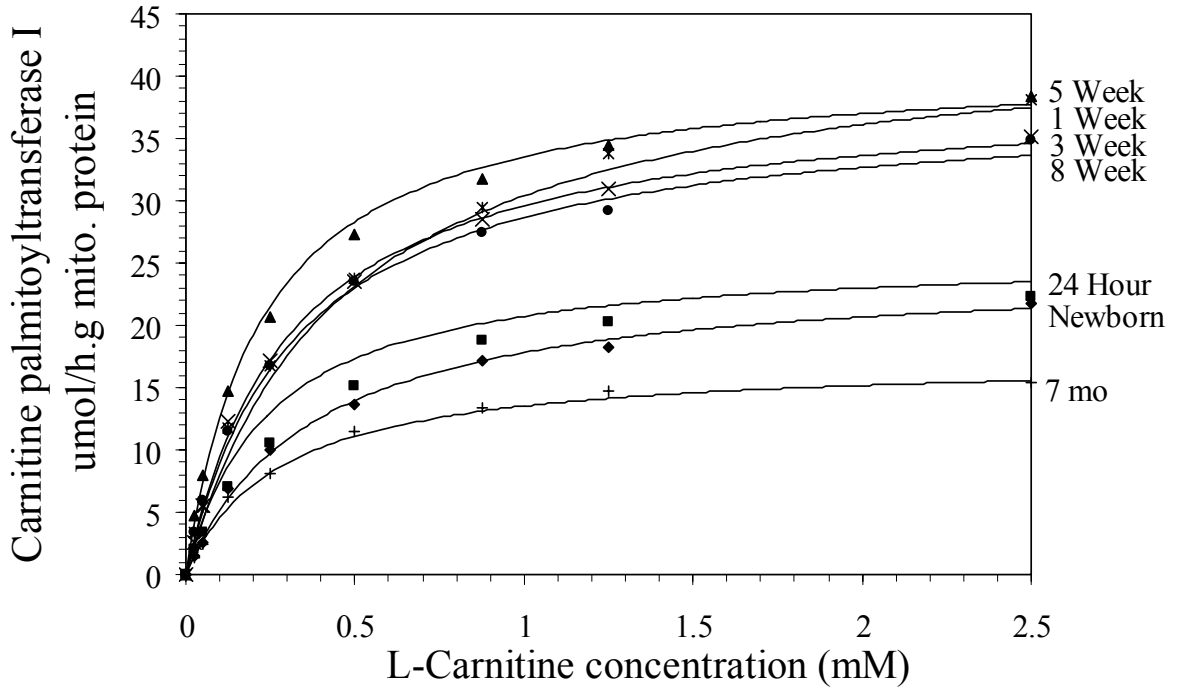
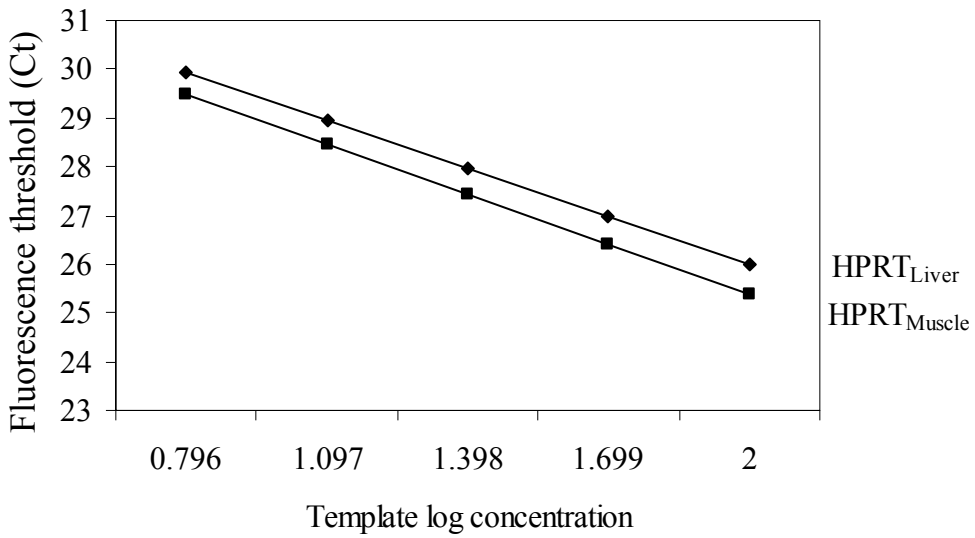


Figure 3.3
Ontogney of carnitine palmitoyltransferase I activity in response to increasing carnitine concentrations in liver (a) and skeletal muscle (b). Values are means, n=2-5.

(a)

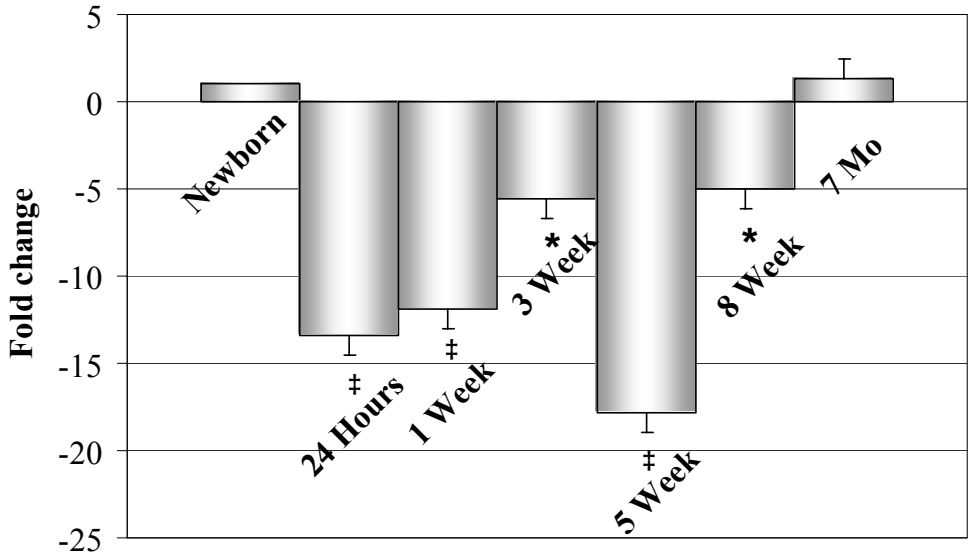


(b)

Gene	Slope	r^2	Efficiency
$HPRT_{Liver}$	-3.23	0.96	1.99
$HPRT_{Muscle}$	-3.40	0.97	1.97

Figure 3.4
Relationship between cDNA template concentration and Ct. (a) Linear regression of log concentration of template versus C_t. Template concentrations were equivalent to 200, 100, 50, 25, and 12.5 ng RNA. Values are means of triplicate readings for the genes hypoxanthine phosphoribosyltransferase (HPRT) expressed in the liver (♦) or kidney (■). (b) Efficiency of amplification for HPRT in liver or kidney. Efficiency calculated as $E = 10^{-1/slope}$.

(a)



(b)

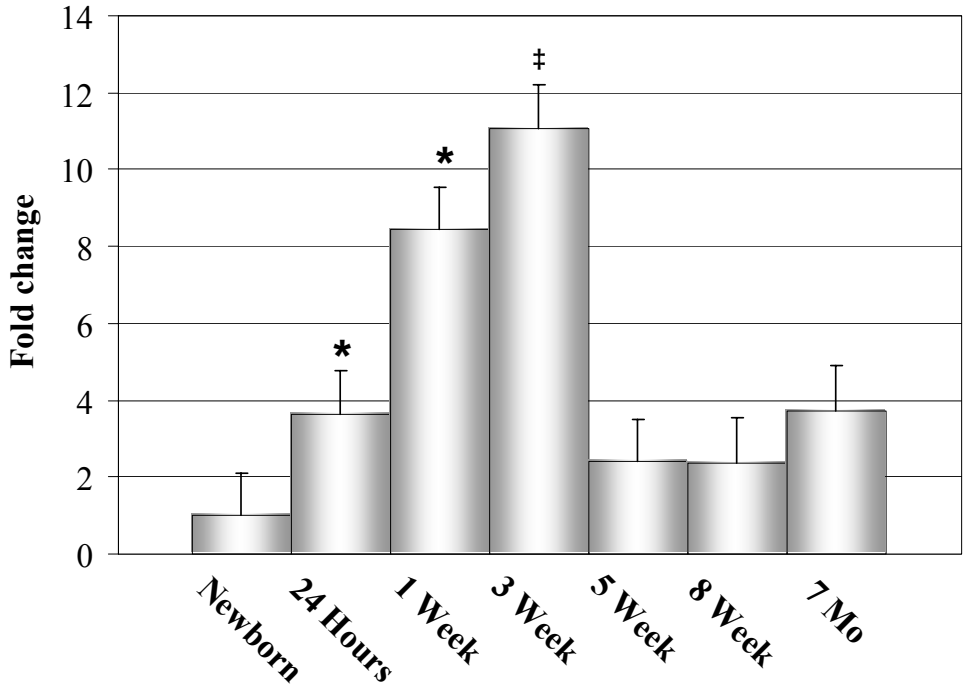
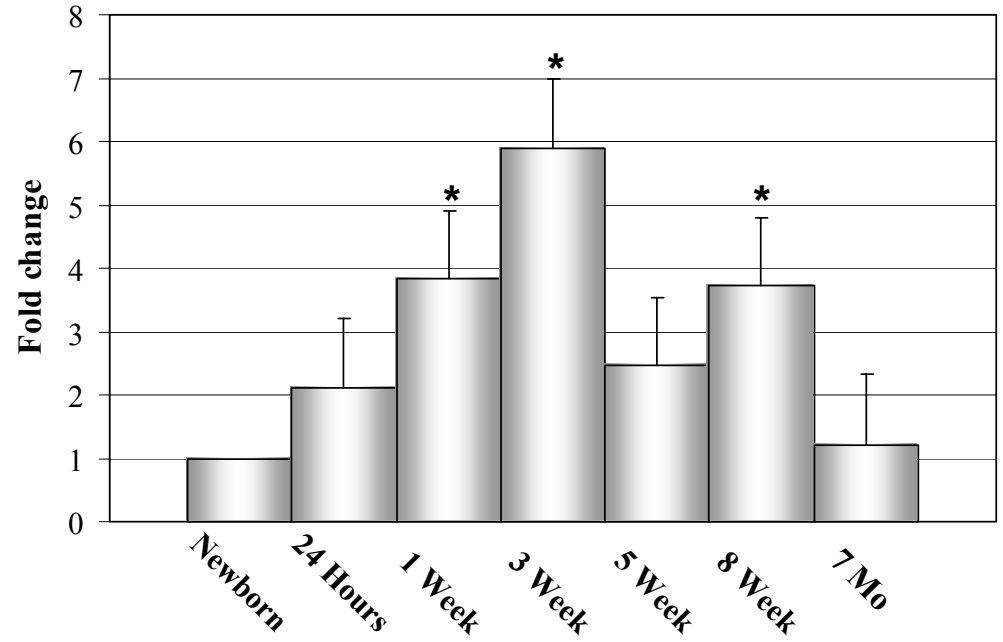


Figure 3.5
Hepatic (a) and skeletal muscle (b) carnitine palmitoyltransferase I mRNA amounts. Data are reported as fold changes relative to the newborn pig. Bars represent means (n=4-5) ± SEM, ‡ ($p < 0.001$) * ($p < 0.05$).

(a)



(b)

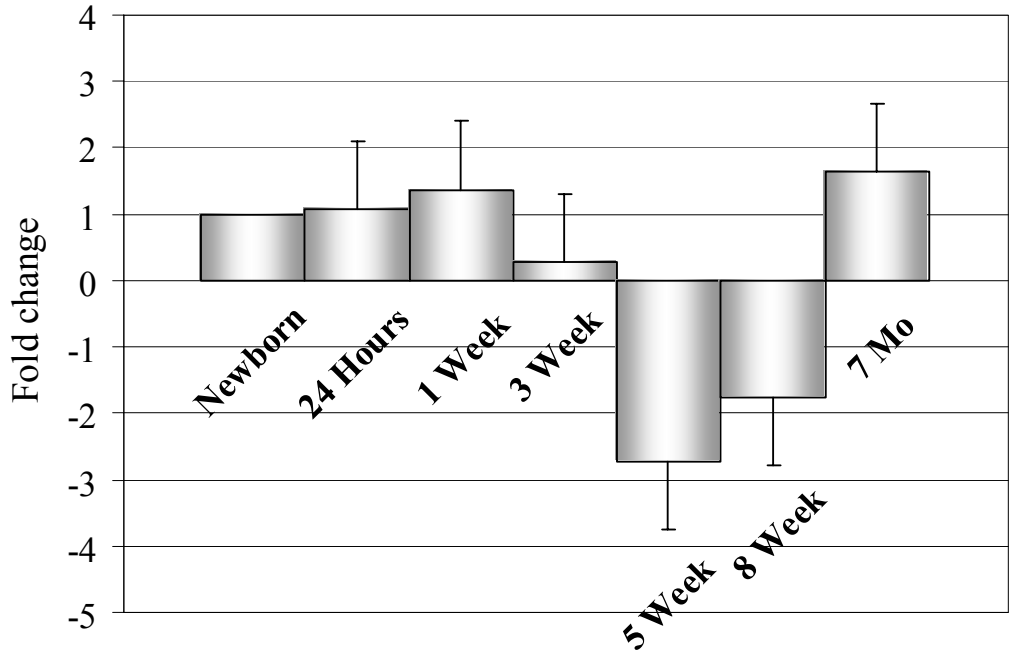


Figure 3.6
Hepatic (a) and kidney (b) γbutyrobetaine hydroxylase mRNA amounts. Data are reported as fold changes relative to the newborn pig. Bars represent means (n=4-5) ± SEM, ‡ ($p < 0.001$) * ($p < 0.05$).

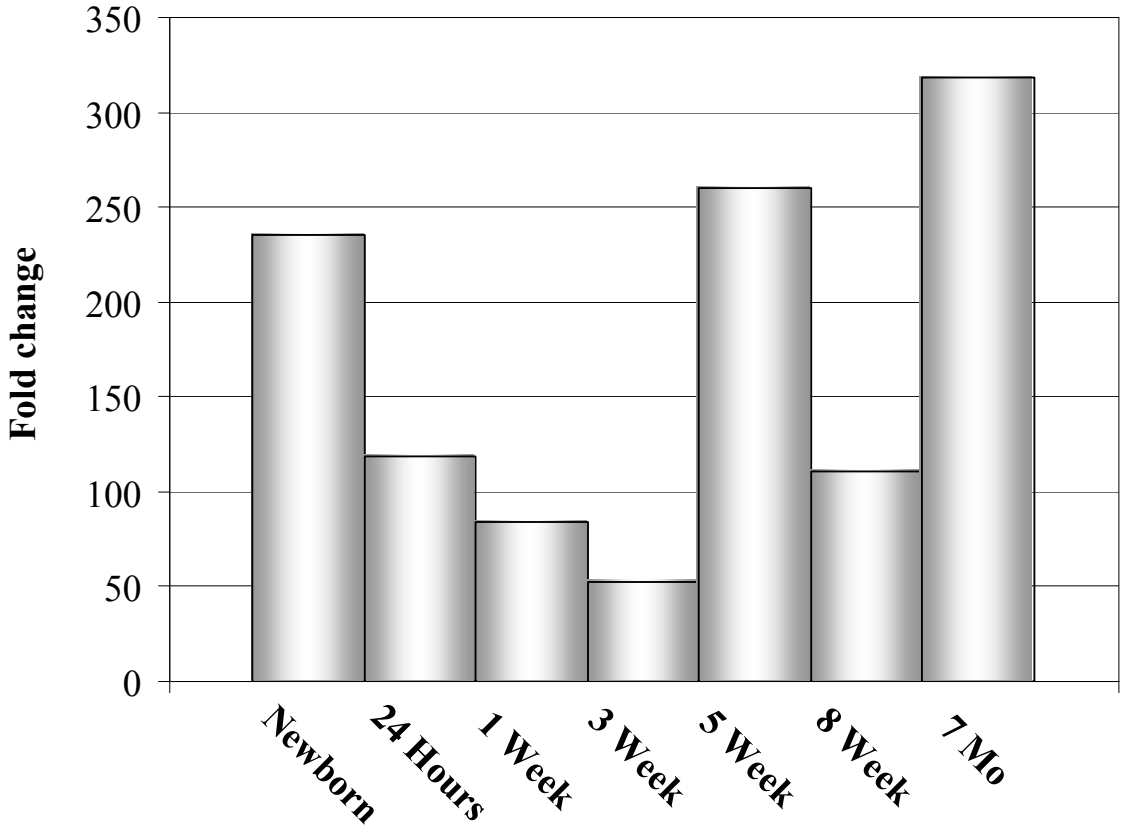
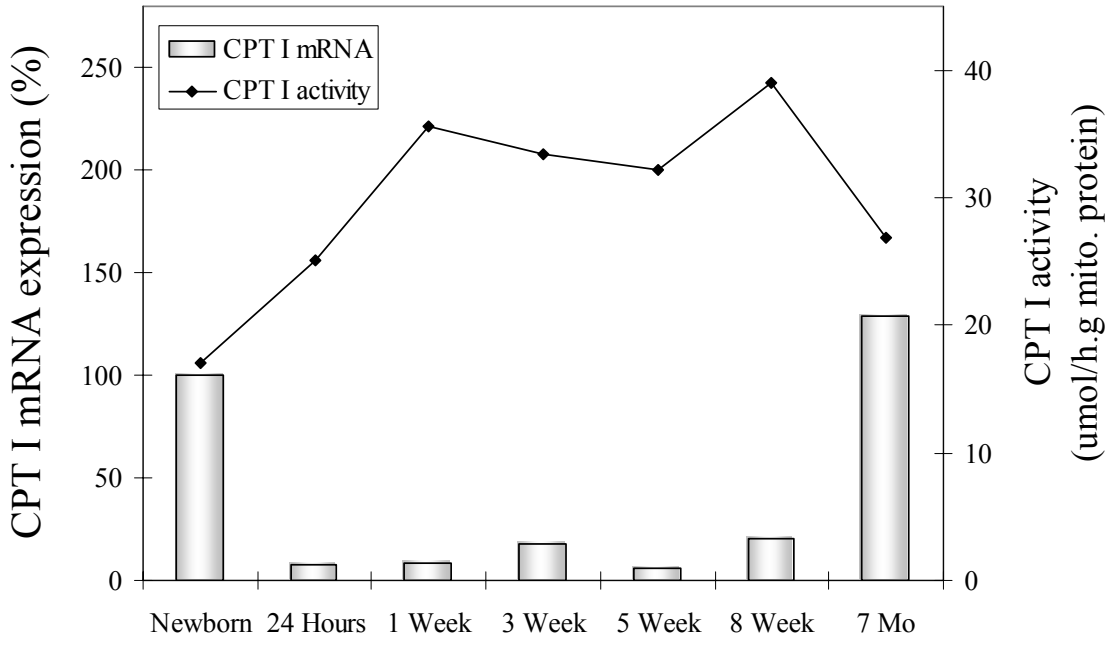


Figure 3.7
Kidney γbutyrobetaine hydroxylase mRNA amounts. Data are reported as fold changes relative to the liver. Bars represent means (n=4-5).

(a)



(b)

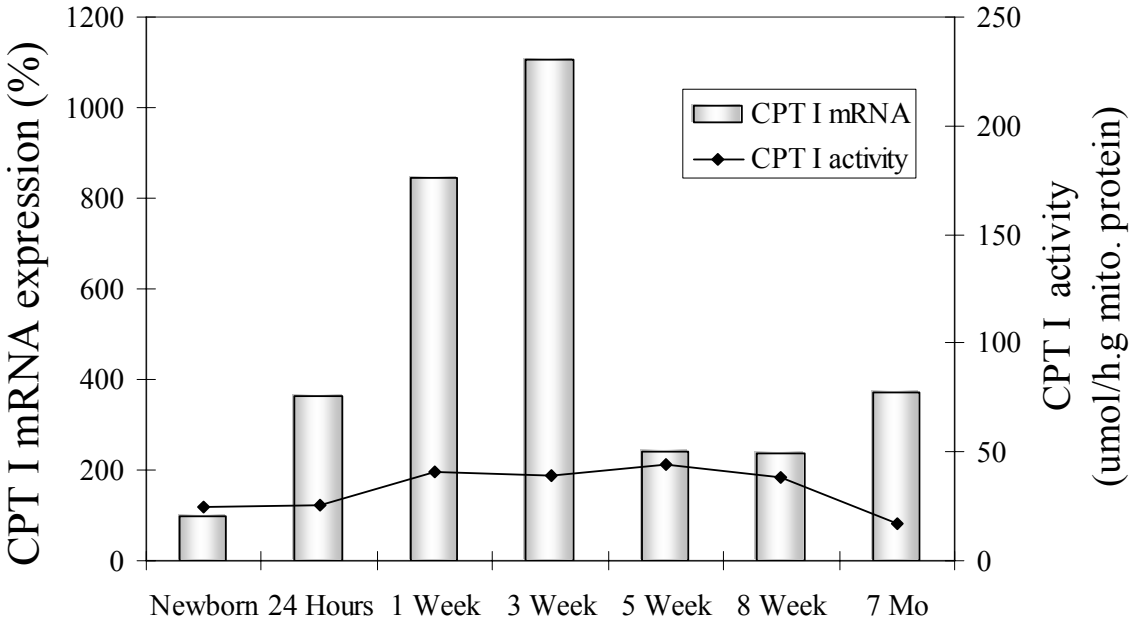


Figure 3.8
Relationship between relative expression of carnitine palmitoyltransferase I mRNA and carnitine palmitoyltransferase I activity in liver (a) and skeletal muscle (b). Amounts of mRNA are presented as % change relative to the newborn. Activity of CPT I is presented as umol/h·g mitochondrial protein.

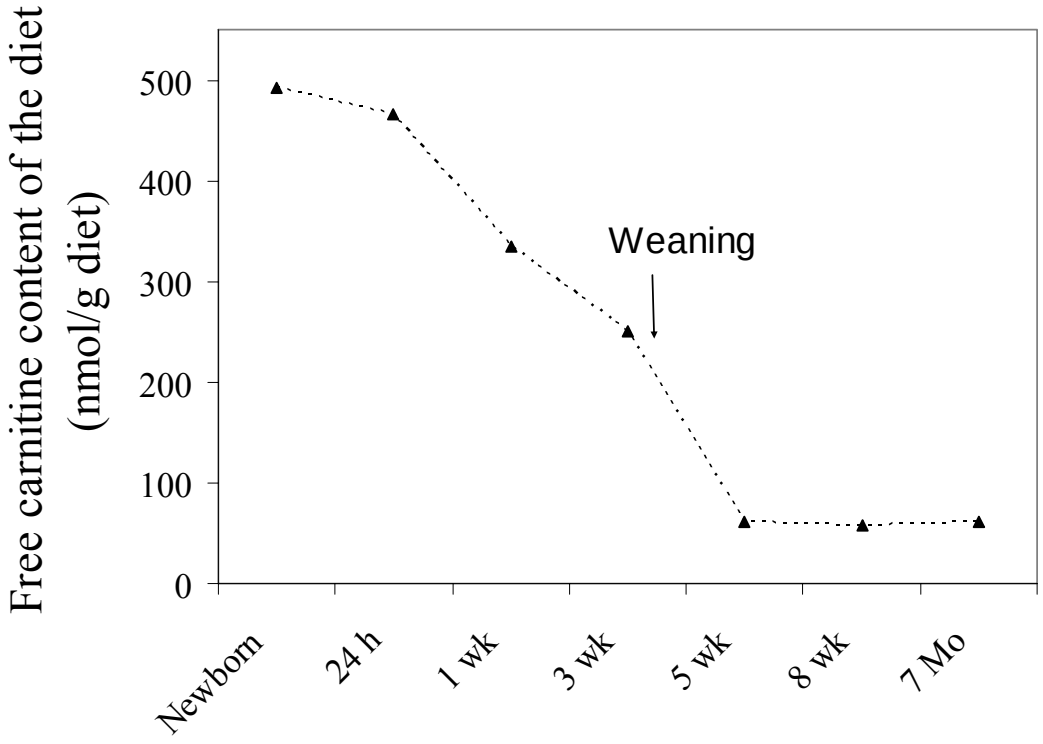
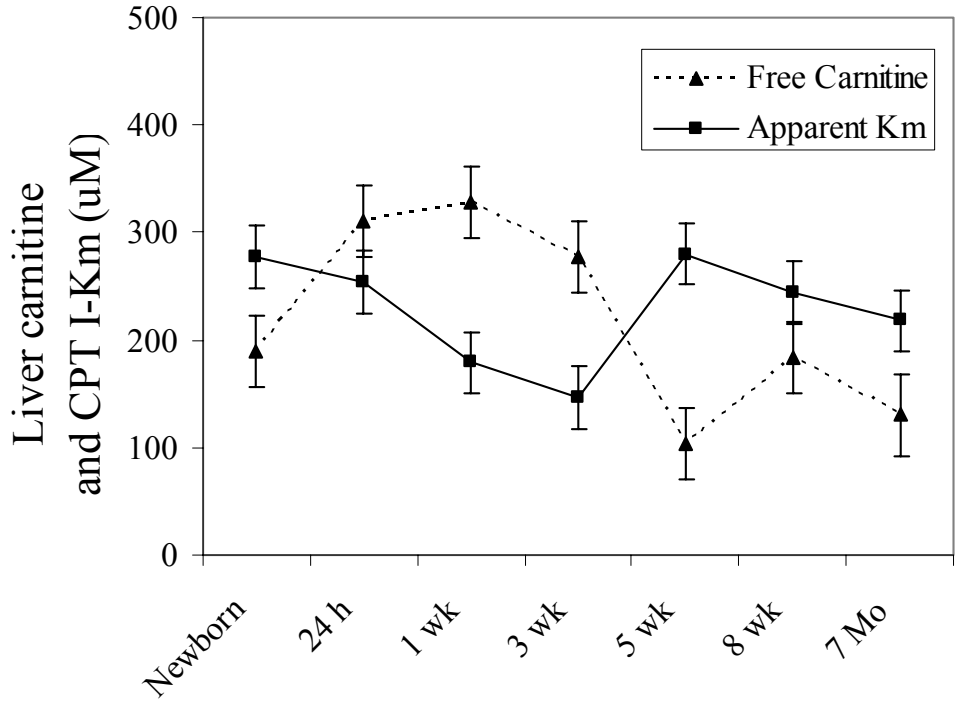


Figure 3.9
Changes in carnitine concentration in milk and diet.

(a)



(b)

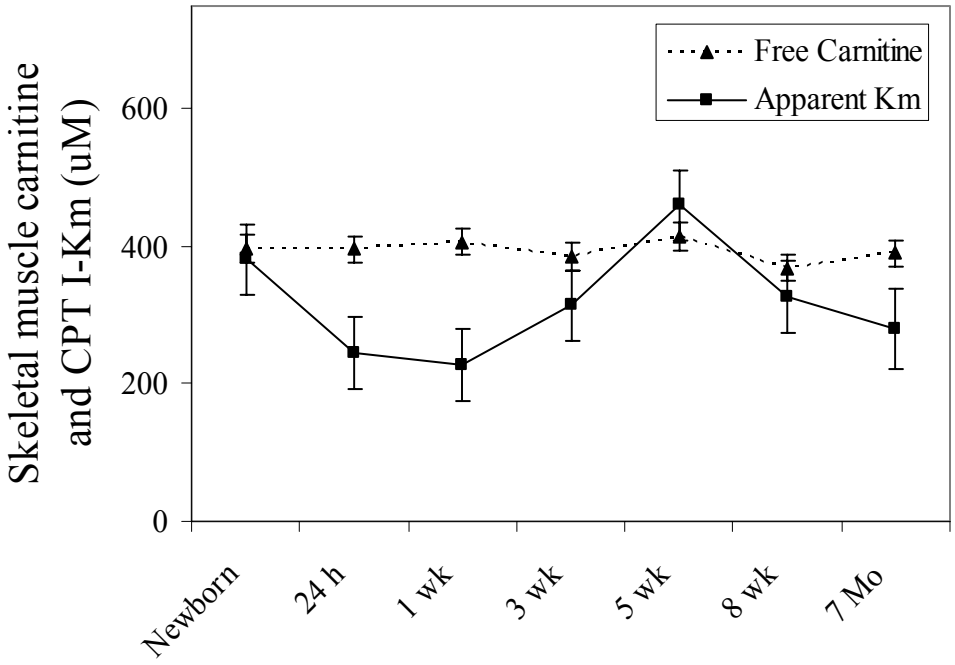


Figure 3.10
Relationship between free carnitine concentrations and the apparent Km of CPT I for carnitine in the liver (a) and skeletal muscle (b) of developing pigs.

APPENDIX

THE EFFECTS OF DIETARY L-CARNITINE SUPPLEMENTATION ON WEANLING PIG PERFORMANCE

SUMMARY

Weanling pigs (n=120; 20.9 ± 1.97 d; 6.42 ± .23 kg) were used to study the effects of L-carnitine on growth performance when fed in conjunction with a diet formulated to contain primarily animal or plant protein. Piglets were provided ad-libitum access to feed and water. Diets were fed in three phases. Phase I diets were fed from d 0 – 7, Phase II diets were fed from d 7 – 14, and Phase III diets were fed from d 14 – 35. Weekly weights, and feed disappearance for each phase was recorded. Subsequently, ADG, ADFI, and G/F were determined within each of the three phases. Dietary L-carnitine had no effect on growth performance during any of the three phases, or on overall performance ($p > .10$). During Phase II, increasing plant protein in the diet decreased ADG and ADFI ($p < .03$, and $p < .02$, respectively). In addition, blood samples were taken at the end of each phase to determine plasma L-carnitine concentrations. The addition of L-carnitine to the diet increased total L-carnitine in the plasma for weanling pigs receiving both animal or plant protein sources during each of the three phases when compared to pigs fed a diet not supplemented with L-carnitine.

INTRODUCTION

The transition from a liquid to a dry diet upon weaning can often result in a growth lag in young pigs. To ease pigs into this diet transition special attention must be payed toward the dietary ingredients. Complex protein sources such as soybean meal are

minimized in the young pig's diet, while animal protein sources are increased. The reason for including more expensive animal proteins is based on the increased digestibility of these proteins, and also to guard against an immunological challenge that often leads to decreased feed intake and gain that may result from feeding of soybean meal. An additional benefit to feeding of animal proteins in the weanling pig diet is their likely contribution of dietary carnitine.

Since its discovery in 1905, research has proven the necessity of carnitine in long chain fatty acid oxidation. Carnitine serves as a co-substrate for carnitine acyl-transferase, and is required for the transport of long chain fatty acids into the mitochondria where they can undergo β-oxidation. Long chain fatty acids are activated to their Co-A esters in the cytosol, since the mitochondrial membrane is impermeable to these Co-A esters, these activated long chain fatty acids cannot enter the mitochondria. Carnitine acyl-transferase exchanges carnitine for the Co-A to produce acyl-carnitine. This acyl-carnitine once inside the inner mitochondrial membrane serves as a substrate for a second carnitine acyl-transferase which completes the final transfer of the fatty acid into the mitochondria by exchanging the carnitine for mitochondrial Co-A. The reformed fatty acyl-CoA is then ready for β -oxidation (McGarry and Brown, 1997). Due to its essential role in fatty acid metabolism carnitine has been classified as a quasi-vitamin, however adult animals can adequately synthesize carnitine and it is therefore not considered an essential dietary component. However, in the young animal attenuated de novo synthesis of carnitine (Borum, 1983) may necessitate demand for dietary carnitine.

Carnitine can be found in both plant and animal products, however its greatest concentrations are found in animal tissues while plants often contain little or no carnitine

(Mitchell, 1978). Milk has been shown to contain carnitine, and therefore may be the source of carnitine which accumulates in tissues early in development (Borum, 1983). It is unknown when de novo synthesis of carnitine begins to sufficiently meet the carnitine needs of the pig. It has been hypothesized that when pigs are transitioned from a mixed diet containing animal products at 7-8 wk of age, to a primarily plant based diet, de novo synthesis is not adequate to compensate for the decrease in dietary carnitine (Heo et al., 2000). Due to varying research results, further research to determine if L-carnitine supplementation can positively impact growth performance in the nursery, and under what conditions these effects can be maximized, is warranted.

EXPERIMENTAL PROCEDURES

Weanling pigs (n = 120; 20.9 ± 1.97 d; 6.42 ± .23 kg) were housed in a nursery facility at the North Carolina State University Swine Educational Unit. Pigs were allotted to one of four dietary treatments according to a 2x2 factorial design (animal versus plant protein; 0 versus 500 ppm supplemental L-carnitine), and provided ad-libitum access to feed and water. Dietary treatments were fed in three phases. Phase I diets were offered from d 0 – 7 (Table 1), Phase II diets were fed from d 7 – 14 (Table 2), and Phase III diets were fed from d 14 – 35 (Table 2). Diets offered during Phases I and II were in pellet form, while diets offered during Phase III were in meal form. Pigs were weighed weekly, and feed disappearance recorded. Subsequently, ADG, ADFI, and G/F were determined within each of the three phases. A subset of pigs from each dietary treatment were selected and bled at the end of each phase. Total L-carnitine concentrations in plasma was determined using a modification to the method of McGarry and Foster (1982). Data was analyzed as a

randomized complete block design with pen as the experimental unit for the analysis of growth performance, and pig as the experimental unit for analysis of plasma samples. Pigs were blocked according to weaning weight, and analysis of variance was performed using GLM procedure of SAS.

RESULTS AND DISCUSSION

Research findings examining the role of supplemental L-carnitine in weanling pigs allowed ad-libitum access to diets has been variable (Rincker et al., 2003; Hoffman et al., 1993; Owen et al., 1996). While Hoffman and co-workers (1993) saw no benefit to feeding L-carnitine on growth performance, Owens and co-workers (1996) reported an increase in feed efficiency by supplementing diets with 500 ppm of L-carnitine. This increase in feed efficiency was due to a decrease in feed intake, and not an increase in ADG. However, subsequent studies by Rincker and co-workers (2003) saw an improvement in both ADG and feed efficiency when diets were supplemented with L-carnitine, with the greatest improvements occurring during Phase II of the nursery.

Results of the current study are summarized in Figure 1 and Table 3. It was hypothesized that the addition of L-carnitine to nursery diets would enhance the growth performance of weanling pigs, and that this would be further accentuated by feeding a diet minimal in animal protein. The addition of 500 ppm L-carnitine to weanling pig diets resulted in similar total plasma L-carnitine concentrations for the supplemented groups during each of the three phases, irregardless of protein source. Furthermore, there were no detectable differences in plasma L-carnitine between weanling pigs receiving animal or plant

protein without L-carnitine supplementation (Figure 1). Despite the elevation in plasma L-carnitine, supplementation had no effect on ADG, ADFI, and F/G in any of the three phases (Table 3). In addition, overall performance was also not affected by the addition of dietary L-carnitine. During phase II, increasing plant protein in the diet decreased ADG and ADFI ($p<0.03$, *and* $p<0.02$, respectively). It was predicted that performance during both Phase I and Phase II would be negatively impacted by feeding a primarily plant based diet, however protein source did not affect Phase I performance. The plant protein source diets were formulated to contain minimal levels of animal protein. The levels included during phase I appear to have been sufficient to maintain growth performance comparable to the controls. However, the further removal of animal protein during Phase II negatively impacted performance.

CONCLUSION

Despite elevating concentrations of total L-carnitine in the plasma, no differences in growth performance were detected following supplementation of weanling pig diets with 500 ppm of L-carnitine.

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Table A.1
Phase I diet composition

Ingredient, %	Animal protein - carnitine	Animal protein + carnitine	Plant protein - carnitine	Plant protein + carnitine
Corn	29.39	29.39	22.72	22.72
Soybean oil	5	5	5	5
Spray dried blood meal	2.5	2.5	1.5	1.5
Spray dried plasma	3	3	3	3
Fish meal, menhaden	5	5	-	-
Soybean meal, w/o hulls	21.81	21.81	38.77	38.77
Whey, dried	20	20	-	-
Lactose	10	10	24.29	24.29
Corn Starch	0.1	-	0.1	-
L-Carnitine[a]	-	0.1	-	0.1
Threonine	-	-	-	-
Methionine, DL	0.16	0.16	0.17	0.17
Dicalcium phosphate	0.874	0.874	1.959	1.96
Limestone	0.39	0.39	0.70	0.70
Salt	0.25	0.25	0.25	0.25
Zinc oxide	0.28	0.28	0.28	0.28
Vit/Min, pmx	0.25	0.25	0.25	0.25
Antibiotic	1	1	1	
Total	100.0	100.0	100.0	100.0
Calculated analysis, ppm				
L-Carnitine	132	511	158	434

[a] 0.1% addition of a 50**%** L –carnitine source. Carnitine was provided by Lonza, Inc. (Fairlaw, NJ 07410)

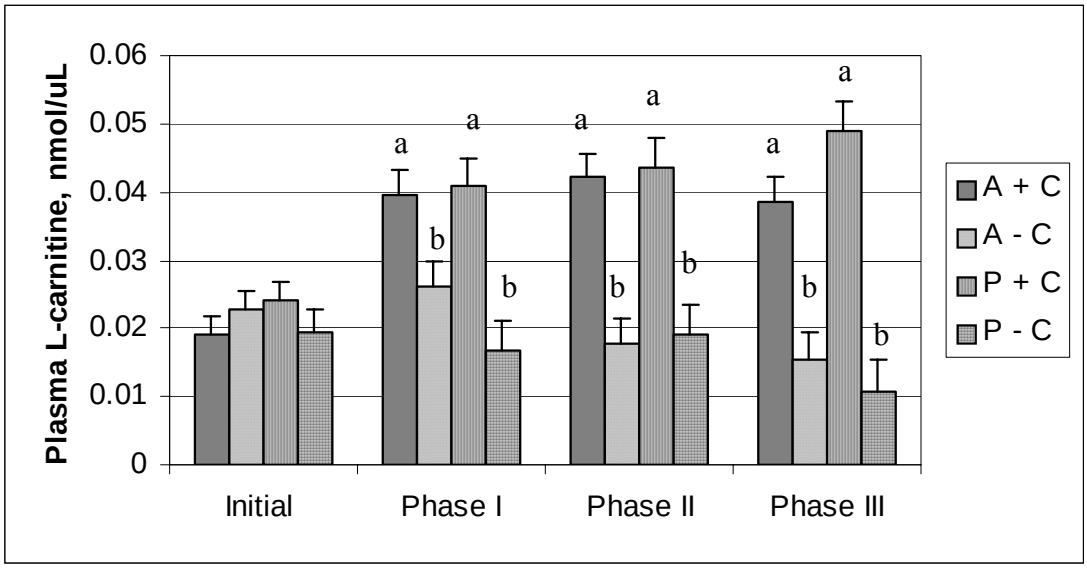
Table A.2
Phase II and Phase III diet composition

	Phase II				Phase III	
	Animal protein		Plant protein		Plant protein	
Ingredient, %	- carnitine	+ carnitine	- carnitine	+ carnitine	- carnitine	+ carnitine
Corn	48.76	48.76	44.26	44.26	56.50	56.50
Soybean oil	5	5	5	5	5	5
Spray dried blood meal	2.5	2.5	1.25	1.25	-	-
Spray dried plasma	-	-	-	-	-	-
Fish meal, menhaden	2.5	2.5	-	-	-	-
Soybean meal, w/o hulls	27.28	27.28	37.71	37.71	34.77	34.77
Whey, dried	10	10	-	-	-	-
Lactose	-	-	7.143	7.143	-	-
Corn Starch	0.1	-	0.1	-	0.1	-
L-Carnitine	-	0.1	-	0.1	-	0.1
Threonine	-	-	0.005	0.005	-	-
Methionine, DL	0.098	0.098	0.093	0.093	0.03	0.03
Dicalcium phosphate	1.455	1.455	1.954	1.954	1.628	1.628
Limestone	0.535	0.535	0.708	0.708	0.793	0.793
Salt	0.25	0.25	0.25	0.25	0.35	0.35
Zinc oxide	0.278	0.278	0.278	0.278	0.08	0.08
Vit/Min, pmx	0.25	0.25	0.25	0.25	0.25	0.25
Antibiotic	1	1	1	1	0.5	0.5
Total	100.0	100.0	100.0	100.0	100.0	100.0
Calculated analysis, ppm						
L-Carnitine	69	432	24	383	16	451

Table A.3
Effects of protein source and dietary L-carnitine on weanling pig performance.

Protein:	Animal		Plant		Probability			
L-*carnitine, ppm:*	0	500	0	500	SE	Protein	L-carnitine	Interaction[a]
Phase I								
ADG, kg	0.225	0.237	0.199	0.210	0.022	0.24	0.60	0.98
ADFI, kg	0.220	0.235	0.208	0.226	0.018	0.59	0.37	0.94
G/F	1.021	1.001	0.948	0.934	0.039	0.09	0.67	0.94
Phase II								
ADG, kg	0.424	0.428	0.378	0.386	0.018	0.03	0.75	0.91
ADFI, kg	0.526	0.554	0.500	0.493	0.017	0.02	0.54	0.32
G/F	0.811	0.774	0.759	0.785	0.036	0.58	0.88	0.39
Phase III								
ADG, kg	-	-	0.485	0.511	0.028	-	0.36	-
ADFI, kg	-	-	0.807	0.862	0.042	-	0.19	-
G/F	-	-	0.600	0.597	0.026	-	0.90	-
Overall								
ADG, kg	0.403	0.422	0.391	0.403	0.013	0.23	0.26	0.79
ADFI, kg	0.579	0.615	0.563	0.587	0.020	0.31	0.16	0.76
G/F	0.698	0.686	0.695	0.693	0.025	0.92	0.79	0.85

[1]Values are LS means, n = 6 pens/treatment. During Phases I and II diets were formulated to include either optimal or minimal levels of animal protein, and two levels of L-carnitine were fed.
[a] Interaction between protein source and L-carnitine level .



[1]A + C (animal protein + 500 ppm L-carnitine), n = 8; A – C (animal protein without L-carnitine supplementation) N=8; P + C (plant protein + 500 ppm L-carnitine) n = 7; P – C (plant protein without L-carnitine supplementation) n = 7.
During Phase III all treatment groups were fed the plant protein either with or without L-carnitine supplementation.

Figure A.1
Plasma L-carnitine concentrations. Data are presented as means ± SEM ($p < 0.05$).