INFLAMMATION AFFECTS ONTGENY OF L-CARNITINE HOMEOSTASIS MECHANISMS IN THE DEVELOPING RAT

A Thesis Submitted to the

College of Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Master of Science

in the College of Pharmacy and Nutrition

University of Saskatchewan

Saskatoon

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ABSTRACT

This thesis research involved investigations into the effects of inflammation on maturation of L-carnitine homeostasis in developing rat neonates. The overall hypothesis was an inflammatory stimulus will alter the ontogeny of L-carnitine homeostasis pathways and this depends upon when the inflammatory stimulus occurs in postnatal development. The objective was to investigate the potential effect of inflammation on carnitine transporter expression in different age groups of neonates and evaluation of effect of inflammation on ontogeny and activity of enzymes involved in carnitine biosynthesis and whether this differs depending upon when in postnatal development the inflammatory stimulus occurs.

Rat pups at postnatal day 3, 7, and 14 received an intraperitoneal injection of lipopolysaccharide (LPS) at a dose known to cause a febrile reaction in rat neonates. L-Carnitine homeostasis pathways underwent significant ontogenesis during postnatal development in the rat. LPS administration caused a significant decrease in free L-carnitine levels in serum and heart tissue and a decrease in mRNA expression levels of the high affinity carnitine transporter, Octn2, in kidney, heart and intestine at all postnatal ages. Furthermore, significant decreases in mRNA expression levels of key enzymes involved in carnitine biosynthesis was observed, while an increase in carnitine palmitoyltransferase mRNA levels were observed at all postnatal ages. Reductions in butyrobetaine hydroxylase mRNA expression were paralleled by reductions in enzyme activity only at postnatal day 3 and 7. Heart creatine phosphate levels were deceased significantly in LPS treated groups in all postnatal ages; however, ADP and ATP levels were unaffected. Collectively, this research provided experimental evidence for a significant effect of inflammation on changes in L-carnitine homeostasis maturation in early neonatal stages. The maturation of physiological processes may be altered by external factors in early postnatal life.

ACKNOWLDEGEMENT

I wish to express my sincere thanks and gratitude to my supervisor Dr. J. Alcorn who provided me with continuous guidance, encouragement, constructive criticism and advice throughout the course of study. This appreciation is also extended to the other members of my advisory committee, Dr. P. Paterson and committee chair, Dr. E. Krol for providing me with encouragement and valuable suggestions.

I am also very appreciative of all my Lab mates who were helpful and supportive to me over the years of study. Uma, Yun, Kali, Jennifer and Puneet, thank you. Thanks for all my friends.

I dedicate this dissertation to my professor Dr. J. Jane Alcorn, and my parents for their support, encouragement and understanding. Without their sacrifice and support, I would not have been able to succeed in my academic career.

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ABBREVIATIONS

ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATB^{0+}	Amino Acid transporter B(0+)
ATP	Adenosine Triphosphate
BBH	Gamma-Butyrobetaine Hydroxylase
BBMV	Brush-Border Membrane Vesicles
BCA	Bicinchoninic Acid
β-ME	beta-Mercaptoethanol
Bsep	Bile Salt Export Pump
BSA	Bovine Serum Albumin
Caco-2	Epithelial colorectal adenocarcinoma cells
CACT	Carnitine-Acylcarnitine Translocase
COT	Carnitine Octanoyltransferase
CPT I	Carnitine Palmitoyltransferase I
CPT II	Carnitine Palmitoyltransferase II
Cr	Creatine
CrP	Creatine Phosphate
CT	Carnitine Transporter
DAD	Diode Array Detector
DOHaD	Developmental Origin of Health and Disease
ELISA	Enzyme-Linked Immunoresorbent Assay

FAS	Ferrous Ammonium Sulfate
FC	Free L-Carnitine
HPLC	High Performance Liquid Chromatography
HQC	High Quality Control
HTML	3-Hydroxy-6-N-Trimethyllysine
IL-1β	Interleukin-1Beta
IL-6	Interleukin-6
jvs	Juvenile Visceral Steatosis
LCFAs	Long-chain Fatty Acids
LPS	Lipopolysaccharide
LQC	Low Quality Control
MQC	Mid Quality Control
NCBI	National Center for Biotechnology Information
Oatp1	*O*rganic anion-transporting polypeptide
OCT1	Organic Cation Transporter
OCTN2	Organic Cation/Carnitine Transporter 2
PCS	Palmitoyl-CoA Synthetase
PEPT1	Peptide Transporter 1
QRT-PCR	Quantitative Reverse Transcription-Polymerase Chain Reaction
SLC	Solute Carrier Family of Transporters
SLC15A1	Solute Carrier Family 15 Member 1
TCA	Citric Acid Cycle

TMABA	4-Trimethylaminobutyraldehyde
TMB	3,3’,5,5’-Tetramethylbenzidine
TML	6-N-Trimethyllysine
TMLH	Trimethyllysine Hydroxylase
TMLD	N’trimethyllysine Dioxygenase
TNF-α	Tumor Necrosis Factor – Alpha

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Chapter 1

1. Introduction

Carnitine (3-hydroxy-4-N-trimethylamino butyrate) is a low molecular weight, water soluble ubiquitous molecule that exists in nearly all plants (1), animals, and microorganisms (1, 2). L-Carnitine is available as free L-carnitine (non esterified molecule; FC) and esterified derivatives (acylcarnitines; AC) in mammals (3, 4). Fatty acids are important energy metabolism substrates and mitochondrial β-oxidation of long chain fatty acids requires adequate amounts of L-carnitine. In neonates, L-carnitine has a crucial role in myocardial β-oxidation of long-chain fatty acids, elimination of toxic metabolites, ketogenesis, and regulation of nitrogen metabolism (5, 6).

Neonates are particularly vulnerable to the development of L-carnitine deficiency and its associated physiological disorders since the biosynthetic enzymes and transporters responsible for maintenance of L-carnitine homeostasis undergo maturation in early stages of postnatal life. Neonates are often exposed to several acute pathological conditions while important physiological systems are still undergoing maturation. While much attention in neonatal therapeutics has focused on appropriate dosage regimens for this age group, less attention has been paid to understanding long-term and short-term effects of acute pathological conditions on the maturation of important physiological processes that may have a specific role in the development of chronic disease in later stages of life. Several pathological conditions can lead to inflammation during the neonatal stage and these conditions can alter the developmental program of physiological processes.

This thesis work endeavours to advance our understanding of the possible effect of inflammation on maturation of L-carnitine homeostasis mechanisms in neonates. The effects of

an inflammatory stimulus on different elements of L-carnitine homeostasis at different stages of neonatal development will identify the influence of inflammatory disease on maturation of L-carnitine homeostasis mechanisms.

1.2. Literature Review

1.2.1. Developmental Programming

Developmental programming refers to characteristics of the environment that appear during fetal and neonatal development, which permanently alter the normal maturation of physiological and biochemical processes (7, 8). Developmental programming has the following characteristics: 1) Susceptibility of organism to external environment is restricted to a critical ontogenetic window during early development; 2) Persistent that continues into adult life; and 3) Unique and quantifiable result that can vary significantly among individuals. Several factors determine developmental programming including nutritional and disease states, environmental conditions, gynecological immaturity, and the maternal or fetal genotype (9).

During early development, a window of plasticity exists, a period of time when the organism may still develop in various directions and has potential to adjust or adapt to its environment. Even though epidemiological research has mainly focused on the detrimental ramifications of programming, it is not predominantly an injurious phenomenon (10). The ability to adopt a phenotype to different conditions prevalent in early life is an important adaptive phenomenon particularly if the environment is comparatively stable. A mismatch between the environment in prenatal or early postnatal life and adult life may culminate in unsuitable adaptations in the organism (11-13). However, adjustments that occur in critical periods of maturation can also guarantee the preservation of homeostasis when the surroundings are jeopardised (10).

Substantial research has focused on prenatal (and to a lesser extent postnatal) nutritional status as this can have significant influence on the developing fetus and neonates (14). The developing system is highly adaptive to the available nutrition supply, a phenomenon referred to as nutritional programming (15, 16). When a discrepancy exists between early life nutrition and availability of macro- and micronutrients in adult life, the permanent alterations induced during nutritional programming may have detrimental consequences in adult life (15, 16).

1.2.1.1 Evidence of programming during postnatal development

Evidence for the ability of the environment in early life to increase risk for chronic disease in adult life emerged in ecological research that investigated the reasons for the north-south division of disease patterns in England and Wales (17). The research uncovered the importance of maternal nutritional state during fetal and neonatal development in coronary heart disease (CHD) (18). An augmented risk of CHD death, high blood pressure, type 2 diabetes, and metabolic syndrome was related to low birth weights (18). This is now termed the Barker theory where it is now widely accepted that fetal programming by nutritional stimuli or glucocorticoids (stress hormones) results in physiological adaptations of the fetus to its environment *in utero* to ensure its readiness for postnatal life (19). Data from the survivors of World War II and the Dutch Hunger Winter also provide clear evidence that malnutrition during neonatal development predisposes the offspring to risk of CHD, metabolic syndrome, diabetes, and other chronic diseases. A multitude of independent research in developed and developing countries have corroborated that chronic disease is connected with low birth weight and malnutrition during pregnancy (20, 21).

The notion of the effects of intrauterine life on adult health and on the incidence of late onset illnesses, such as hypertension and type II diabetes, is a comparatively more recent idea. Forsdahl and coworkers in 1977 reported that the incidence of arteriosclerotic heart disease in a particular age group could be associated with the degree of infant mortality of that same population (22). A few studies conducted in the 20th century showed that a relationship between birth weight and tendency of developing cardiovascular and metabolic diseases in adulthood (23) (24).

Studies in primates demonstrate that over-nutrition during infancy in the female results in development of obesity in early adult life (25). Therefore, the question has been raised: "what is the place of epigenetic memory being stored in the intervening period?" Further research was conducted on baboons, which evaluated cholesterol levels on specific diets (26, 27). Higher plasma LDL and VLDL cholesterol, lower HDL cholesterol, and increased cholesterol was revealed in adult baboons that were breastfed in the early life as compared to bottle fed baboons suggesting primates may be programmed by breast-feeding (27). In fact, post-mortem evaluations showed that breastfed baboons had profoundly more atherosclerosis (26, 27).

1.2.2 Epigenetics and its Importance during Postnatal Development

It has become increasingly evident in recent years that development is under epigenetic control. Epigenetics explains the genetic modification in functions of genes which are independent of gene code or DNA sequences (28). Epigenetics defines changes in expression levels of functional genes over a particular period of time. According to the National Institutes of Health's definition, epigenetics is the study of changes in the regulation of gene activity and expression that are not dependent on gene sequence (29, 30). Evidence is available that shows

changes in developmental programming due to altercations in epigenetics following from numerous external stimuli (29, 31-34).

The relationship between the environmental conditions during development and developmental programming is very complex (35). Limited research has been conducted to identify the factors affecting the complex relationship between these mechanisms. The identification of links between external signals and the epigenetic machinery with specific physiological outcomes is an area of increasing importance because of its impact on developmental programming and child health (30, 36). Histone modifications and DNA methylation were two well studies mechanism in epigenetics. Only two of many factors, nutritional conditions and stress, have been studied and well documented. It has been reported that these factors influence adult risk of developing metabolic disease, such as type 2 diabetes mellitus and cardiac diseases (37). High risk to develop cardiovascular morbidity and mortality during adult life is associated with individuals born small for gestational age (4 –7). This epidemiological evidence is now supported by an extensive experimental literature in animals. Evidence on the importance of prenatal and early postnatal growth for later morbidity suggests the existence of a link between developmental responses to early environments and adult biology. For instance, the roles of histone demethylases in the context of whole body physiology are now being uncovered (38, 39). The histone demethylase, Jhdm2a, for instance, was recently identified as a crucial regulator of genes involved in energy expenditure and fat storage and, therefore, Jhdm2a may be a key factor in obesity and metabolic syndrome (38, 39).

It is common knowledge in human history that a child's development during the fetal and neonatal period is influenced by the environment and life events. In particular, inappropriate nutrition is an important causal element precipitating a developmental programming event that

may contribute to the risk of chronic disease (14). The foremost mechanistic hypothesis is that when a critical period of maturation is progressing under suboptimal circumstances, the organism adapts to ensure survival and to maintain vital functions at the expense of other less immediately critical functions (40). Nevertheless, the fetus' metabolic *economy mode* that secures survival in response to prenatal adverse conditions becomes maladaptive in a postnatal world where nutritional resources are not limiting (40). Continued adaptations in postnatal life also play a role in further modulating antenatal programming.

There are three intriguing and fascinating facets about the epigenetic state that are important to relate in the context of developmental programming (41). First, epigenetic states can be paradoxically both reversible and heritable. Second, epigenetic states can be both heritable across cell divisions in somatic cells and potentially "inherited" across several generations. Third, epigenetic states can be both carriers of "memory" of early life experiences and "triggers" of disease susceptibility in later life. Because of the reversible nature of epigenetics, it can be modified by many external factors, which may cause the development of an unusual phenotype (42). Standard physiological responses to these different stimuli also may be changed by epigenetic mechanisms (42). The Developmental Origin of Health and Disease (DOHaD) hypothesis proposes that some disorders, such as type 2 diabetes mellitus and cardiovascular disease, can result from an imbalance between the environments that are experienced *in utero*, in early infancy, and later in life (43). The key feature of the "epigenotype model" of DOHaD is largely based on the finding that the environment can modulate epigenetic states. Indeed, there is mounting evidence that links environmental stimuli and the epigenome. Examples include behavioral programming by maternal care in rats, and divergence between genetically identical inbred mice (44, 45). Furthermore, environmental influences in early life can induce permanent

alterations in the epigenotype and determine adult phenotypes and disease susceptibility. Epigenetic misprogramming may occur throughout development; however environmentally induced changes that affect programming during early development have a greater and more widespread effect than those that occur during less “plastic” times in development (46).

1.2.3 Critical window of susceptibility

Sensitive periods in fetal and postnatal developmental exist during which even minor interventions can have long lasting effects on adult physiology (47). Fetal or neonatal exposure to chemicals is an important concern since developing organisms are tremendously susceptible to perturbation by chemicals. Harmful effects may happen at concentrations of the chemical that are far below levels considered toxic in the adult (48). Furthermore, the timing of exposure is important as developing systems show critical periods of susceptibility to various chemicals such that an exposure during this critical period will lead to abnormalities in organ function, which may not occur when the exposure occurs at some other time of development (48). These critical windows of susceptibility can be explained by the fact that the protective techniques that are available to the adult, such as DNA repair mechanisms, a competent immune system, detoxifying enzymes, liver metabolism, and the blood/brain barrier as examples, are not completely functional in the prenatal or early postnatal period (49). Additionally, the developing organism has an enhanced metabolic rate as compared with an adult, which, in some cases, can result in increased toxicity (49). During a critical window, exposure to environmental factors will modify normal cellular and tissue development and function resulting in developmental programming and a risk for metabolic and hormonal ailments later in life. Therefore, exposures during critical windows of prenatal advancement usually do not manifest until much later in life (50).

Since each system of organs has various developmental trajectories, and the sensitive window for exposures to different factors varies relative to the stage of tissue development, the consequences of exposures depend not only on the type of exposure, but on the time when the exposure occurs (50). A number of studies illustrate that the *in utero* developmental period is a critically sensitive window of vulnerability (51, 52). Disturbances during the critical window of susceptibility can lead to subtle functional switches that may not appear until later in life. Mounting evidence also indicates that brief systemic inflammation during critical periods of development may results in long-lasting cerebral and peripheral vulnerability (programming or sensitization) well into adulthood (53).

Heindel and Newbold showed several significant principles that depict how early life environmental vulnerabilities contribute to high risk of adult disease (54). Chemical exposures can have both tissue-specific and time-specific ramifications on growth and development (55). As long as tissue is developing, it is susceptible to disturbances from environmental vulnerabilities. These disturbances can result from alterations in gene expression, protein activity, cell communication or other mechanisms (54). The consequences of environmental chemical vulnerabilities can be transgenerational, therefore influencing future generations.

1.2.4 L-Carnitine

1.2.4.1 General aspects of L-carnitine

In early twentieth century, carnitine was first discovered in skeletal muscle extracts (56). Carnitine is a low molecular weight, quaternary ammonium compound and it is present in both Levo (L) and Dextro (D) forms. However, L-carnitine is the biologically active form. L-Carnitine is a trimethylated amino acid that plays an important role in the transfer of long-chain

fatty acids (LCFAs) across the inner mitochondrial membrane. L-Carnitine is biosynthesized in mammals from two amino acids, L-lysine and L-methionine, and also is supplied from dietary sources (57). In mammals, L-carnitine is present as free L-carnitine, the major form in the total L-carnitine pool, and as esterified derivatives, which are the catalytic products of carnitine acyltransferases. The acylcarnitines appear as the result of the reaction when acyl moieties (short chain or long chain) are transferred to carnitine from acyl-CoA (58).

1.2.5 L-Carnitine Functions

L-Carnitine has a number of direct and indirect roles in many mammalian physiological systems and these essential functions directly or indirectly regulate overall energy metabolism (59). L-Carnitine plays a major role in mitochondrial β-oxidation of long-chain fatty acids by facilitating their transport across the inner mitochondrial membranes and is of utmost significance for maintaining normal mitochondrial function (3, 60, 61). It is also involved in the transport of peroxisomal β-oxidation products to the mitochondria, restoration of normal acyl-CoA/CoA balance, and regulation of toxic effects of metabolites produced in fatty acid metabolism (61).

Recent experiments and clinical studies have shown that mitochondrial dysfunction secondary to a disruption of L-carnitine homeostasis cause in decreased nitric oxide signaling and the advancement of endothelial dysfunction (62). L-Carnitine is transported via the circulation and is then taken up by other tissues through active transport systems. In the kidney, L-carnitine and butyrobetaine are reabsorbed efficiently to minimize urinary loss. Sodium-dependent cationic transporter (OCTN2) is the primary known transporter responsible for both the active transport of L-carnitine to other tissues and reabsorption in kidney (63, 64).

1.2.5.1 Long-chain fatty acid oxidation

The mitochondrial β-oxidation of long-chain fatty acids is the cardinal energy producing process for organs that require long-term energy. Long-chain fatty acids cannot cross the inner mitochondrial membranes for further metabolism due to structural limitations. Palmitoyl-CoA synthetase (PCS) is the first enzyme in the fatty acid transport process (65). PCS is located in the outer mitochondrial membrane and activates fatty acids that are present in the cytosol to produce coenzyme A-derivatives (acyl-CoA) of fatty acids. Carnitine palmitoyltransferase I (CPT I) located in the outer mitochondrial membrane facilitates the transesterification of acyl-CoA and produces acylcarnitines (66, 67). In transesterification, the acyl moiety of the long-chain fatty acids is transferred from CoA to the hydroxyl group of carnitine. Specific transporters mediate the transport of the long chain fatty acids into mitochondrial matrix such as carnitine-acylcarnitine translocase (CACT), which exchanges one long-chain acylcarnitine for one carnitine. Carnitine palmitoyltransferase II (CPT II) facilitates the back conversion of acylcarnitine to carnitine and the particular long-chain acyl-CoA. Within the mitochondrial matrix acyl-CoAs undergo β-oxidation and enter the respiratory chain. L-Carnitine is subsequently translocated by translocase or modified to acylcarnitines in the matrix of mitochondria. Carnitine acyltransferases transfer the acyl group from acyl-CoA to L-carnitine. These enzymes can be divided into three types (58) : 1) carnitine palmitoyl transferase (CPT), which transfers medium and long-chain acyl-CoA with more than 12 acyl groups to L-carnitine with a preference for long-chain acyl-CoA, at least at physiological concentrations of L-carnitine; 2) carnitine octanoyltransferase (COT), which uses both medium and long-chain fatty acids with 5-12 acyl groups having a preference for medium-chain acyl-CoA; and 3) carnitine

acetyltransferase (CrAT), on the basis of their specification to acyl groups and their cellular settling employs short-chain acyl groups (C1–C4) as substrate. The CPT system is a significant feature of *the carnitine shuttle,* which facilitates the transfer of long-chain fatty acids from cytosol into the mitochondrial matrix, the site of β-oxidation.

The CPT system includes carnitine palmitoyltransferase 1 (CPT-1) localized in the outer mitochondrial membrane, the carnitine–acylcarnitine translocase (CACT), an integral inner membrane protein, and carnitine palmitoyltransferase 2 (CPT-2) localized on the inner mitochondrial membrane (68). CPT-1 conjugates L-carnitine with LCFAs, CACT transfers the acylcarnitine across the inner plasma membrane, and CPT-2 conjugates the fatty acid back to coenzyme A for subsequent β-oxidation. CPT-1 has various isoforms with tissue-specific expression, CPT-1A (liver), CP T-1B (muscle) and CPT-1C (brain) (69). Finally, CrAT, which is situated in the mitochondrial matrix, has the ability to reconvert the short and medium chain acyl-CoAs into acylcarnitines using intramitochondrial carnitine. Decreased CrAT activity results in increased levels of acyl-CoA, which causes in inhibition of many enzymatic processes involved in oxidative metabolism (70) (71-73).

1.2.5.2 Regulating the mitochondrial acyl-CoA/CoA ratio and toxic effects

In the process of long chain fatty acid transport, carnitine acyletransferase produces acylcarnitines and free coenzyme A (CoA) in the inner mitochondrial matrix. L-Carnitine acts as a buffer for free CoA (74). Through the action of CAT, L-carnitine and CoA pools are in close relationship. This reversible exchange allows the cell to regulate its levels of free CoA using L-carnitine as a buffer, and since L-carnitine is present in most tissues at a much higher concentration than CoA, the extra mitochondrial acetylcarnitine/carnitine ratio will prevent

significant fluctuations in the mitochondrial acetyl-CoA/CoA ratio by formation of acyl-CoA (75, 76). L-Carnitine prevents the toxic effects of acyl groups, which are produced by poor metabolism of fatty acid oxidation, by converting those acyl groups into carnitine esters. Since L-carnitine exists as either free L-carnitine (nonesterified molecule; FC), or acylcarnitines (esterified form; AC), the AC/FC ratio is an important criterion for assessment of a functional L-carnitine shortage (76). Enzymatic alterations in L-carnitine metabolism can result in higher levels of acylated carnitines and, hence, an elevated AC/FC ratio (76, 77).

L-Carnitine mediated transport of LCFAs has 3 other distinct functions. A free CoA pool, both in the cytosol and within the mitochondrial membrane, is necessary for many metabolic processes and pathways, including the citric acid cycle, ketogenesis, and gluconeogenesis. When the acyl group of acyl CoA combines with carnitine, acylcarnitine esters are formed which can then be removed from the mitochondrial membrane and cell and thereby eliminated by liver or kidney. This transport of acylcarnitine esters out of the mitochondria serves 2 purposes. First, it maintains a pool of free CoA at the mitochondrial membrane, and second, it protects cells against high, and potentially toxic, concentrations of acyl CoA compounds that could inhibit enzyme activity in metabolic processes, thereby ultimately altering cellular ATP production (60).

The interruptions in mitochondrial function have been identified as the main cause of several pathological conditions diabetes (58). L-Carnitine also removes the acyl groups from mitochondria as acylcarnitines. The increase in free fatty acid levels can induce mitochondrial dysfunction resulting in cell death and/or enhanced secondary generation of reactive oxygen species (ROS) (78). These effects can be attenuated with L-carnitine treatment. Studies have also shown that L-carnitine has a protective effect both on mitochondria and in whole cells by inhibiting free fatty acid induced mitochondrial membrane damage and/or its secondary effects

(79). The protective effects of L-carnitine and related metabolites have been postulated to be due to improved energy metabolism and the inhibition of electron leakage from mitochondrial electron transport systems.

1.2.5.3 L-Carnitine Importance and Systemic Deficiency

L-Carnitine deficiency was first described as a new syndrome in humans in 1973 (80). Until now, two types of L-carnitine deficiency have been observed: primary and secondary L-carnitine deficiencies (81). These are commonly classified by the etiopathogenesis and organ involvement. Primary L-carnitine deficiency is caused by disturbances in the L-carnitine system itself and leads to impaired fatty acid oxidation. Primary L-carnitine deficiency is an autosomal recessive disorder of fatty acid oxidation, which can be associated with a novel mutation in SLC22A5, the gene encoding Sodium-dependent cationic transporter OCTN2 (81, 82, 83). Patients with primary L-carnitine deficiency have increased renal excretion of L-carnitine, and patients experience recurrent hypoketotic hypoglycemia and/or dilated cardiomyopathy.

Secondary L-carnitine deficiencies are associated with an impaired mitochondrial oxidation of acyl-CoA intermediates, a limited supply of substrates for L-carnitine biosynthesis, or a dietary insufficiency (84). Secondary L-carnitine deficiency is characterized by free plasma L-carnitine concentrations less than 20 μmole/L or 0.39 mg/dL. Toxicity caused by L-carnitine deficiency results from the accumulation of fatty acids or decreased energy production (85, 86). Impairment in mitochondrial fatty acid oxidation can lead to severe hypoglycemia and dilated cardiomyopathy. In addition, patients with inborn errors of long chain fatty acid oxidation present with a variety of severe clinical problems, such as cardiomyopathy, retinopathy, and peripheral neuropathy, presumably due to the accumulation of toxic long chain acyl CoA esters.

Symptoms related to a defect in OCTN2 may include fatty liver, growth retardation, hypoglycemia, and hyperammonemia (87). Many individuals with L-carnitine deficiency are generally normal at birth and may appear healthy for several years until overt clinical signs appear. Early identification of L-carnitine deficiency can be lifesaving because L-carnitine supplementation during the early stage of a L-carnitine deficiency can reverse certain pathological changes associated with cardiac, neural, and hepatic systems (87).

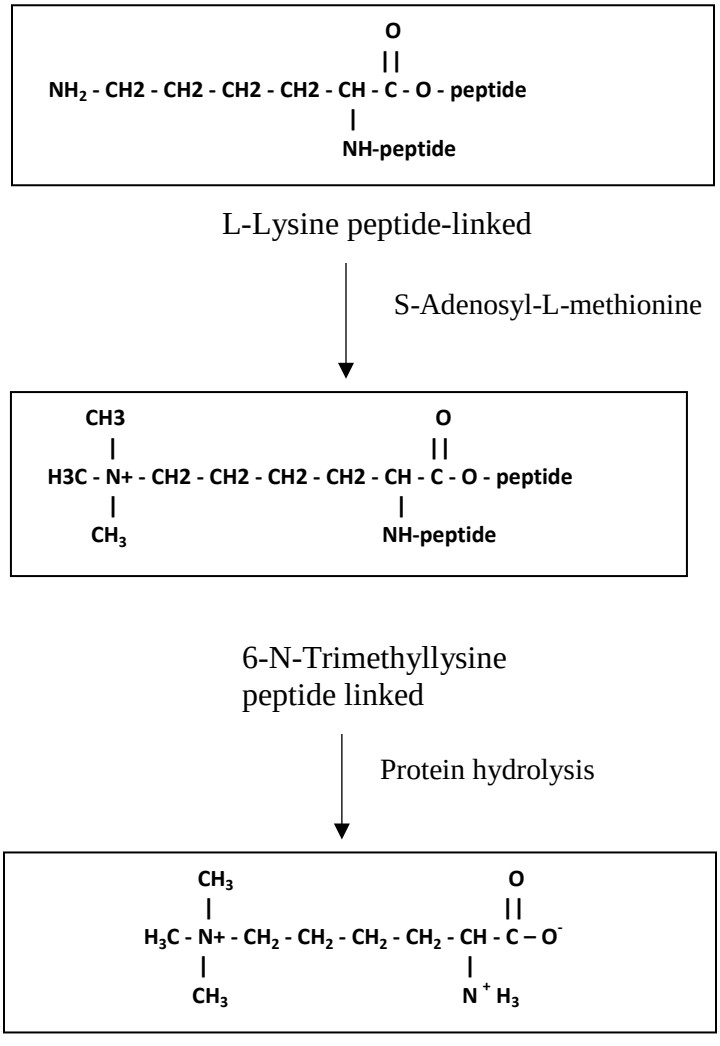
1.2.6 L-Carnitine Homeostasis

1.2.6.1 *De novo* biosynthesis

Human L-carnitine may derive from dietary sources or biosynthesis employing lysine and methionine. The main dietary sources of L-carnitine are red meat, fish and dairy products, which can provide 2–12 mmol per day per kg of body weight and 1–2 mmol of carnitine is endogenously synthesized (58). The kidney, liver, and brain are the main sites of L-carnitine synthesis. After synthesis, L-carnitine is transported via the circulation and is then taken up by other tissues through carrier-mediated transport systems. In the kidney, L-carnitine and butyrobetaine are reabsorbed efficiently to minimize urinary loss (63). OCTN2 is the primary known active transporter responsible for both the transport of L-carnitine to other tissues and re-absorption in kidney (63).

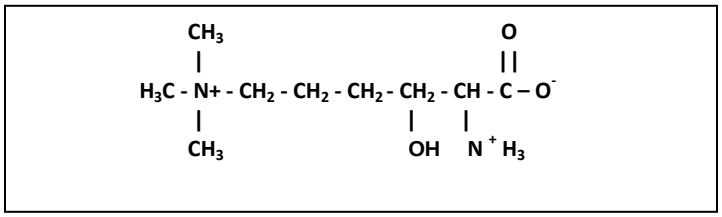
Endogenous biosynthesis of L-carnitine involves a number of enzymatic steps (Figure 1.1) (88, 89); however, this pathway consists of the rate-limiting enzymes, i.e N'trimethyllysine dioxygenase (TMLD) and beta-butyrobetaine dioxygenase (BBH). TMLD mediates the hydroxylation of trimethyllysine (TML) to 3-hydroxy trimethyllysine (HTML) in mitochondria of liver, kidney, heart, muscle, and brain. TMLD is a non-haem ferrous-iron dioxygenase

requiring 2-oxoglutarate, Fe^{+2} and molecular oxygen as supporting factors (90). Limited information is available on HTML aldolase (HTMLA) that catalyzes the aldolytic cleavage of HTML into 4-trimethylaminobutyraldehyde (TMABA) and glycine. TMABA dehydrogenase (TMABA-DH) requires NAD^{+} as a cofactor and liver and kidney express the highest level of TMABA-DH, which is low in brain, heart and muscle (90). BBH's function is to catalyse the stereospecific hydroxylation of butyrobetaine to L-carnitine. BBH requires molecular oxygen, Fe^{+2} and ascorbate for activity. BBH is localized in the cytosol of mammalian cells and is primarily expressed in the liver, kidney, brain, testis, and epididymis (91).



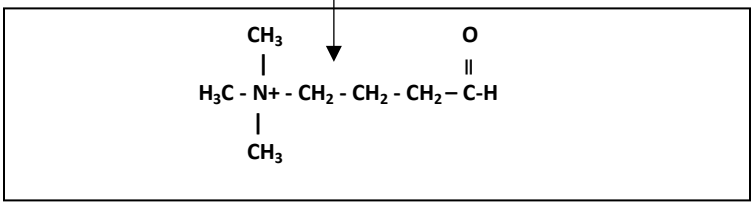
6-N-Trimethyllysine

6-N-trimethyllysine hydroxylase



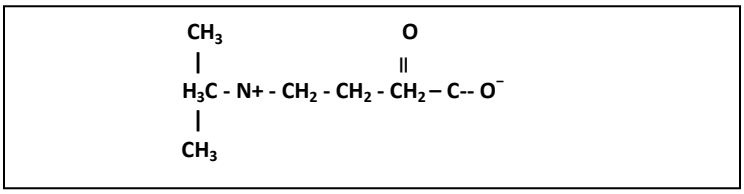
3-Hydroxy-6-N-trimethyllysine

3-hydroxy-6-N-trimethyllysine aldolase



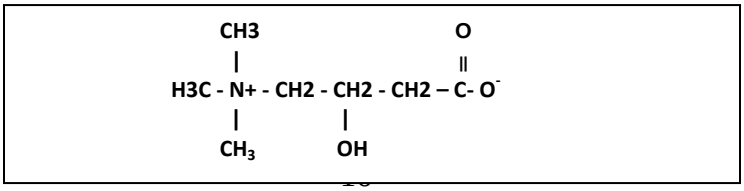
4-Trimethylaminobutyraldehyde

4-trimethylaminobutyraldehyde dehydrogenase



Butyrobetaine

butyrobetaine hydroxylase



Carnitine

Figure 1.1 *De novo* synthesis of L-carnitine.

1.2.6.2 L-Carnitine absorption

Dietary L-carnitine intake by humans ranges from less than 1 up to 15 μmol/kg body wt/day. Vegans typically acquire very little L-carnitine from diet, usually less than 1 μmol/kg body wt/ day. Individuals consuming dairy products obtain 1 to 8 μmol L-carnitine/kg body wt/day and 6 to 15 μmol L-carnitine/kg body wt/day for individuals that consume red meat (92) (93). The bioavailability of dietary L-carnitine ranges from 54–86% of dietary intake. *In vivo* and *in vitro* studies on L-carnitine uptake in the intestine reported two absorption modes, linear absorption and nonlinear absorption (92). Linear absorption of L-carnitine represents the unsaturable passive diffusion of L-carnitine across the intestinal epithelium, whereas nonlinear absorption represents the carrier mediated saturable and active uptake process by transporters. Studies of L-carnitine absorption and its components in humans, experimental animals, and cell culture models suggest that L-carnitine is transported from the intestinal lumen into the enterocyte by carrier-mediated processes, but passes across the serosal membrane into the circulation by simple diffusion (94). Consequently, L-carnitine is rapidly taken up from the gastrointestinal lumen, but is slowly released into the portal circulation. Studies with Caco-2 polarized epithelial cell cultures and human muscle biopsy specimens suggest high-affinity, saturable, sodium-gradient-driven uptake of carnitine (95). Extensive intracellular acetylation of L-carnitine (50–60%) occurs in rat and guinea pig intestine and in isolated guinea pig

enterocytes. In rat and guinea pig intestine, acetyl-L-carnitine is more readily released across the serosal membrane than is nonesterified L-carnitine (96). Intracellular acetylation of L-carnitine may facilitate its diffusion across the serosal membrane. In guinea pig intestine, luminal acetyl-L-carnitine was taken up by intestinal mucosa at about the same rate as was nonesterified L-carnitine, but the calculated efflux of total L-carnitine into the circulation was 4-fold greater for acetyl-L-carnitine compared to nonesterified L-carnitine (92, 97).

1.2.6.3 Tissue Distribution and Renal Elimination

L-Carnitine is distributed in various parts of the body with extensive distribution into skeletal muscle and heart. There are three distinctive pools in L-carnitine distribution in the human body (88). The principal pool represents muscle tissues and averages 95% of total body L-carnitine. The second pool consists of liver and kidneys and corresponds to total of 4% of total body L-carnitine. The small pool corresponds to extracellular fluids and it accounts for the remaining 1% of total body L-carnitine (88). The concentration in tissues such as muscle is 25-50 fold higher than in plasma levels due principally to the activity of OCTN2 transporter that actively takes up L-carnitine from blood into tissues against a concentration gradient (63).

The kidneys have a crucial and definitive role in the maintenance of L-carnitine homeostasis in mammals by minimizing the renal loss of L-carnitine. L-Carnitine is eliminated from the body primarily by renal excretion of nonesterified carnitine and acylcarnitine esters (63). As a small, water-soluble non protein-bound compound, L-carnitine is readily filtered by the renal glomerulus. The Na^+ dependent active reabsorption of L-carnitine by OCTN2 occurs in the proximal renal tubule and helps to prevent the renal clearance of L-carnitine. The total renal

elimination of L-carnitine per day is estimated at approximately 100-300 μmol, which is 2-10% of total filtered L-carnitine (92, 98).

L-Carnitine reabsorption and excretion rates are affected by diet and physiological and pathological factors (99, 100). Less efficient reabsorption of acylcarnitines and renal acylation of L-carnitine followed by leakage of the locally formed acylcarnitine products into urine can lead to the high acylcarnitine to carnitine ratio in urine compared to that of plasma (99, 100). The efficiency of carnitine reabsorption increases as dietary intake of L-carnitine decreases, independent of glomerular filtration rate and filtered load (98). This adaptive response serves to maintain circulating L-carnitine concentration in the face of decreased input from dietary intake. Conversely, the rate of L-carnitine excretion increases rapidly, and the efficiency of L-carnitine reabsorption decreases as the filtered load of L-carnitine increases above normal, as for example following ingestion of a dietary L-carnitine supplement or by intravenous infusion of L-carnitine (98). A "threshold" effect is observed at near-normal plasma L-carnitine concentrations, above which the rate of L-carnitine excretion soon parallels the increase of filtered load. This homeostatic mechanism serves to maintain circulating L-carnitine concentrations in a narrow "normal" range (98).

L-Carnitine is required as a cofactor in the transport of long-chain acyl groups (fatty acids) across the mitochondrial membranes to facilitate the β-oxidation of LCFA and hence ATP production. L-Carnitine uptake into tissues is mediated through different transporters; 1) organic cation/carnitine transporters (OCTNs), 2) neutral and cationic amino acid transporter, $ATB^{0,+}$, and 3) carnitine transporter 2 (CT2). Polyspecific organic cation transporters belong to the SLC22 family and the MATE family (101). The SLC22 family contains the three subtypes of passive diffusion organic cation transporters called OCT1 (SLC22A1), OCT2 (SLC22A2) and

OCT3 (SLC22A3); Organic cation/carnitine transporter OCTN1 (SLC22A4) that may be a proton cation exchanger; the Na^+-carnitine cotransporter OCTN2 (SLC22A5), which facilitates the Na^+ independent transport for organic cations and L-carnitine; and organic cation transporter OCT6 (SLC22A16) (101).

Apart from OCTNs, $ATB^{0,+}$ also transports L-carnitine across cellular membranes. This transporter belongs to the SLC6A family of transporters (102). The letter B of $ATB^{0,+}$ represents broad substrate specificity of the transporter system and the superscript represents both neutral and cationic amino acids as substrates to this transporter (102). $ATB^{0,+}$ transports amino acids depending on Na^+ and Cl^- ion concentrations across the cell membrane. $ATB^{0,+}$ has low affinity for L-carnitine and is expressed in the lung, colon, mammary gland, pituitary gland and ocular tissue (103-105). Furthermore, L-carnitine is transported by L-carnitine transporter 2 (CT2). This novel transporter belongs to the SLC6A family of transporters and is expressed in human testis. The localization of these transporters in tissues and at epithelial barriers is critical in understanding their endogenous role in cellular metabolism and function. For example, expression of OCTN3 in peroxisomal membranes plays a critical role in peroxisomal β-oxidation pathways (106).

1.2.6.3.1 Organic Cation/Carnitine Transporters

Organic Cation/Carnitine Transporters (OCTNs) are members of the *Solute Carrier Transporter 22 (SLC22)* family (107, 108). Although SLC22 members can transport cationic chemicals, OCTNs are most notably known for their ability to mediate the cellular uptake of L-carnitine (107). OCTN class has three different transporters, OCTN1, OCTN2 and OCTN3. OCTN1 was first cloned from a human liver cDNA library, and rodent rat and mouse isoforms

were later isolated (109). OCTN1 transporter protein consisted of 11 transmembrane domains and one-nucleotide binding domain. OCTN1 is a sodium-independent organic cation transporter that transports cations in a bidirectional, pH dependent manner. It is a multispecific transporter and has low affinity for L-carnitine in species including rat and humans. It is abundantly expressed in intestine, liver, and kidney (110, 111).

OCTN2 was cloned from a human kidney cDNA library and OCTN2 possibly has 12 transmembrane domains (112). OCTN2 has high affinity for L-carnitine and transports L-carnitine in a sodium-dependent manner. The unique aspect of this transporter is its dual mode of transport: it transports organic cations via a sodium independent process and L-carnitine via a sodium dependent process (113). It is widely expressed in tissues such as heart, skeletal muscle, kidney, intestine, placenta, brain and mammary gland. A mutation in the OCTN2 gene causes secondary L-carnitine deficiency (114). OCTN1 and OCTN2 transporters show more than 70% similarity in their amino acid sequence; however, it is identified that both transporters demonstrate differences in their function (115). OCTN3 was first detected in mouse kidney, and OCTN3 protein has later been detected in a human cell line (116). OCTN3 transports L-carnitine in a passive, sodium and pH independent manner. It has higher specificity for L-carnitine transport than OCTN1 and OCTN2. It is predominantly expressed in testis and in peroxisomes (117).

1.2.6.3.2 OCTN2 (Slc22A5) transport

OCTN2 has a relatively ubiquitous distribution and is expressed in epithelial cells, muscle cells, glial cells, macrophages, lymphocytes and sperm. In humans, the strongest expression of OCTN2 was found in liver (118), kidney (119) (120), skeletal muscle (121), heart, placenta,

brain (122), mammary glands (123), and blood-retinal barrier (124). As well OCTN2 is expressed in intestinal tissues (125) and contributes to the absorption of L-carnitine. In heart, OCTN2 protein is present in the vascular endothelium (124). OCTN2 transporter expression was also found in some cancer cell lines suggesting that L-carnitine has a crucial role in maintaining energy metabolism in cancer cells. In rodents, Octn2 was wider distribution than in human (126). Octn2 was found in blood vessels, and astrocytes. In kidney of rat Octn2 transporter is localized to the apical membrane of proximal tubular epithelial cells (126). In mice, octn2 has localized to the apical membrane of cells lining the epididymal ductules and of proximal tubular epithelium in kidney (126).

Sodium-dependent, high-affinity carnitine transporter, OCTN2, is responsible for the active transport of L-carnitine into several tissues (Figure 2). Skeletal muscle and cardiac muscle utilize fatty acids as an energy source; therefore, a deficiency of L-carnitine in these tissues leads to cardiomyopathy and muscle weakness. In juvenile visceral steatosis (*jvs*) mice, which exhibit a Secondary Carnitine Disease (SCD) phenotype, alterations in intestinal absorption, tissue distribution, and elimination of L-carnitine have been observed (85, 127, 128). Pathologically, *jvs* mice display severe lipid accumulation in the liver, hypoglycaemia, hyperammonaemia, and growth retardation (129). It was confirmed that L-carnitine transport activity is impaired due to mutation of the octn2 gene in jvs mice and mutation of the OCTN2 gene in SCD patients. In addition, Japanese individuals and familial members who are affected by L-carnitine deficiency have various mutations in the OCTN2 gene, all of which are associated with a loss or decrease of L-carnitine transport activity (130, 131). OCTN2 is now generally accepted to be the causal gene of the genetic disease SCD (130, 131).

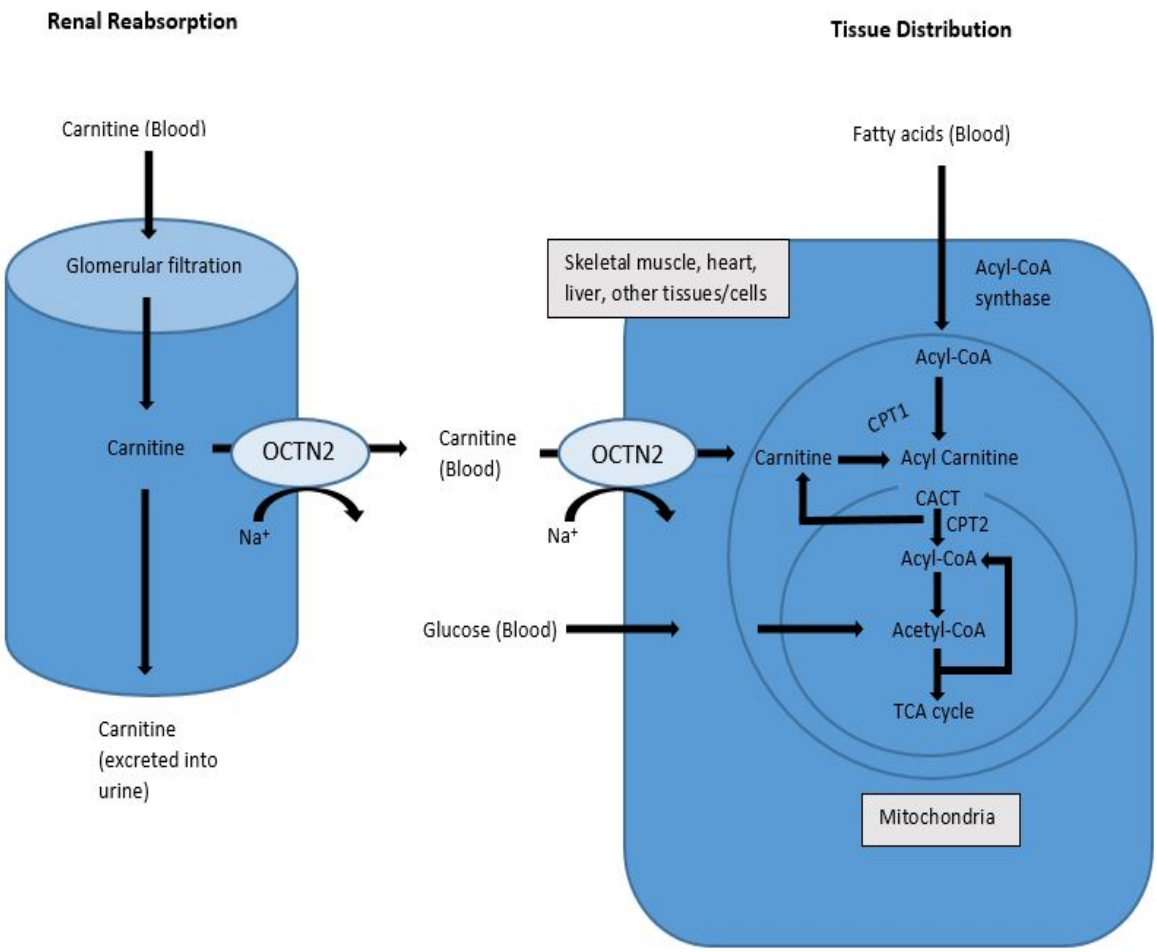


Figure 1.2 Schematic of the distribution and role of Octn2 in L-carnitine disposition and importance of L-carnitine in long-chain fatty acid oxidation. Octn2 facilitates the reabsorption of L-carnitine into the systemic circulation and also Octn2 mediated transport of L-carnitine from blood to various tissues.

1.2.7 L-Carnitine Homeostasis in Neonates

The shift in nutritional source from the maternal umbilical blood supply to milk causes important physiological changes in newborns. The source for metabolic energy for the fetus comes from carbohydrate sources, mainly glucose, whereas neonates rely less on carbohydrate

sources and more on fatty acids (132). The newborn must adapt to the shift in energy substrate supply. Development of fatty acid utilization mechanisms is requisite to guarantee appropriate physiological development. Moreover, fatty acid oxidation is the part of thermal homeostasis in neonates, and disorders in fatty acid oxidation predispose to a risk for sudden infant death syndrome (133). In neonates, L-carnitine is essential for long-chain fatty acid oxidation. Fatty acid oxidation rates increase in early stages of neonatal development and these results correlate with the evidence that show increased levels of L-carnitine concentrations in neonates (134).

In neonates, the concentration of free L-carnitine in the plasma characterizes the balance between intestinal uptake, biosynthesis, and reabsorption by the kidney. The plasma L-carnitine concentration increases from postnatal day 8 of life and reaches adult levels at 4-7 months of age in human (134, 135). In early postnatal development, neonates depend on an exogenous source as enzymes required for L-carnitine biosynthesis are very immature. Hepatic γ-butyrobetaine hydroxylase (γ-BBH) levels are extremely low in early stages of life and the activity of BBH enzyme increases with age (136). Expression of BBH mRNA, however, was undetectable in foetuses and newborn rats (136). Gene expression of Bbh enzyme in rat shows an increase between postnatal day 4 and 20, but significant increases have been seen between postnatal day 4 and 11. In addition to the maturation of Bbh enzyme, Cpt and trimethylamino hydroxylase (Tmlh) expression and enzyme activities increase during postnatal development (136).

The kidneys play a dual role in body L-carnitine homeostasis. The kidneys efficiently reabsorb filtered L-carnitine via OCTN2 and, at least in humans, the kidneys are involved in L-carnitine biosynthesis. Foetal rat kidneys express Octn2 mRNA and the same transcript is present in all ages studied (91). In human kidneys, however, neither BBH nor TMABA-DH activities were affected by age. Plasma L-carnitine concentration is maintained within a narrow range in

spite of the continuous supply of L-carnitine to the plasma under normal physiological conditions (137). Renal clearance is the determining factor in the maintenance of plasma levels of L-carnitine and renal clearance is largely determined by the kinetic properties of the brush-border Na^+/L-carnitine cotransporter, OCTN2. Renal reabsorption of free carnitine is 98% and 99% of the filtered load, unless the transporters become saturated. Hence, the kidney has a dual role in L-carnitine homeostasis: it minimizes clearance of L-carnitine in the urine and also the kidney is involved in biosynthesis of L-carnitine in human (138-141). To our knowledge, limited data are available regarding: (i) at what stage of nephrogenesis the OCTN2 transporter is first apparent and at what stage its activity corresponds to that in adults, and (ii) whether renal expression of BBH and TMABA-DH mRNAs are affected by ontogeny. A systematic study on the ontogeny of Octn2, Bbh and Tmlh in foetuses, newborn, suckling, weaning and adult rats was conducted to develop concrete knowledge on ontogeny of L-carnitine mechanisms (136). The results suggest that the kidneys of rat foetuses have a functional OCTN2 transporter and that with development increases in the activity and mRNA expression of OCTN2 and the expression of BBH and TMABA-DH mRNAs is observed.

1.2.7.1 Ontogeny of Bbh and Tmlh

The renal expression of TMABA-DH and BBH undergoes a developmental maturation in human and rats (91). TMABA-DH mRNA was detected in foetuses and its abundance significantly increased from neonatal to adult rats. Renal BBH mRNA expression, however, was not detected until the suckling period and thereafter it was significantly modified by age in rats (91). Therefore, the kidney and liver tissue of foetuses and newborn rats cannot synthesize L-carnitine or at least the turnover of L-carnitine during these periods is not sufficient to conduct

normal physiological functions. Although adult rat kidney expresses BBH and TMABA-DH mRNAs, this expression was much lower than that measured in liver, suggesting that in the rat at least, L-carnitine biosynthesis occurs mainly in the liver. In adult rat, it is reported that the activity and expression levels of these two enzymes in liver and kidney were increased at least by several fold suggesting clear ontogenic changes in L-carnitine biosynthesis maturation (91). In humans neither renal BBH nor TMABA-DH, activities were affected by age, whereas liver BBH activity and mRNA levels were increased by maturation.

1.2.7.2 Ontogeny of Octn2

The shift in nutritional source in neonatal diet necessitates the ontogenic changes in L-carnitine homeostasis mechanisms. The neonatal diet has a considerably higher content of fatty acids originating from milk. Hence, neonates require maturation of fatty acid oxidation mechanisms to generate the energy from a fatty acid rich diet. Transporters, mainly OCTN2, involved in L-carnitine homeostasis, undergo changes in neonates to utilize the fatty acid rich diet during the postnatal development (126). In newborns, the uptake of exogenous L-carnitine from intestine in early postnatal days is necessary to maintain systemic L-carnitine levels as endogenous synthesis is limited. *In vivo* studies reported the postnatal development of key factors involved in L-carnitine homeostasis (136). Na^+-Dependent L-carnitine uptake rate in intestine is significantly higher in both pre-term foetuses and newborn rats than in suckling rats. After weaning, Na^+ dependent L-carnitine uptake is no longer measurable in any intestinal regions examined. This is consistent with the peak in Octn2 expression in intestine in newborn rats with expression levels decreasing with age. Octn2 expression in the ileum was higher than in jejunum. In addition to the transporter expression data, the rate of Na^+ independent L-carnitine

uptake also increased after birth, and thereafter it decreased during the suckling period until it reached adult values (91). Developmental maturation is associated with a decrease in the number (and/or activity) of the intestinal L-carnitine uptake carriers.

Decreases in rat intestinal Octn2 expression with the age indicates that in rats, homeostatic control of serum L-carnitine concentration switches from dietary intake of L-carnitine in the intestine to *de novo* biosynthesis in liver and kidney (142) (143). During the late gestation period and early stage of life, the rat L-carnitine biosynthetic pathway is immature and the demand for L-carnitine may exceed its endogenous biosynthesis (142) (143). Foetuses and neonates would therefore depend upon external sources of L-carnitine to meet their metabolic needs. During these periods, the mother's liver L-carnitine biosynthesis and intestinal Octn2 is up-regulated, and rat pups may receive most of their L-carnitine from their mother. Several studies suggested that foetal L-carnitine is derived from the mother via transplacental transfer of L-carnitine, which is supported by the observation that OCTN2 is expressed in human placenta (144). Age-dependent changes in Octn2 transporter expression levels in rat kidney were reported previously in addition to the studies on intestinal Octn2 expression levels (91, 136). Foetal rat kidneys express Octn2 mRNA and the same transcript is present in all the ages studied. As previously reported, Octn2 mRNA abundance is up-regulated in kidney by maturation, mainly in the renal cortex (126, 145). These findings suggest that from the end of the gestation period to adulthood, Octn2 mediates renal Na^+/L-carnitine transport and this corroborates the view that maturation increases the number of Octn2 transporters at the apical membrane.

The findings of ontogenic changes of intestinal L-carnitine uptake and Octn2 transporter expression correlate with the maturation of L-carnitine biosynthesis mechanisms. In the early stage of life, when the demand for systemic L-carnitine exceeds the endogenous capacity for its

biosynthesis, rat pups receive most of their L-carnitine from exogenous sources by active uptake by OCTN2 transporters. In the growing rat, however, the endogenous capacity for L-carnitine synthesis is increased sufficiently, and its intestinal Na^+/L-carnitine transport activity is no longer measurable. Therefore, in the same individual, up-regulation of the liver L-carnitine biosynthesis is accompanied by the down-regulation of its intestinal Na^+/L-carnitine transport activity (143, 146). In isolated brush-border membrane vesicles (BBMV), OCTN2 transporter expression was increased significantly in the postnatal stage. Between the postnatal day 5 and 11, heart, renal and intestinal OCTN2 mRNA levels showed significant changes and the expression increased to 80% of its adult levels (126). The observed changes in activity of transporters and enzymes involved in L-carnitine biosynthesis demonstrate the significant maturation of L-carnitine hemostasis in neonates during the postnatal day 1 and 20. In addition, evidence suggests the ontogeny of transporters and enzymes can be modified by various effects, even though expression is genetically programmed (147). Furthermore, intestinal membrane lipid composition, fluidity and activity of Na^+-dependent D-glucose transport are affected by dietary fat. It is inferred that the modifications in normal neonatal health during neonatal development may influence the ontogeny of transporters and enzymes.

1.2.8 Inflammation and its effect on transporter expression and function

The immune system is primarily responsible for the control or eradication of pathophysiological conditions that are established in mammalian system by a disease-causing organism or by processes that elicit an inflammatory response (148). The response of the immune system to pathogenic and pathophysiological conditions is activation of a series of events denoted as the inflammatory/immune response. The inflammatory response is coordinated

by an array of diverse mediators originating from diverse cell types. There is a large body of evidence in pharmacology suggesting that changes associated with the inflammatory response are responsible for significant changes in drug disposition (149).

A considerable amount of attention has been given to the effect of inflammation on drug disposition mechanisms in recent years (150). In the adult, effects of inflammation on transporters involved in uptake and disposition of various nutrients, mainly L-carnitine and drugs have been investigated (151, 152). In recent studies, it is reported that administration of endotoxin significantly decreased bile flow due to the effect of an inflammation-mediated down-regulation of hepatic organic anion and renal glucose transporters (153, 154). As well, interferon-γ (IFN- γ), a principle cytokine, is responsible for induction of the inflammatory response by increased expression of the di/tri-peptide transporter h-PEPT1 (SLC15A1) in cultured human intestinal cells (Caco-2) (155, 156). Pro-inflammatory cytokines, Tissue Necrosis Factor-Alpha (TNF)-alpha, Interklukin-6 (IL-6) and Interlukin-1 β (IL-1 β), decrease solute carrier (SLC) and multi drug resistant transporter (MRP) expression in primary human hepatocytes (157, 158). The expression profile of OCT1 (SLC22A1) is of particular interest because of its close relationship to OCTN family transporters (159, 160). OCT1 expression was decreased several fold when hepatocytes were exposed to IL-6 and IL-1β. It is reported that one of the major components responsible for developing the inflammatory response, TNF-α, induced a sustained decrease in Ntcp, Oatp1/Oatp1a1, and Bsep mRNA expression as well as a transient effect on expression of multidrug resistance associated protein 2 (Mrp2) (161). Studies reported that the decrease in mRNA expression of Octn2 transporter in rat colon is a significant factor for development of inflammatory bowel disease (162). Many transporters have been shown to be down regulated with LPS administration and in inflammatory diseases. Moreover, the effects of inflammation on

various drug transporters and the pharmacokinetic consequences of altered drug transporter expression have undergone some investigation (163).

The identification of the complex relationship between inflammation and regulation of transporter expression is in its initial stages. However, evidence suggests the involvement of class II nuclear hormone receptors in transcriptional regulation in inflammatory conditions (164, 165). The effects of endotoxin treatment on transporter expression and the activity of corresponding transcription factors have been studied in detail (166, 167). Endotoxin treatment leads to down regulation of sodium dependent taurocholate cotransporter Ntcp (*Slc10a1*) protein expression, mRNA levels, transcription initiation, and reduces nuclear levels of its key transactivators, hepatocyte nuclear factor 1 (HNF-1; *TCF1*) and the nuclear receptor heterodimer retinoid X receptor:retinoic acid receptor (RXR:RAR) (168, 169). The mechanism of pro-inflammatory cytokine-regulated regulation of transporter expression has been clearly investigated (170, 171). However, the ability of the inflammatory stimulus to alter the ontogeny of enzymes and transporters involved in nutrient homeostasis in neonates has not received much attention.

1.3. Rationale and Purpose:

An important and yet to be answered question is the potential external factors that are likely to affect developmental programming of L-carnitine homeostasis. Many approaches are used to understand those factors involved in epigenetic regulation, but few studies evaluate importance of nutrition and macro- or micronutrients. Previous work in my laboratory assessed the impact of a drug on L-carnitine homeostasis pathways to provide perspective on the impact

of exogenous factors on the maturation of L-carnitine homeostasis processes and the possible long-term results of a drug exposure on the maturing physiological system.

At present, very little information exists on the impact of disease on normal developmental programming. Inflammation is a common condition of neonatal disease and the adult literature clearly indicates that inflammation alters expression of various nutrient and xenobiotic transporters and enzymes. The purpose of this thesis is to resolve an important question, whether an inflammatory stimulus in neonates can cause significant changes in the ontogeny of L-carnitine homeostasis and change the maturing physiological processes involved in L-carnitine homeostasis. Regardless of the fact that we know much about L-carnitine homeostasis and biosynthesis and its physiological importance, we have much to learn about the mechanisms and regulatory signals of ontogeny of L-carnitine homeostasis mechanisms. Information about how factors associated with ontogeny change relative to each other under inflammatory conditions will offer an improved understanding of the processes involved in L-carnitine homeostasis and physiological maturation of organs crucially associated with fatty acid metabolism. The information which will be gathered from this research will have clinical significance.

4. Hypothesis and Objectives

At present, there is very little information on the potential ability of inflammation in neonates to cause alterations in the normal ontogeny of L-carnitine homeostasis mechanisms. The effect of inflammatory stimuli in neonates could differ depending on age, intensity of exposure, and the timing of that exposure during neonatal development. Such inflammatory stimulus effects may lead to adaptive approaches that alter the normal ontogenic pattern of L-carnitine homeostasis in neonates. The overarching hypothesis of this thesis is as follows:

Hypothesis: An inflammatory stimulus will alter the ontogeny of L-carnitine homeostasis pathways and this depends upon when the inflammatory stimulus occurs in postnatal development.

Objectives

i. Evaluation of the effect of inflammation on Octn2 transporter expression in different age groups of neonates.

ii. Evaluation of effect of inflammation on ontogeny and activity of enzymes involved in carnitine biosynthesis and whether this differs depending upon when in postnatal development the inflammatory stimulus occurs.

Chapter 2

2. Materials and Methods

2.1 Animals:

Female Sprague-Dawley (SD) rats ordered at gestation day 14 (for pups at postnatal day 3, 7 and 14) (n=4 of each postnatal stage) were obtained from Charles River Canada (St. Constant, PQ) and were housed singly in a room temperature and humidity controlled facility (22°C ± 2°C) on a 12-hour light:dark cycle (0700 h – 1900 h). All rats had free access to food (Prolab® RMH 3000, Purina, Inc., Richmond, IN) and water throughout the study and were allowed a 7-day acclimatization period. All animal experimental procedures were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane and animal use. A MilliQ Synthesis (Millipore, Bedford, MA) water purification system provided purified deionized water.

2.2 Materials

Sterile 15 mL and 50 mL polypropylene centrifuge tubes, 12 well plates, 96 well plates, eppendorf tubes, bicinchoninic acid (BCA) protein assay kit, and 4 mL scintillation vials were purchased from Thermo Fisher Scientific (Ottawa, Ontario, Canada). Quantitative reverse transcription-polymerase chain reaction (QRT-PCR) tubes and two-step SYBR green RT-PCR kits were acquired from Applied Biosystems (Foster City, California, USA). Ribonucleic acid (RNA) isolation mini kit and reverse transcription kit were purchased from Qiagen Inc. (Toronto, Ontario, Canada). Enzyme-Linked Immuno Sorbent Assay (ELISA) kits for tumour necrosis factor alpha were purchased from Sigma-Aldrich (Toronto, Ontario, Canada). Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), creatine (Cr), creatine phosphate (CrP), L-carnitine, horse serum, trypsin, phosphate buffered saline, and

lipopolysaccharide (LPS: Escherichia coli strain O55:B5) were purchased from Sigma-Aldrich (Toronto, Ontario, Canada). High performance liquid chromatography (HPLC) grade methanol was purchased from Fisher Laboratories (Toronto, Ontario, CA). Highly purified deionized water was obtained from a MilliQ Synthesis water purification system (Millipore, Bedford, MA). All other solvents and reagents used were of the highest analytical grade available.

2.3 Acute LPS Administration in Rat Pups

Pregnant SD rats (14 days gestation) were obtained from Charles River and housed in pairs according to usual animal husbandry procedures. Two days prior to expected parturition, dams were housed individually. Pups culled from litters were humanely killed by isoflurane anaesthesia and exanguination by cardiac puncture. At postnatal day 3, 7 and 14, pups were removed from their dams for short duration (maximum 15 minutes). During this time body weight was measured. For each litter, half of the pups were randomly selected to receive an intraperitoneal injection (0.1 mL) of LPS and the other half saline (0.1 mL). LPS was administered at 0.1 mg/Kg bodyweight in 1 mL/Kg pyrogen-free saline. This dose (0.1 mg/Kg LPS) was reported in the literature to cause a febrile reaction in neonatal rat pups but not death due to endotoxaemia (172). The litter is considered an individual sample and four litters were used per postnatal treatment day (to give a sample size of n=4/group) and male/female pups from a single litter are considered a replicate within that individual sample.

At 3 and 16 hours post-LPS administration, pups were humanely killed following isoflurane anesthesia and blood, heart, intestine, kidney and liver rapidly collected. The dosing schedules were considered from results obtained from careful review of literature (173) and from a pilot study to monitor short-term and long-term inflammatory effects and developmental maturation of L-carnitine biosynthesis pathways in rat pups (postnatal day 3, 7 and 14). Blood

samples were collected in glass tubes and left for 1 hour at 37°C to allow them to clot. The serum was separated from blood by centrifugation at 1700 x g for 20 minutes @ 4°C, and transferred to new eppendorf tubes with storage at -20°C. All tissues were snap frozen in liquid nitrogen and stored at -80ºC. Blood and tissues were pooled from 3-4 pups from a single dam at each time points such that the dam was considered the experimental unit.

2.4 Serum Tumour Necrosis Factor – Alpha (TNF-α) Levels

TNF-α activity in serum was measured using enzyme-linked immunosorbent assay (ELISA). Quantitative analysis of TNF-α was performed for pooled serum samples according to the manufacturer's protocol. In brief, specific antibodies for rat TNF-α was coated on 96-well plate well. In the first step, standard samples were added to wells to develop a standard curve to calculate the TNF-α levels in samples. Standard samples were washed four times with wash buffer and then biotinylated anti-rat TNF-α antibody was added. The washing step was repeated to wash biotinylated anti-rat TNF-α antibodies, after washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted into the wells. A 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was added to the wells and after one more washing step was employed, the color develops in proportion to the amount of TNF-α bound. The Stop Solution (0.2M Sulphuric acid) changes the color from blue to yellow, and the intensity of the color was measured at 450 nm. The same procedure was employed for serum samples. The slope and coefficient of determination values were found to be 0.2089 and >0.986, respectively. The standard curve range was linear (r^2 >0.98) between 0.082-20.00 ng/mL.

2.5 L-Carnitine Analysis in Serum and Heart

Free L-carnitine present in rat serum and heart samples was measured using an HPLC-UV based pre column derivatization method that was previously established in our lab (136, 174).

The pooled heart samples were homogenized with phosphate buffer (50 mM, pH 7.4) and the homogenate was centrifuged (Accuspin Micro 17, Fisher Scientific) at 2500×*g* for 10 min at 4ºC. Proteins present in the heart homogenate supernatant or in serum samples were precipitated using acetonitrile:methanol in 9:1 v/v ratio. For every 20 µL heart homogenate or serum, 180 µL of acetonitrile:methanol mixture was added. 300 mg mixture of Na_2HPO_4 and Ag_2O (9:1 wt/wt) and then 300 mg of KH_2PO_4 was added and vortexed for 1 hour. Derivatization reagent (40 mg/mL ρ-bromophenacyl bromide with 50 µL 40% tetrabutylammonium hydroxide solution) was added into the organic extract. The reaction mixture was incubated at 60ºC for 120 minutes in a water bath. The resulting reaction mixture was cooled immediately in cooled tap water and then centrifuged at 12,000×*g* for 15 min. Resulting final samples were filtered through micro filters into HPLC vials. L-Carnitine was analyzed using an Agilent 1200 HPLC system with Diode Array Detector (DAD), quaternary pump system and autosampler. Sample size of 10 µL was injected using an auto sampler onto a CN (cyano) column (HyperClone 5 µm, 250 × 4.6 mm, Phenomenex, Torrance, CA) with detection wavelength set at 260 nm. The mobile phase (90% acetonitrile/10% mM citrate phosphate buffer, pH 3) was delivered at a flow rate of 1 mL/min. Intra- and interassay accuracy and precision ranged from 0.4%- 9.6%.

2.5.1 HPLC method validation parameters

An HPLC method for free L-carnitine was validated using the standard addition method. The linearity of measurement was evaluated by analyzing different concentrations of the standard solutions of the L-carnitine hydrochloride in blank serum. The calibration curve was generated by subtracting peak area values obtained for standard samples from the peak area values of blank samples. The calibration curve was constructed by plotting average peak area against concentration and a regression equation was computed. The slope and coefficient of

determination values were found to be 12.602 and >0.99, respectively. The standard curve range was linear (r^2 >0.99) between 1.03-66.7 μmol/L.

2.6 Total mRNA Isolation and Quantitative RT-PCR Analysis

Total RNA was extracted using RNeasy Midi Kits according to the manufacturer's instructions. Frozen tissues were thawed for 15-20 min at room temperature. Cell lysate was homogenized with 350 μL of buffer RLT containing beta-mercaptoethanol (β-ME). To this homogenate, 1 volume of 70% ethanol was added to precipitate the nucleic acids. The cell lysate was applied to the RNeasy Midi column, and a series of buffers were added to remove the cellular contaminants according to the manufacturer's instructions. The purified RNA was isolated from the column using a RNase-free water (30 μL). The concentration and purity of the isolated RNA was determined by using Nanoview spectrophotometer (GE Healthcare Life Sciences, Quebec, Canada). Total RNA was quantified by measuring the absorbance of a diluted RNA (RNA: RNase-free water) at 260 nm according to the following formula:

Concentration of RNA = 40 μg/mL × A260 × Dilution factor

RNA purity was assessed by measuring absorbance ratio (A260/A280) of a diluted sample of RNA (RNA: 10 mM TrisCl (pH-7.5)). Pure RNA has a ratio between 1.9- 2.1. All samples used for QRT-PCR were of high purity. Total RNA was stored at -80°C until analysis.

2.6.1 Primer Design

Gene sequences for Octn2 transporter, Tmlh, Cpt1b, Cpt2 and Bbh enzymes and β-actin were obtained from the National Center for Biotechnology Information Gene bank (NCBI) and specific primers were designed using Primer3 software (http://frodo.wi.mit.edu/) (Table 2.1). The primer pairs were selected using the following criteria: primer Tm (58–60°C), primer length (18–24 mer), limit repeats of guanosine and cytosine, and amplicon size (80–200 bp).

Table 2.1. Forward and reverse primer sequences, amplicon size, and predicted melt temperatures (Tm) for gene expression analysis of various transporters and enzymes important in the maintenance of L-carnitine homeostasis using QRT-PCR.

Gene	Accession number	Forward primer	Reverse primer	Tm (ºC)	Base pair
β-actin	NM_001101.3	GGGAAATCGTGCGTGCATT	GCGGCAGTGGCCATCTC	86.1	76
Octn2	NM_0006000.3	GATGGTGGAGAAAGCCCAACGG	GAGAGCATTTCTGACTACACGCAGG	81.3	104
Bbh	NM_000576.2	ATTGAGCTGCATCCTCTGGT	CATTCTGCCCCACCCTTCAT	80.5	142
Tmlh	NM_001166496	CAGCCCTCCTACAACTCCTG	AGAGGTTCCTGGACGATGTG	81.6	190
Cpt 1b	NM_145913.3	GGGCGTCAGATTTGGGGGTT	TTGAAGATTAGGAATGGGGAGCG	79.3	91
Cpt2	NM_178498.3	GCCAGGTGAACCCTGCCACTTT	GCTGGTTTGGGGCTGGZGTGT	83.2	145

Octn2: Organic cation/carnitine transporter 2; Bbh: Gamma-butyrobetaine hydroxylase; Tmlh: Trimethyllysine hydroxylase; CPt1b: Carnitine palmitoyltransferase 1b; Cpt2: Carnitine palmityolytransferase 2.

2.6.2 Validation of the $2^{-\Delta\Delta CT}$ Method

Primer pairs for QRT-PCR assays for Octn2 transporter, Tmlh, Cpt1b, Cpt2 and Bbh enzymes was initially validated to give PCR efficiency between 1.9 and 2.1 (standard curve using 3 serial dilutions of RNA). Serial dilutions of primer pairs (3 mM, 0.3 mM, 0.03 mM and 0.03 M) were tested against different concentrations (2000 µg/mL, 1000 µg/mL, 500 µg/mL and 250 µg/mL) of RNA samples. The best possible combination of primer concentration and template concentrations that produced the lowest C_t was selected. The PCR efficiency was determined from the slope of the standard curve generated with serial dilutions of cDNA template for each gene. It was calculated using the equation $Ex = (10^{-1/slope}) - 1$, where Ex is the PCR efficiency. A slope of -3.32 is indicative of a PCR efficiency of 1 (100%). The PCR products of respective genes were verified by 2% agarose gel electrophoresis. The reactions were further optimized for usage of the $2^{-\Delta\Delta CT}$ method using β-actin as an endogenous standard. Only primers giving a PCR amplification close to 100% and the relative efficiencies between the target and β-actin that were approximately equal were used (i.e. the slope from log RNA concentration versus C_T were < 0.1). Fold differences in mRNA expression between control and treated samples were calculated.

QRT-PCR reactions consisted of two steps: 1) Reverse transcription (1 cycle at 48ºC for 30 min) in order to make cDNA from RNA. 2) Real time PCR (Initial activation step (1 cycle at 95ºC for 15 min) was followed by a three step thermal cycling (40 cycles; denaturing at 94ºC for 15 s, annealing at 60ºC for 30 s, and extension at 60ºC for 30 s). Finally, a melt curve analysis from 65ºC to 95ºC at 0.5ºC/s was performed.

2.7 Liver γ-Butyrobetaine Hydroxylase (Bbh) Enzyme Activity

γ-Butyrobetaine hydroxylase (Bbh) enzyme portion was extracted from homogenates of liver samples by employing two successive centrifugation steps. 100 mg of liver tissue was first homogenized in 100 mL of homogenization buffer (300 mM sucrose, 1 mM EGTA, and 50 mM Tris, pH 7.5). In the first centrifugation step, the liver homogenate was centrifuged at 13,000 ×g for 30 min at 4ºC. The supernatant of the first centrifugation step was transferred to new tubes and then centrifuged at 100,000 ×g for 1 h at 4ºC. The cytosolic fraction containing Bbh was then subjected to dialysis (molecular weight cutoff of 12,000 Da, D9777, Sigma, USA), and was placed in 5 L of dialysis buffer (75 mM KCl, 0.1mM DTT and 0.5 mM EDTA in sodium phosphate buffer, pH 7.4) in a glass beaker covered with parafilm for 12 hours at 4°C (175). The dialyzed samples (20 µL), which remains in dialysis tube, were tested for L-carnitine residue and the remaining dialysate was stored at -80ºC for Bbh testing (176). The optimal protein concentration, substrate concentration, and reaction time to give linear product formation were determined for each age group. For determination of Bbh activity in rat pup livers, 1 mL reaction buffer consisting of 0.2 mM γ-butyrobetaine, 20 mM potassium chloride, 3 mM 2-oxoglutarate, 10 mM sodium ascorbate, 0.4 mg/mL catalase in 20 mM potassium phosphate buffer (pH 7.0) was prepared. The reaction was initiated by addition of 2 µL ferrous ammonium sulfate (FAS) (final concentration was 0.25 mM) and 20 µL enzyme into 78 µL reaction buffer and incubated for 25 min at 37ºC using a Boekel/Grant Orbital and Reciprocating Water Bath (Model ORS200, ExpotechUSA, Houston, TX). The reaction was then terminated by adding 10× volume of acetonitrile:methanol (9:1). The mixture was centrifuged at 13,000×g for 2 min and the supernatant was used for L-carnitine analysis by HPLC as described above.

2.8 Heart Carnitine Palmitoyltransferase (Cpt) Enzyme Activity

Heart Cpt enzyme activity was measured using the spectrophotometric method previously established in the laboratory (177). Frozen heart tissue was homogenized in 10% (wt/v) homogenization buffer (20 mM HEPES, 140 mM KCl, 10 mM EDTA and 5 mM $MgCl_2$, pH 7.4) supplemented with 3 mg nagarse using a Polytron homogenizer (Brinkmann Instruments, Rexdale, Canada). The homogenate was then centrifuged at 500×*g* for 10 min at 4°C. The supernatant was collected in new tubes and centrifuged at 9000×*g* for 35 min at 4°C. The pellet was then washed with the homogenization buffer without nagarse and centrifuged at 9000×*g* for 35 min at 4°C. The pellets obtained from the above steps were resuspended in 200 µL isolation buffer without nagarse. Protein concentration was measured using the Advanced Protein Assay kit (Bio-Rad Laboratories Canada, Mississauga, ON) with bovine serum albumin as standard. The optimal protein concentration and reaction time to give linear product formation were initially determined. To determine total Cpt activity 20 µg protein was assayed in 200 µL ml reaction buffer containing 20 mM HEPES, 1 mM EGTA, 220 mM sucrose, 40 mM KCl, 0.1 mM 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), 1.3 mg/mL BSA, and 40 µM palmitoyl-CoA, pH 7.4. The reactions were initiated by adding 1 mM L-carnitine and read at 412 nm after 5 min incubation at 37ºC using Synergy HT Multi- Mode Microplate Reader (Biotek instrument, USA).

Cpt2 activity was determined using the same reaction conditions as total Cpt except 10 µL Cpt1 inhibitior, malonyl-CoA, was added into 200 µL of the reaction mixtures to obtain a final concentration of 10 µM. Cpt1 activity was calculated by subtracting the Cpt2 activity from the total Cpt activity. The Cpt activity was calculated as amount of CoASH released per min per mg protein, which is based on the 5-thio-2-nitrobenzoate formation from CoASH-DNTB reaction. The extinction coefficient for 5-thio-2-nitrobenzoate was 13.6 mM/cm (178, 179).

2.9 Heart High Energy Phosphate Substrate Determination

High energy phosphate substrate levels (adenosone triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP)) and creatine compound levels **(**creatine (Cr) and creatine phosphate (CrP)) in heart samples were measured using HPLC-UV as described previously by Olkowski et al (180). The 1200 series High Pressure Liquid Chromatography (HPLC) from Agilent Technologies (Mississauga, ON, Canada) was employed to carry out the following assays. The HPLC system is consisted of a series 1200 quaternary pump with an online degasser, autosampler and diode array detector. Processed samples (10 μL) were injected onto a C18 column (Pursuit XRs 5C18 250×3.0 mm) using auto sampler. The analytes were eluted under gradient conditions (table 5) at a flow rate of 0.7 mL/min and absorbance was monitored at 210 nm. The mobile phase was filtered through a 0.22 μm Nylon filter (Pall Scientific, Mississauga, ON) and degassed in an ultrasonic bath for 30 min prior to use. The column was maintained at 20°C and washed with water:methanol (50:50) after every use.

Table 2.2. Gradient assay of HPLC-UV method to measure Cr, Cr P, ATP, ADP and AMP levels in rat pup heart sample.

Time (min)	**20 mM Phosphate buffer (pH-5.5)**	**Methanol**	**Water**	**Flow rate (mL/min)**
0.0	100	0	0	0.7
17.0	100	0	0	0.7
17.5	0	95.0	5.0	0.4
19.5	0	95.0	5.0	0.4
20.0	0	5.0	95.0	0.4
22.0	0	5.0	95.0	0.4
23.0	100	0	0	0.5
28.0	100	0	0	0.7

For preparation of the standards, primary stock solutions for Cr, CrP, ATP, ADP, and AMP were prepared at a concentration of 1 mg/mL by dissolving in phosphate buffer (pH-5.5). From this stock solution, 10 μg/mL of Cr, CrP, ATP and ADP, and 20 μg/mL of AMP were prepared and diluted serially 2 times to get a standard curve for 6 different concentrations (10 to 0.312 μg/mL for Cr, CrP, ATP, & ADP, and 20 to 0.625 μg/mL for AMP). High quality control (HQC), mid quality control (MQC) and low quality control (LQC) samples at 8, 4, and 0.5 μg/mL for Cr, Cr P, ATP, and ADP and 16, 8, and 1.5 μg/mL for ATP were prepared using the stock solution independent of those concentrations used for the standard curve. Primary stock

solutions were stored at -20°C. The standard curve range was linear (r^2 >0.99). Intra- and interday accuracy and precision was within 12%.

For preparation of the samples, 100 mg of heart samples were homogenized in 1 mL 0.7 M ice cold perchloric acid (MW 100.46) with a final concentration 100 mg/mL. The homogenate was centrifuged at 12,000 rpm for 5 minutes. The supernatant was collected and neutralized with 2M potassium hydroxide to bring pH near to 7.0. The supernatant was then filtered through 0.45 μm filter (Nonsterile Syringe Filter Nylon, Chromatographic Specialties Inc. Brockville, Ontario, Canada) and 10 μL was injected onto a 3μ Luna C-18 (Phenomenex, Torrance, CA) column using gradient flow conditions. Two mobile phase components used included 20 mM potassium phosphate buffer (pH 7.0) and 100% methanol. The gradient was 100% phosphate buffer from 0–6.5 min, 100% methanol from 6.5–12.5 min, and 100% phosphate buffer from 12.5 to 25 min for column re-equilibration, which was sufficient to achieve stable baseline conditions. The high energy phosphate substrates including CrP, Cr, ATP, ADP, and AMP were monitored at 210 nm.

2.10 Statistical Analysis:

The data received from different analysis were reported as mean ± standard error of the mean (SEM). Comparisons for all the parameters at different ages (Postnatal Days) and time points (treatment time) were analyzed using two-way ANOVA with Bonferroni post-test. The effects of LPS on rat pups in each treatment duration period were analyzed by two-way ANOVA. Data used for this purpose include serum L-carnitine levels, mRNA expression level of Octn2, Tmlh mRNA expression, and Bbh mRNA expression and activity in control rats from postnatal day (PD) 3, 7, and 14.

Chapter 3

3. Results

To determine whether acute inflammatory stimuli can change the normal ontogeny of L-carnitine homeostasis in neonates, rat pups at different age (PD3, 7 and 14) were treated with LPS. mRNA and enzyme activity expression levels of important components of L-carnitine homeostasis were evaluated at 3 h and 16 h post injection to capture early mRNA expression changes (3 h) and changes in enzyme activity that takes time to occur (16 h) following the inflammatory stimulus. The results section is divided into two parts: i) Ontogeny of L-carnitine homeostasis, and ii) Effect of inflammation on L-carnitine homeostasis.

3.1 Ontogeny of L-Carnitine Levels, Octn2, Tmlh and Bbh

The ontogenesis of critical components of the L-carnitine homeostasis was examined by evaluation of serum and heart free L-carnitine concentration as well as mRNA expression of Octn2, Bbh and Tmlh in different tissues of rat pups of different postnatal ages. Compared to PD3, (average 20 mmol/L), serum free L-carnitine concentrations were significantly higher at PD7, (average 30 mmol/L), which was further increased to an average value of 34 mmol/L at PD14 (Figure 3.1). Heart free L-carnitine levels also increased with postnatal age, but showed a significant increase between PD7 and PD14 (Figure 3.2).

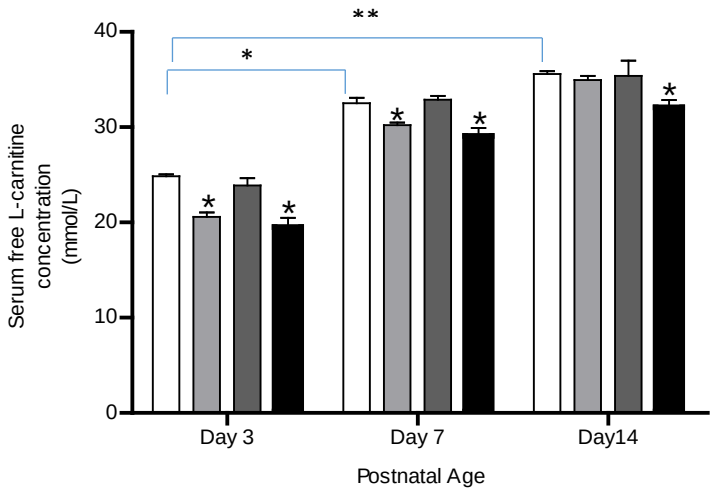


Figure 3.1 Mean ± SEM free L-carnitine concentration in rat serum (n = 4) at postnatal day 3, 7 and 14 (3 hour control – white bar; 3 hour treatment – light grey bar; 16 hour control – dark grey bar; 16 hour treatment – black bar). L-Carnitine concentration was determined using HPLC-UV as described in the text. Two-way ANOVA with Bonferroni post hoc test was used for the comparisons between treatment groups and one-way ANOVA was used for the comparisons between ages and treatment. Bars with an asterick indicate significant difference relative to control, $P<0.05$.

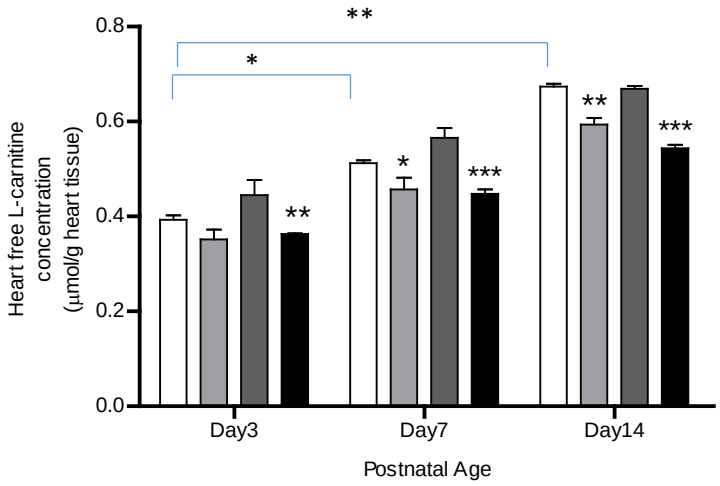


Figure 3.2 Mean ± SEM free L-carnitine concentration in rat heart samples (n = 4) at postnatal day 3, 7 and 14 (3 hour control – white bar; 3 hour treatment – light grey bar; 16 hour control – dark grey bar; 16 hour treatment – black bar). L-Carnitine concentration was determined using HPLC-UV as described in the text. Two-way ANOVA with Bonferroni post hoc test was used for the comparisons between ages and treatment and bars with astericks indicate significant difference relative to controls, $P<0.05$.

Heart and kidney Octn2 expression was increased with increasing postnatal age. Kidney and heart Octn2 mRNA expression were increased 8- and 13-fold between PD 3 and PD 14, respectively, although increases in expression in heart between PD 3 and PD 7 were not statistically significant (Figure 3.3). In the intestine, Octn2 expression was low at PD 3 and expression decreased insignificantly at PD 7 (Figure 3.3). Kidney and liver Tmlh mRNA expression increased between PD 3 and PD 14 (Figure 3.4). Kidney Tmlh mRNA expression significantly increased 24-fold between PD 3 and PD 7, whereas liver Tmlh mRNA expression increased 18-fold between PD 7 and PD 14. Liver Bbh mRNA expression and activity

significantly increased postnatally (Figure 3.5). Bbh mRNA expression levels were increased insignificantly between PD 3 ad PD 7; however, significant increase (35-fold), in Bbh mRNA expression was found at PD 14. Bbh enzyme activity showed 2.5 and 5 times increase at PD 7 and PD 14 relative to PD 3, respectively ($P<0.05$) (Figure 3.5 B).

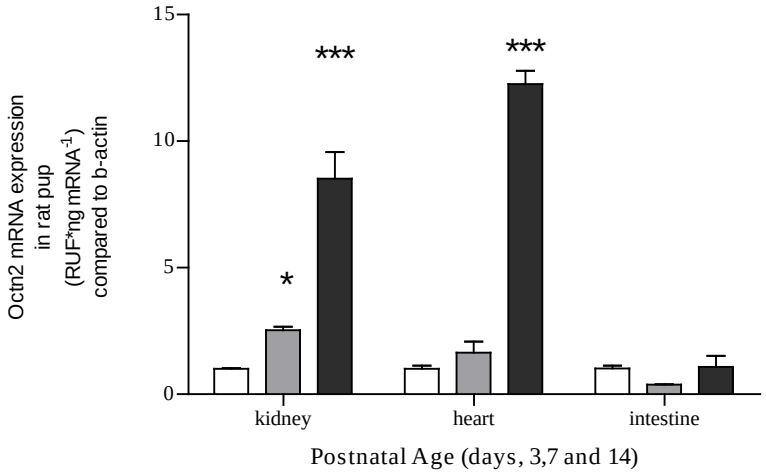


Figure 3.3 Mean ± SEM organic cation/carnitine transporter 2 (Octn2) (PD 3 – white bar; PD 7 light grey bar; PD 14 – black bar) mRNA expression levels in rat pup kidney, heart, and intestine at different postnatal ages (n=4). mRNA expression was normalized to ß-actin. Bars with no star indicate no significant difference relative to PD 3, $P<0.05$.

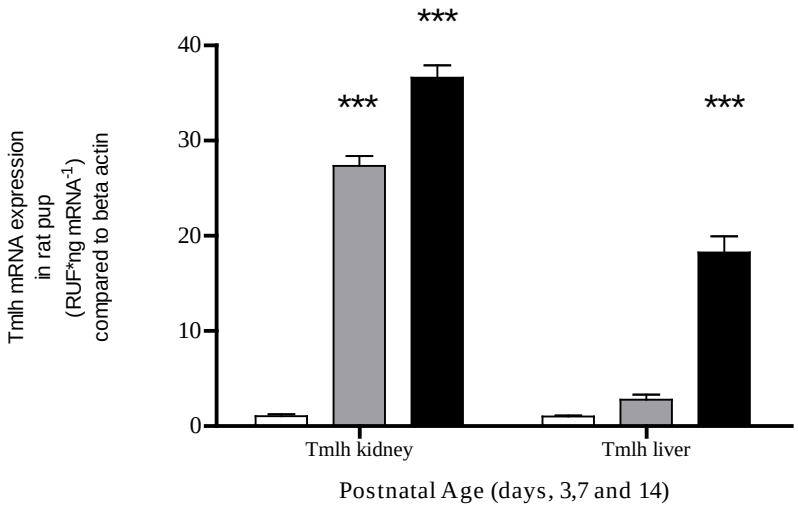


Figure 3.4 Mean ± SEM Tmlh (PD 3 – white bar; PD 7 light grey bar; PD 14 – black bar) mRNA expression levels in rat pup kidney and liver at different postnatal ages (n=4). mRNA expression was normalized to ß-actin. Bars with no star indicate no significant difference relative to PD 3, $P<0.05$.

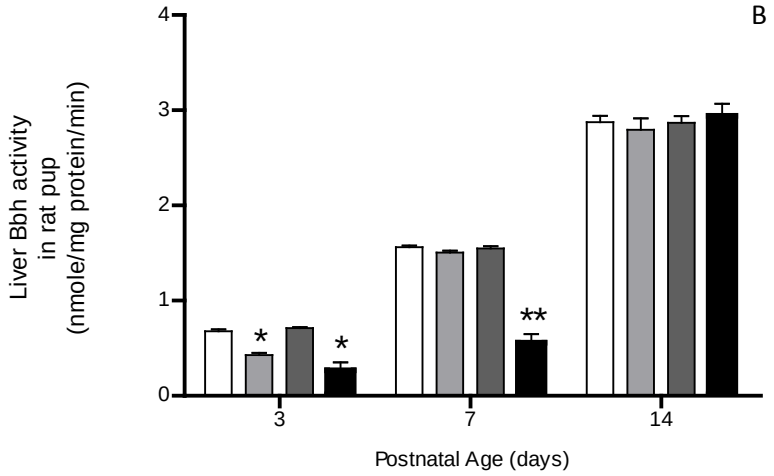


Figure 3.5 Mean ± SEM Bbh (PD 3 – white bar; PD 7 light grey bar; PD 14 – black bar) mRNA expression levels in rat pup liver (A) and liver Bbh activity (B) at different postnatal ages (n=4). mRNA expression was normalized to ß-actin. Bars with no star indicate no significant difference relative to PD 3, $P<0.05$.

3.2 TNF-α Levels:

To confirm the induction of inflammation, serum samples obtained from LPS and saline treated rat were evaluated for TNF-α activity. Serum TNF-α at 3 h was tested in treatment and control animals (Figure 3.6). Significant increases in serum TNF-α concentration were observed at 3 hours post LPS administered treatment groups at all age groups of rats. The TNF-α concentration was increased approximately 25-, 22-, and 42-times in 3 hours post LPS administered in PD 3, PD 7, and PD 14 rat pups, respectively.

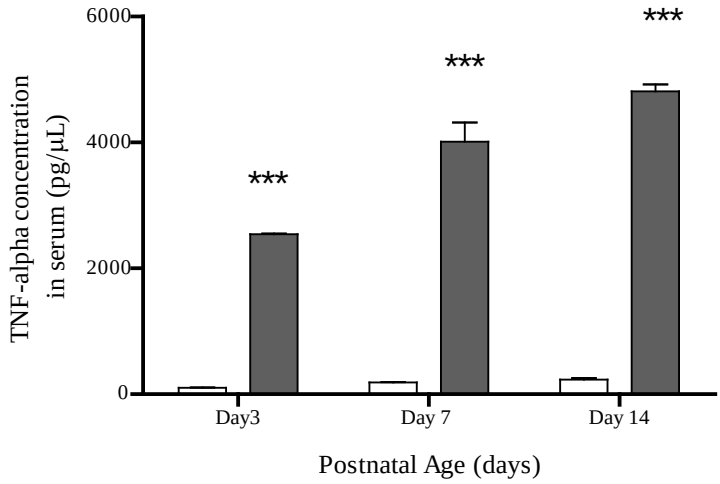


Figure 3.6 Mean ± SEM TNF-α concentrations in rat serum (Control Group – white bar; Treatment Group – grey bar). Student T-test was used for the comparisons between treatment groups and bars with three astericks indicate significant difference, $P<0.05$.

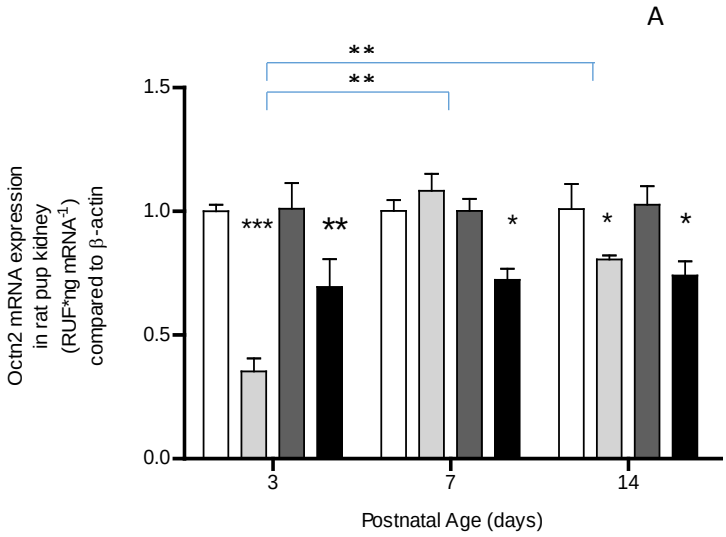
3.3 Effect of acute inflammation on ontogeny of L-carnitine homeostasis mechanism

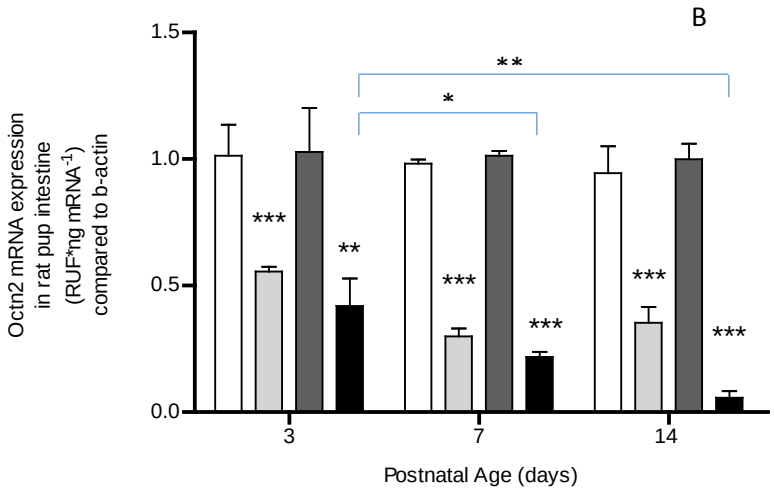
LPS administration caused changes in free L-carnitine levels in serum and heart (Figure 3.1). Serum free L-carnitine levels were significantly decreased in all LPS treated groups of all postnatal age rat pups. LPS caused no change in free L-carnitine levels in 3 h post LPS administration treatment group of PD 14 rat pups. Free L-carnitine levels in heart were significantly decreased in all LPS treated groups of all postnatal age rat pups. LPS caused no change in heart free L-Carnitine levels in 3 h post LPS administration treatment group of PD 3 rat pups (Figure 3.2).

3.4 Effect of LPS on mRNA expression of Octn2 transporter in kidney, liver, heart and intestine

To examine the ontogenesis of critical components of the L-carnitine homeostasis pathway we conducted an mRNA expression of one of the main components of the homeostasis pathway, Octn2, in different tissues of rat pups at 3 h and 16 h post LPS administration at PD 3, PD 7 and PD 14. LPS treatment caused a significant decrease in kidney, heart and intestinal Ocnt2 expression ($p<0.05$) (Figure 3.7). Kidney Octn2 expression was decreased by 26, 25 and 24 percent relative to control in 16 h post LPS administration in rats at PD 3, PD 7, and PD 14, respectively (Figure 3.7 A). Except in the 3 h post LPS administration treatment group at PD14 rat pups, mRNA levels decreased by 20 percent, Octn2 expression levels in kidney did not show statistically significant changes in any remaining age groups (Figure 3.7 A). Intestinal Octn2 expression was decreased by 45, 75 and 60 percent at 3 h post LPS administration in PD 3, PD 7 and PD 14 rat pups, respectively (Figure 3.7 B). Intestinal Octn2 expression is decreased by 60, 75 and 90 percent in 16 h post LPS administration in rat pups at PD 3, PD 7 and PD 14, respectively. Octn2 expression levels in heart and intestine were decreased significantly in 3 h post LPS administration and 16 h post LPS administration treatment groups of all postnatal age groups (figure 7.3 C). Heart Octn2 expression was decreased by 25, 50 and 27 percent at 3 h post LPS administration in rat pups at PD 3, PD 7 and PD 14, respectively (Figure 7.3 C). Heart Octn2 expression was decreased by 40, 80 and 70 percent at 16 h post LPS administration in rat pups at PD 3, PD 7 and PD 14, respectively (Figure 3.7 C). Except in the 16 h post LPS administration treatment group for PD 7 and PD 14, where a 64 percent decrease in Octn2 mRNA expression levels was observed in liver, expression changes were not statistically

significant in both treatment groups (3 h post LPS administration and 16 h post LPS administration) at all postnatal ages (Figure 3.7 D).





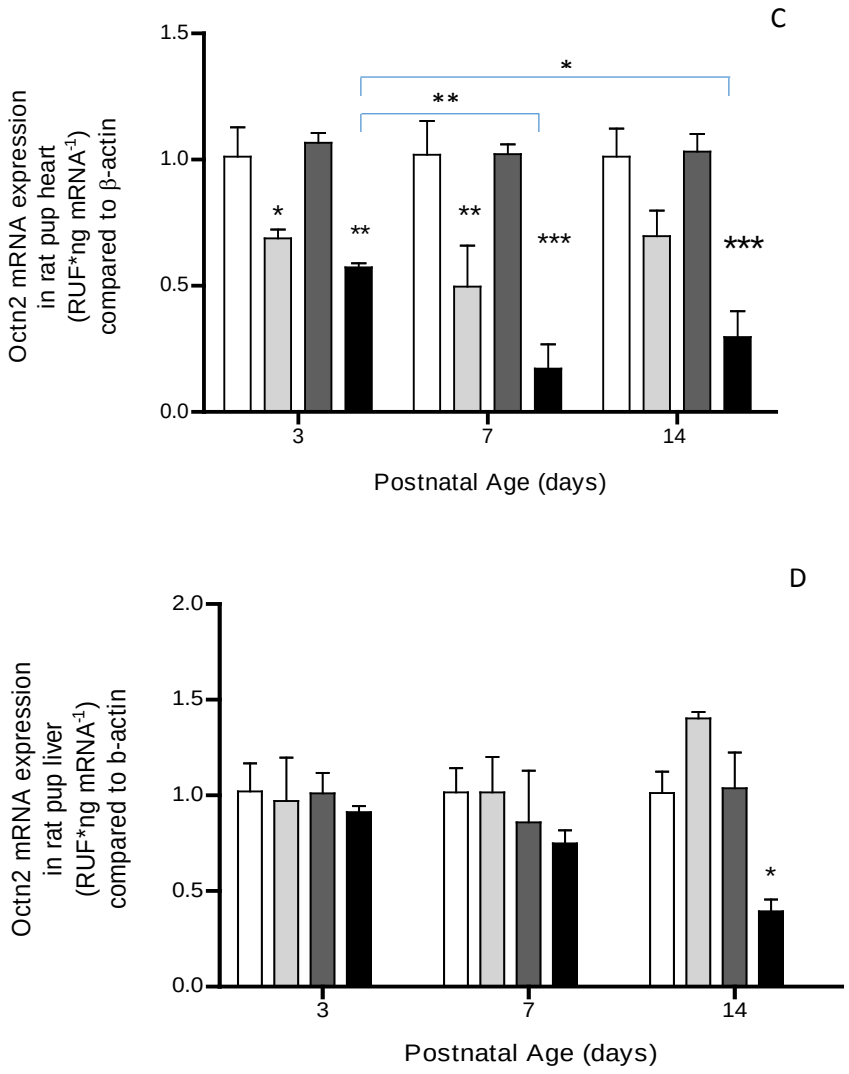
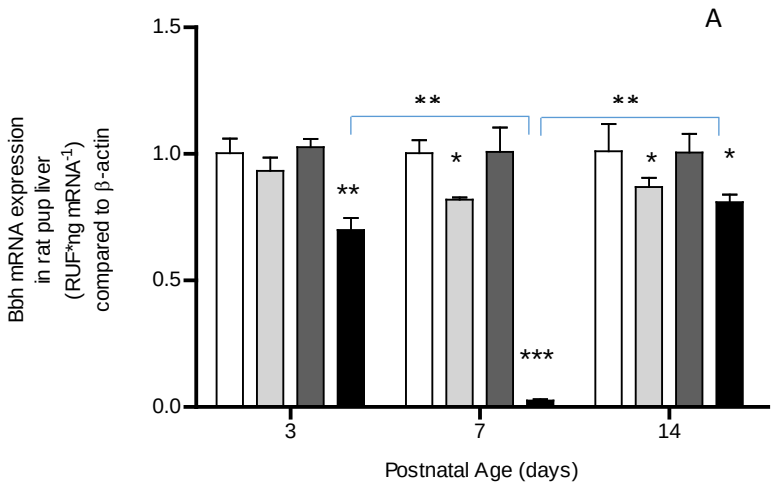


Figure 3.7 Mean ± SEM organic cation/carnitine transporter (Octn2) mRNA expression (white bar – control; light grey bar – 3 h post LPS administration; dark grey bar – 16 h control; black bar – 16 h post LPS administration) in rat kidney (panel A), intestine (panel B), heart (panel C), and liver (panel D) at postnatal day 3, 7 and 14 (n = 4). mRNA expression was normalized to β-actin and fold difference (FD) was determined by using $^{2\text{-}\Delta\Delta}$CT method. Two-way ANOVA with Bonferroni post hoc test was used for the comparisons between ages and treatments and bars with the same symbols indicate no significant difference, $P<0.05$.

3.5 Effect of LPS on mRNA expression and activity of Bbh in liver

LPS caused changes in Bbh mRNA expression levels and Bbh enzyme activity in all treatment groups ($P<0.05$) (Figure 3.8). Liver Bbh mRNA expression was decreased significantly ($p<0.05$) in all LPS treatment groups of all age groups, except in the 3 h post LPS treatment group at PD 3 and PD 14 ($p>0.05$) (Figure 3.8 A). Liver Bbh mRNA expression was significantly decreased by 32 and 25 percent in the 16 h post LPS treatment groups at PD 3 and PD 14, respectively, while it was significantly decreased 26 and 95 percent in both 3 h and 16 h post LPS treatment groups of PD 7, respectively. LPS caused no changes in liver Bbh activity in all treatment groups at PD 14 (Figure 3.8 B). At PD 3, LPS caused a significant decrease in Bbh activity in 3 h post LPS administration group (57 %) and in the 16 h post LPS administration treatment group (73 %). At PD 7, LPS only significantly decreased the 16 h post LPS administration treatment group (44 %) (Figure 3.8 B).



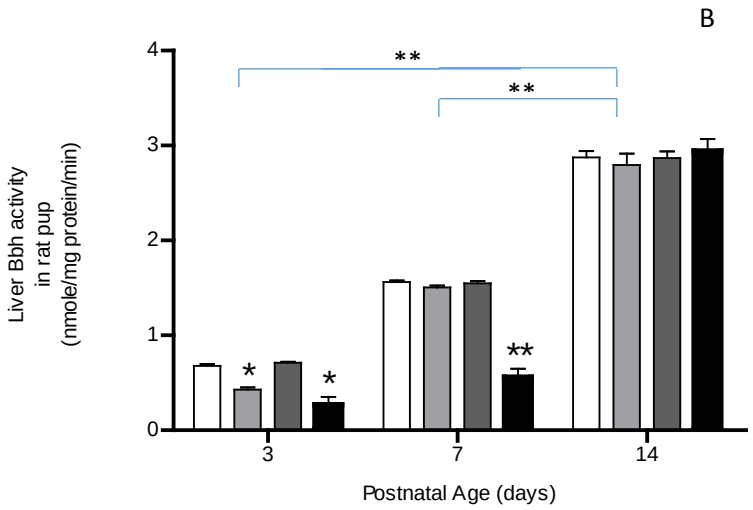


Figure 3.8 Mean ± SEM rat liver gamma-butyrobetaine hydroxylase (Bbh) (A) mRNA expression and (B) activity at postnatal day 3, 7 and 14 (n = 4) (white bar – control; light grey bar – l3 h post LPS administration; dark gray bar – 16 h control; black bar – 16 h post LPS administration). mRNA expression was normalized to β-actin and fold differences (FD) were determined by using $^{2-\Delta\Delta}$CT method. Bbh activity was measured by quantifying the conversion of γ-butyrobetaine to L-carnitine by HPLC-UV. Two-way ANOVA with Bonferroni post hoc test was used for the comparisons between ages and treatments and bars with the same letters indicate no significant difference, $P<0.05$.

3.6 Effect of LPS on mRNA expression and activity of Tmlh in liver and kidney

LPS administration significantly decreased liver Tmlh mRNA expression levels in all age groups ($P<0.05$) (Figure 3.9 A). Liver Tmlh mRNA expression was decreased by 40 and 85 percent at 3 h and 16 h post LPS administration at PD 3, respectively. Liver Tmlh mRNA

expression was decreased by 30 and 90 percent at 3 h and 16 h post LPS administration at PD 7, respectively. Liver Tmlh mRNA expression was decreased by 60% at 3 h post LPS administration at PD 14. LPS caused no changes in kidney Tmlh mRNA expression in all treatment groups at PD 3 and at 3 h post LPS treatment group at PD 7 (Figure 3.9 B). Tmlh mRNA expression in kidney was decreased by 45% at 3 h post LPS administration in in 16 h post LPS administration treatment groups at PD 3 by 35% in 16 h post LPS administration treatment groups at PD 14, and by 90% at 16 h post LPS administration treatment group at PD 7 (Figure 3.9 B).

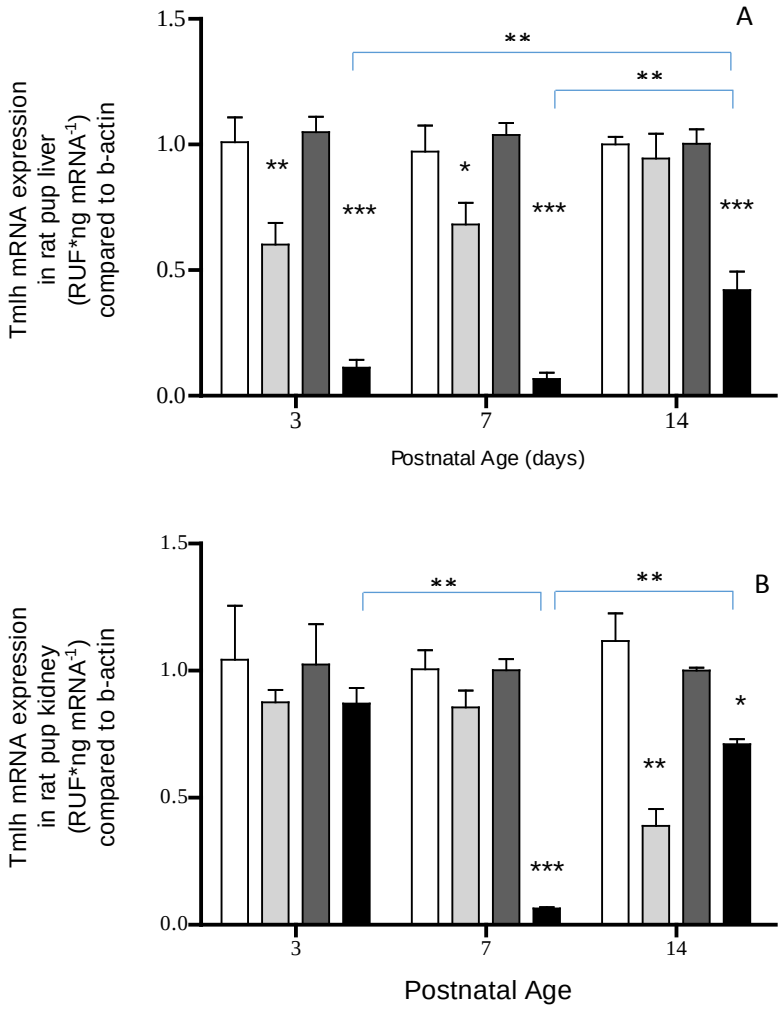
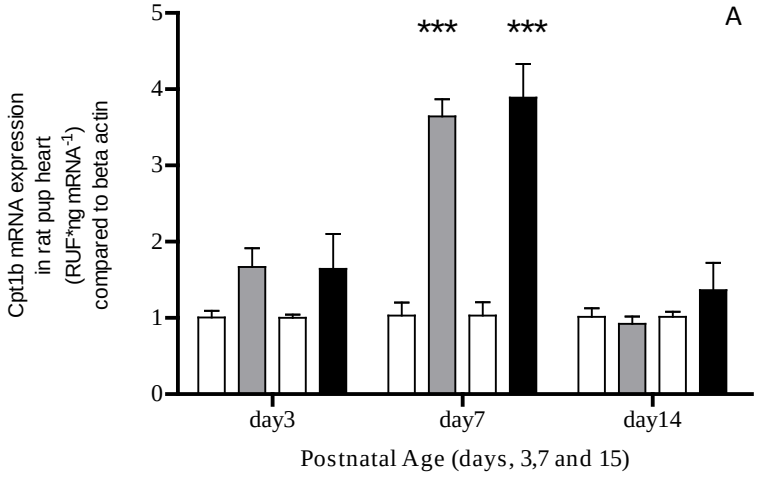


Figure 3.9 Mean ± SEM mRNA expression of Tmlh in rat pups (in liver (A) and kidney (B)) at postnatal day 3, 7, and 14 (n = 4) (white bar – control; light grey bar – 3 h post LPS administration; dark gray bar – 16 h control; black bar – 16 h post LPS administration treatment). mRNA expression was normalized to β-actin and fold difference (FD) was determined by using 2-ΔΔCT method. Two-way ANOVA with Bonferroni post hoc test was used for the comparisons between ages and treatment bars with astericks indicate significant difference relative to control, $P<0.05$.

3.7 Effect of LPS on mRNA expression and activity of Ct1b and Cpt2 in heart

LPS caused a significant increase (3.6 fold in 3 h post LPS administered group and 3.8 fold in 16 h post LPS administered group) in heart Cpt1b mRNA expression levels at PD 7 (Figure 3.10 A). Except in PD 7 treatment groups, heart Cpt1b mRNA expression levels did not show significant changes at all postnatal ages (figure 3.10 A). LPS caused no significant changes in heart Cpt2 mRNA expression in any of the treatment groups (figure 3.10 B)).



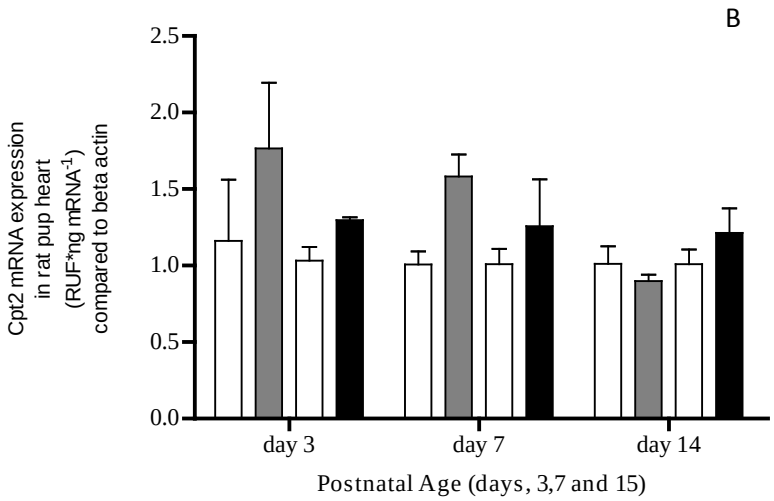


Figure 3.10 Mean ± SEM mRNA expression of heart carnitine palmitoyltransferase 1b (Cpt1b) (A) and carnitine palmitoyltransferase 2 (B) in rat pups at postnatal day 3, 7, and 14 (n = 4) (white bar – control; light grey bar – 3 h post LPS administration; dark grey bar – 16 h control; black bar – 16 h post LPS administration). mRNA expression was normalized to β-actin and fold difference (FD) was determined by using $^{2\text{-}\Delta\Delta}$CT method. Two-way ANOVA with Bonferroni post hoc test was used for the comparisons between ages and bars with astericks indicate significant difference relative to control, $P<0.05$.

LPS caused no significant changes in heart Cpt1b and Cpt2 enzyme activity levels at PD 3, PD 7 and PD 14. (Figure 3.11).

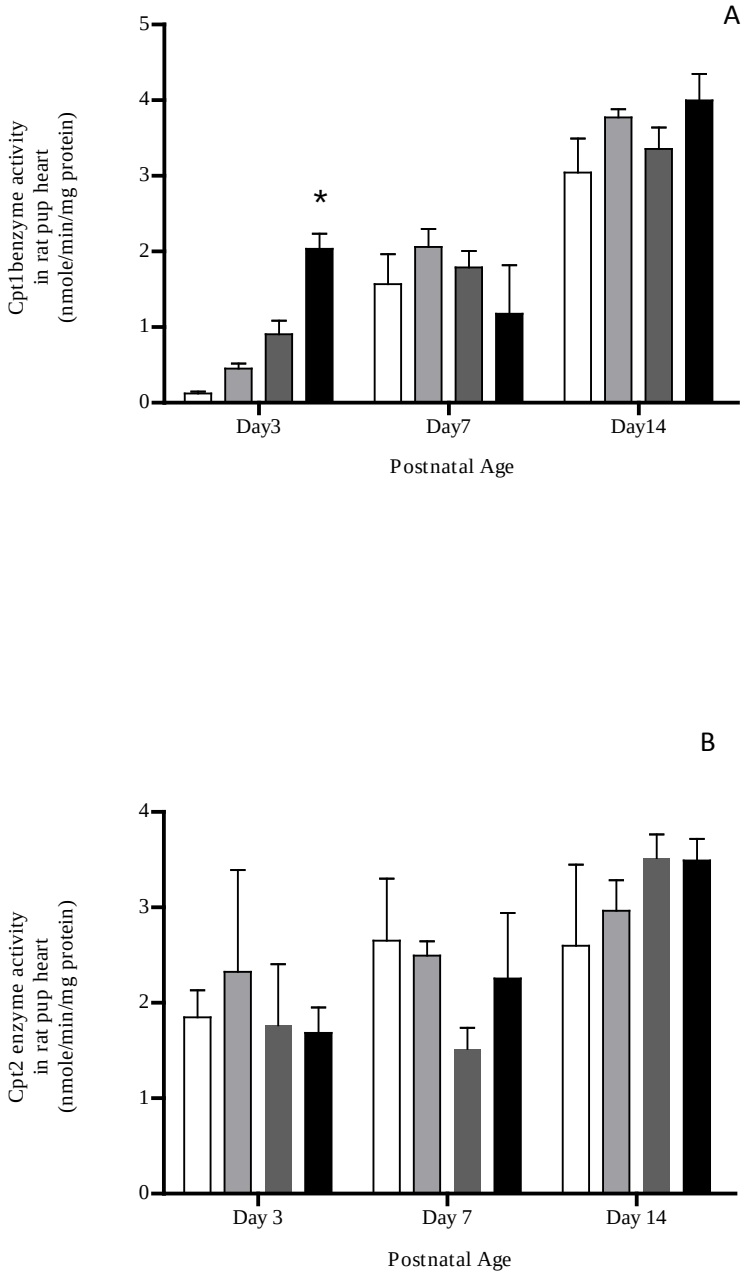
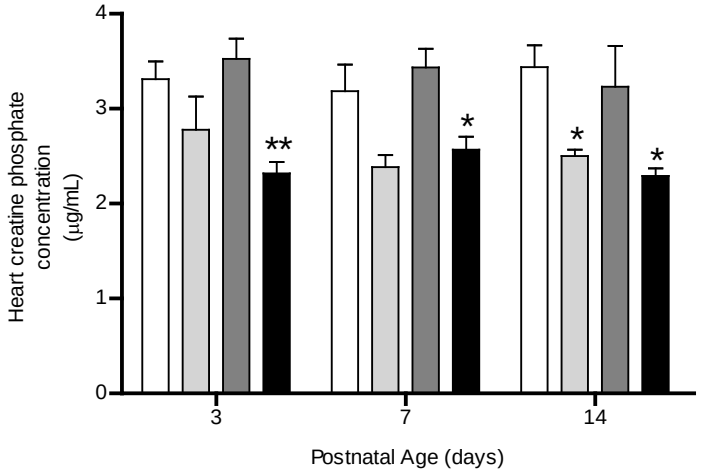


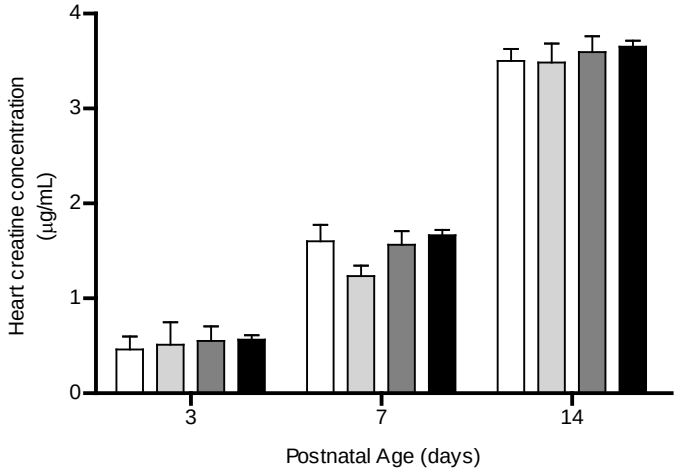
Figure 3.11 Mean ± SEM enzyme activity of heart carnitine palmitoyltransferase 1b (Cpt1b) (A) and carnitine palmitoyltransferase 2 (Cpt 2) (B) in rat pups at postnatal day 3, 7, and 14 (n = 4) (white bar – control; light grey bar – 3 h post LPS administration; dark gray – 16 h control; black bar – 16 h post LPS administration treatment). Two-way ANOVA with Bonferroni post

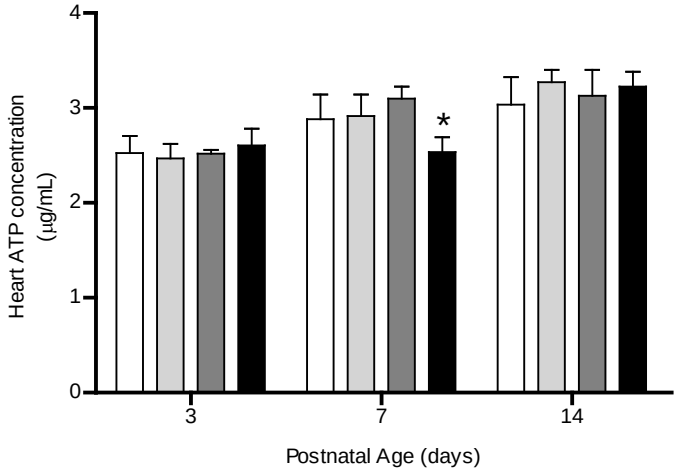
hoc test was used for the comparisons between ages and treatment and bars with asterisks indicate significant difference, $P<0.05$.

3.8 Effect of LPS on high energy phosphate substrate concentration in heart

Significant changes in heart creatine phosphate was observed in 16 h post LPS administered treatment groups in all age groups of rats. However, significant changes in heart creatine, ATP, and ADP levels were not observed in LPS treated rat pups. At 16 h post LPS administration, creatine phosphate was decreased by 34% in PD 3 rat pups, 25% in PD 7 rat pups, and 30% in PD 14 rat pups (Figure 3.12).




Heart creatine concentration (µg/mL)
4
3
2
1
0
3
7
14
Postnatal Age (days)


Heart ATP concentration (µg/mL)
4
3
2
1
0
*
3
7
14
Postnatal Age (days)

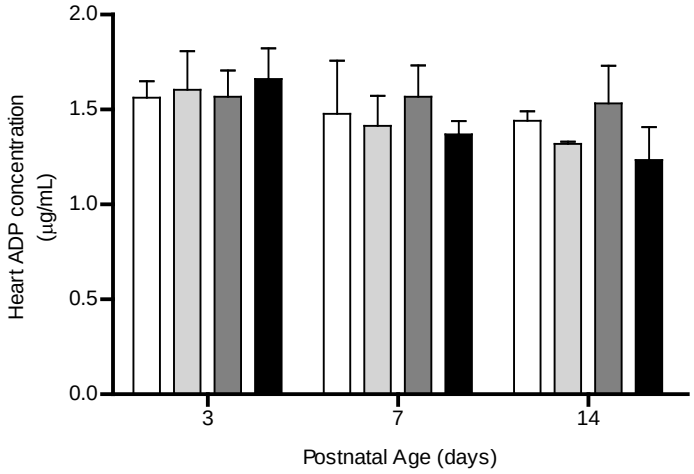


Figure 3.12 Mean ± SEM heart high-energy phosphate substrate concentration in rat pups at postnatal day 3, 7 and 14 (n = 4) (white bar – control; light grey bar – 3 h post LPS administration; dark gray bar – 16 h control; black bar – 16 h post LPS administration treatment). The heart high energy phosphate substrate levels including creatine (Cr), creatine phosphate (CrP), ATP, and ADP were measured with HPLC-UV method as described by Olkowski et al. [24]. Two-way ANOVA with Bonferroni post hoc test was used for the comparisons between ages and treatment and bars with an asterisk indicate significant difference, $P<0.05$. (Cr – creatine; CrP -creatine phosphate).

Chapter 4

4. Discussion

The nutritional requirements for normal growth and development are often affected by ontogenic processes of several nutritional pathways during postnatal development. The developmental changes in these nutrient homeostasis pathways are usually genetically programmed (181). However, these processes can be affected by exogenous factors such as dietary components, environmental factors including chemicals, pathological conditions, and therapeutic drugs. Since my laboratory is interested in understanding how external factors influence the ontogeny of important nutrient homeostatic processes, my thesis work involved the evaluation of the effect of acute inflammation on the developmental maturation of L-carnitine homeostasis pathways.

I used the rat in my work as a previous study in the lab provided the ground work for the ontogeny of a number of L-carnitine homeostasis mechanisms in the rat (136). Previous studies on evaluation of key pathways in the L-carnitine homeostasis pathway provided the basis from which I conducted further evaluations regarding the effects of pathological factors on L-carnitine status during postnatal development and possible long-term consequences of any disturbance in the normal ontogeny of these pathways (182). My laboratory's previous study did not assess potential gender effects on the ontogeny of and effects of a drug on L-carnitine homeostasis pathway development. Ethical considerations precluded an evaluation of gender effects in the current study as more that twice as many animals would be necessary in the study design. This may represent an important limitation of study design that we acknowledged at the start of the study. The information gleaned from my thesis work will begin the journey in understanding

how exogenous factors may alter maturation and the possible long-term consequences of such alterations.

As mammalian foetuses transition to extrauterine life, the umbilical supply of essential nutrients is cut off, including glucose which serves as the major energy fuel of the fetus. During the neonatal period, fatty acid oxidation becomes the main source of energy for a number of tissues (183). A sufficient supply of L-carnitine is essential for efficient mitochondrial fatty acid utilization, as this nutrient facilitates the movement of long-chain fatty acids across the mitochondrial membrane making them available for β-oxidation. The foetus liver stores significant levels of L-carnitine during late gestation, and such stores assure adequate levels in the immediate postpartum period (136). However, these tissue stores quickly deplete, and the L-carnitine homeostasis mechanisms begin to undergo maturational changes to ensure the physiological system has adequate L-carnitine to meet the energy needs of various tissues.

Postnatal increases in serum free L-carnitine is reported in the literature and these increases correlate with maturation of a number of enzymes and transporter systems that critically determine L-carnitine levels in the body (136). Previous studies on evaluation of key pathways in the L-carnitine homeostasis pathway (136) provided a basis from which we conducted further evaluations regarding the effects of inflammation on L-carnitine status during postnatal development and this concept may be extended to evaluate possible long-term consequences of any disturbance in the normal ontogeny of these pathways. This corroboration was essential as it shows ability to reproduce previous work in the laboratory and suggests that outcomes of my evaluations regarding LPS administration have important value. Since very little work has been published in the literature regarding the effects of inflammation on the transporters and ontogeny of L-carnitine homeostasis, I have no basis for comparison. One study conducted in my

laboratory evaluated the effect of inflammation on activity of nutrient transporter (manuscript submitted), but my thesis work represents novel information. I was not able to repeat the evaluations to ensure reproducibility of experimental outcomes during my graduate program.

Lower serum free L-carnitine levels in the early postnatal period is, in part, due to the limited capacity for endogenous biosynthesis by the young neonate (135). As noted in other studies (90), my study showed that hepatic γ-Bbh and Tmlh mRNA expression and γ-Bbh activity was significantly lower at early postnatal development in rat pups, consistent with the lower serum free L-carnitine concentrations observed at younger neonatal ages. The increase in serum free L-carnitine levels in rat pups with advancing age was matched with the maturation of hepatic γ-Bbh and Tmlh, suggesting that enhancements in endogenous L-carnitine biosynthesis contributes, in part, to the postnatal increase in L-carnitine levels in the body. However, evaluation of other critical mechanisms responsible for L-carnitine homeostasis suggests that maturation of other processes, namely renal reabsorption of L-carnitine, additionally contributes to the postnatal rise in serum L-carnitine.

Absorption of dietary sources of L-carnitine requires the function of several transporter systems expressed at the gastrointestinal epithelial barrier. Expression of duodenal Octn2 did not change with postnatal development. This was not consistent with increases in Octn2 expression noted in the literature, but could be the result of different rat strains (Sprague Dawley vs. Wistar) or different portion of the small intestine (jejunum or ileum vs. duodenum) (143). I did not evaluate protein expression levels nor measure transporter activity in the intestine; hence, I cannot conclude with certainty whether the lack of changes in mRNA expression with development may suggest that the absorptive capacity of L-carnitine by these transporter systems remains unchanged.

In my study renal Octn2 expression was increased during postnatal development in the rat, which was consistent with my laboratory's previous study and the literature (184). The increase in renal Octn2 expression was matched with the postnatal increase in serum L-carnitine levels. Hence, in addition to maturation of endogenous biosynthesis, maturation of renal Octn2 also has a significant role in the postnatal pattern of serum L-carnitine development. Overall, our data suggests the developmental changes in hepatic γ-Bbh and Tmlh expression and renal Octn2 expression may contribute to the postnatal increase in serum L-carnitine levels.

The highest concentrations of L-carnitine are primarily found in serum (136). In my study, heart L-carnitine levels increased during postnatal development (Figure 3.2). Evaluation of the expression of Octn2 in pup heart tissue also suggested that this postnatal increase corresponds to enhanced heart Octn2 expression with advancing postnatal age. The neonatal and adult heart relies on fatty acids as a principal energy substrate (185). Hence, L-carnitine has a significant role in energy production in neonatal cardiac tissue. The dramatic increase in fatty acid oxidation rates in early heart development after birth has been attributed to an increase in L-carnitine levels. Although we observed a significant increase in L-carnitine levels in the heart with advancing age of the neonate, cardiac ATP levels remained constant through postnatal development. My studies showed a trend towards increasing ATP levels but this was not significant. Interestingly, I found that Cpt1b and Cpt2 enzyme levels as well as creatine phosphate and creatine levels increased with postnatal development, which was consistent with my laboratory's previous study (136). Unfortunately, I was unable to determine AMP levels with reliability due to HPLC assay interference as such information is important in understanding the energy charge of a tissue. These ontogenic changes suggest an increasing capacity of the heart to utilize fatty acids for energy production with advancing neonatal age. This maturation does

suggest that exogenous impacts (such as disease and drugs) that influence these systems, either through direct inhibition or altered genetic regulation, may have significant impacts on the ability of the heart to generate ATP through fatty acid oxidation. The overall significance of such developmental changes is not clear and requires investigation.

In addition to my evaluation of the ontogeny of L-carnitine homeostasis mechanisms, the main objective of the work carried out in my thesis was to evaluate the effect of inflammation, a significant clinical factor, on the ontogeny of L-carnitine homeostasis mechanisms in neonates. The pathogenesis of inflammation due to the administration of LPS may involve different inflammatory stimuli, which in turn may alter the natural ontogenic process of L-carnitine homeostasis. Specifically, inflammation-mediated alteration in the expression and function of Octn2, enzymes involved in endogenous biosynthesis, and the enzymes involved in mitochondrial shuttling of acylcarnitines may have significant consequences on serum and tissue levels of L-carnitine and on ability of organs to utilize fatty acids for ATP generation. In my study, I conducted a systematic comparison of the gene expression and activity, where possible, of Octn2, Bbh, Tmlh, Cpt 1b and Cpt 2 enzymes between control and LPS treated rat pups at three different postnatal ages. LPS is a commonly employed substance to elicit an inflammatory response (186), and I utilized a neonatal rat inflammatory model well established in the literature to meet this objective (173). We have used SD rats at postnatal day 3, 7 and 14, as these ages provide several advantages when monitoring the ontogenic changes of L-carnitine mechanisms. In the rat, endogenous biosynthesis matures at PD 8 (91, 187, 188). Hence, these age groups capture the ontogeny of the L-carnitine biosynthetic enzymes and other key L-carnitine homeostasis mechanisms prior to and following maturation of endogenous L-carnitine biosynthesis. My findings can be used to postulate and predict the potential outcomes of the

effect of inflammation on ontogenic mechanisms. However, my work cannot elucidate the exact impact of the changes caused by an inflammatory stimulus and their overall contribution to L-carnitine homeostasis during development and further studies will be required to clarify contributions.

Several findings were generated in my work, which are summarized in the following discussion. My thesis work suggests that development of inflammation in neonates can alter the normal ontogeny of L-carnitine homeostasis. My findings suggest that inflammation has statistically significant effects on the main elements of L-carnitine homeostasis and that inflammation is likely to have significant clinical relevance and relevance to the overall development of the neonate as it proceeds towards adult life stages.

Acute inflammation caused a significant decrease in free L-carnitine levels in serum and heart tissue. Based on the results obtained from Octn2 gene expression experiments, the decrease in mRNA expression levels of renal and intestinal Octn2 might be the principle cause of depletion of serum and heart L-carnitine levels. Renal reabsorption of L-carnitine from the urinary filtrate plays a significant role in maintenance of L-carnitine levels in the body. Almost 95% of the excreted L-carnitine is reabsorbed by transporters expressed in the proximal tubules of the kidney with Octn2 as the principal transporter involved in this process (189, 190). In my study renal Octn2 expression decreased following LPS administration in the rat pup. The decrease in renal Octn2 expression corresponded strongly with decrease in serum L-carnitine levels suggesting that renal Octn2 plays a significant role in the postnatal pattern of serum L-carnitine development. In addition to kidney Ocnt2 levels, gene expression levels of intestinal Octn 2 might be an additional cause of the reduction in serum L-carnitine concentration. Absorption of dietary L-carnitine requires the function of several transporter systems expressed

at the gastrointestinal epithelial barrier. Expression levels of duodenal Octn2 were decreased with LPS administration and this might result in the decrease in L-carnitine absorption from the milk following suckling. The decrease in heart Octn2 gene expression (in conjunction with lower serum L-carnitine levels) likely explains the reduction in heart L-carnitine levels with LPS administration, since Octn2 mediates the uptake of L-carnitine into heart tissue.

My study corroborated previous investigations in that hepatic γ-Bbh mRNA expression and activity levels were low in the first days of life and matured with postnatal age (187, 188). The maturation of hepatic γ-Bbh contributes to the postnatal increase in L-carnitine levels in the body. However, my study on the effect of acute inflammation suggests that maturation of hepatic γ-Bbh was disturbed and this might play a significant role in the reduction of serum L-carnitine levels. Although inflammation caused alterations in hepatic γ-Bbh mRNA levels at all three postnatal age groups, hepatic γ-Bbh enzyme activities were not affected at PD 14. These results correlate with the serum L-carnitine levels at PD 14 post LPS administration suggesting that the γ-Bbh enzyme had matured to maximum levels prior to PD 14. Hepatic and renal Tmlh mRNA expression levels were significantly lower at early postnatal development in rat pups and matured with postnatal age. My study on the effect of acute inflammation suggests that maturation of hepatic and renal Tmlh mRNA expression changed with an inflammatory stimulus at all postnatal age groups. Without evaluation of Tmlh activity, it is difficult to speculate whether reduction in Tmlh mRNA expression translates into reductions in Tmlh activity. The literature suggests Tmlh is a rate-determining enzyme in the biosynthesis of L-carnitine (191). Consequently, any reduction in Tmlh activity following inflammation may result in a lowered capacity to synthesize L-carnitine *de novo*. This could contribute to inflammation mediated reductions in serum L-carnitine levels noted in my study.

Since LPS causes differential changes in transporters that mediate the transport of critical substrates used in the generation of cellular energy, I next evaluated whether such changes could result in differences in mitochondrial ATP production. LPS significantly decreased CrP and caused a non-significant decrease in ADP levels. Reductions in CrP is generally considered a pathological indicator. Interestingly, in a previous study in the lab, chronic cefepime administration (cefepime is a competitive inhibitor of Octn2) caused histopathological changes in the heart at an early postnatal age, but not at older postnatal ages (136). This study also indicated reductions in CrP with chronic cefepime administration. Consequently, reductions in heart CrP may suggest early pathological changes with LPS administration. Furthermore, the decreased expression of Octn2 in LPS treated rats may suggest a reduction in the intracellular supply of L-carnitine and reduction in fatty acid oxidation. To maintain cellular ATP levels, heart tissue may have to increase glycolysis (and thus oxidative phosphorylation) to compensate for the lowered energy production by fatty acid oxidation. This may explain the minimum changes in ATP levels in LPS challenged rats.

Based on the results obtained from the present study, inflammation shows time dependent effect on the ontogeny of L-carnitine homeostasis in early neonatal stages. The overall results from the current study suggests that LPS induced inflammation has profound effect on ontogeny of L-carnitine homeostasis in early postnatal stages (PD 3 and PD 7). Since most of the elements in L-carnitine homeostasis are undergoing ontogeny before PD 14, inflammation caused less effects on PD 14 rats when compared to earlier postnatal age groups.

L-carnitine homeostasis pathways underwent significant ontogenesis during postnatal development in the rat. However, the exact relationship between these pathways and their contribution to L-carnitine homeostasis during development is not completely known and further

studies are required to clarify their contributions. Such a clarification is necessary to understand the impact of exogenous and endogenous factors on L-carnitine status during development.

4.1 Future Directions

- The individual role of pro-inflammatory cytokines in LPS induced inflammation and its role in modulating the transporter expression at different stages of L-carnitine ontogenesis will be known by performing *in vitro* studies with radiolabelled substrates.
- More experiments should be carried out to estimate the time required for translational changes in mRNA to provide a clearer picture on dosing and time elapse in similar experiments in future.
- Experiments to assess cardiac oxygen consumption and high energy substrate levels should be carried for understand how inflammation modulates substrate energy levels and L-carnitine dependent energy producing pathways at the molecular level.
- Radiolabelled studies are required to assess the Octn2 transporter activity in heart and kidney to determine whether inflammation-mediated reduction in transporter mRNA expression correlates with reduction in transporter activity.
- In the neonate, my work suggested that inflammation resulted in changes in L-carnitine homeostasis pathways depending on timing and duration of inflammation to postnatal age. However, whether these changes are permanent or transient are unknown. Future studies are needed to investigate the potential for metabolic programming of L-carnitine and other nutrient homeostasis pathways following inflammation and the possible long-term consequences of such interactions on the risk for adult onset disease.

- Separate experiments of similar kind should be conducted on male and female animals to classify unknown functional differences between the two genders.

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