AMINO ACID METABOLISM IN THE LIVER AND PANCREAS

ROBIN DA SILVA


CENTRE FOR NFLD. STUDIES
MEMORIAL UNIVERSITY
OF NEWFOUNDLAND

Amino Acid Metabolism in the Liver and Pancreas

by

© Robin da Silva, B.Sc. (with Honours)

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Abstract

This thesis presents four research papers dealing with the metabolism of creatine and amino acids in the liver and pancreas. Creatine is synthesized in two enzymatic reactions that utilize the amino acids arginine, glycine and methionine. The first step in creatine synthesis is the transfer of the amidino group of arginine onto the amine of glycine resulting in ornithine and guanidinoacetate, catalyzed by the enzyme arginine:glycine amidinotransferase. The guanidinoacetate can then be methylated by the universal methyl donor S-adenosylmethionine to form creatine and S-adenosylhomocysteine. The first two papers investigate the role of the rat liver and pancreas in creatine biosynthesis respectively, using both *in vivo* and *in vitro* techniques. I provide evidence that the liver does not perform the first step of creatine synthesis but does perform the second step and has the capacity to methylate all of the guanidinoacetate that is synthesized in the kidney. The third paper examines the effects of creatine-supplementation in high-fat diet fed rat model of non-alcoholic fatty liver disease. This paper shows that dietary creatine-supplementation prevents the accumulation of lipids in the livers of rats fed high-fat diets as well as a number of the negative observations associated with non-alcoholic fatty liver disease. The fourth paper deals with the fate of perivenous hepatocytes in rats that have undergone portacaval shunts. It is known that the expression of hepatic glutamine synthetase is markedly down-regulated in portacaval anastomosis and it has been suggested that there is a loss of the perivenous hepatocytes which contain this enzyme activity. I provide evidence that

ornithine aminotransferase activity and the expression of an ammonium transporter RhBG, proteins known to be localized in the perivenous cell population, are increased and unchanged respectively in rats with portacaval anastomosis. The papers presented in this thesis a) show the major role of the liver in creatine synthesis, b) demonstrate a possible role for the pancreas in creatine synthesis, c) introduce creatine as a potential treatment for fatty liver disease, and d) demonstrate that perivenous hepatocytes are not lost during portacaval shunting. These constitute important contributions to the current body of literature relating to creatine metabolism and hepatic zonation.

Acknowledgements

First of all I would like to thank my parents for giving me the life that I have today. They bestowed upon me good values that many parents hand down but more than that they have instilled the importance of education and taught me to always ask questions. Without them I would not be.

I would like to thank my supervisors Dr. John T. Brosnan and Dr. Margaret E. Brosnan for sharing their knowledge and experience and for constantly supporting me over the years. I cannot hope to repay the kindness that they have shown. I only hope that one day I can do for others as they have done for me.

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I would also like to thank the CIHR and the School of Graduate Studies for funding and support for the studies that I have conducted.

Copyright Clearance

Figure 1.3 Diagram of proposed hepatic zonation of metabolism. Taken from: Hepatic glutamate metabolism: a tale of 2 hepatocytes, Am J Clin Nutr 90(3):857S-861S, 2009. Copyright License #: 2837160111865

Table 1-1 Clinical and biochemical characteristics of GAMT, AGAT, CRTR deficiency and diagnostic tests. Taken from: Clinical characteristics and diagnostic clues in inborn errors of creatine metabolism, J. Inherit. Metab.Dis. 26:299-308, 2003.
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Chapters 2: Creatine Synthesis: Hepatic Metabolism of Guanidinoacetate and Creatine in the Rat, *in vitro* and *in* vivo. Am. J. Physiol Endo. Metab. 296:E256-E261, 2009. Copyright permission is not required for use in a thesis or dissertation.

Chapter 4: Creatine supplementation prevents the accumulation of fat in the livers of rats fed a high-fat diet. J. Nutr. 141:1799-1804, 2011.
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Authors Contributions

The experiments in chapters 2, 3 and 5 were conducted primarily by me in the labs of Dr. John T. Brosnan and Dr. Margaret E. Brosnan at Memorial University of Newfoundland. Personal aknowledgments are given at the end of each manuscript where assistance was provided. Notably, the mass spectrometry analysis in chapter 2 was performed by Itzhak Nissim at the University of Pennsylvania School of Medicine. The experiments presented in chapter 4 were done in collaboration with Rafael Deminice in the labs of Dr. John T. Brosnan and Dr. Margaret E. Brosnan at Memorial University of Newfoundland. In addition, the experiments measuring phospholipids and mRNA expression in chapter 4 were done in collaboration with Karen Kelly and Dr. René Jacobs at the University of Alberta. Specific contributions of each author are given in the acknowledgments section of chapter 4.

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List of Abbreviations

ADP: adenosine diphosphate

AGAT: arginine:glycine amidinotransferase

AIN93-G: American Institute of Nutrition 93 diet for growth and lactation

AMP: adenosine monophosphate

ANOVA: analysis of variance

Art: arterial blood

ASH: alcoholic steatohepatitis

ATP: adenosine triphosphate

BHMT: betaine—homocysteine methyltransferase

CβS: Cystathionine β-synthase

CγL: Cystathionine γ-lyase

C: control

CD36: fatty acid transporter (cluster of differentiation 36)

CDP: cytidylyl diphosphate

bCK: brain type creatine kinase

mCK: muscle type creatine kinase

UmiCK: ubiquitous mitochondrial creatine kinase

CMP: cytidylyl monophosphate

CNS: central nervous system

CPT1a: carnitine palmitoyltransferase 1a

CRT1 or CRTR or SLC6A8: sodium-dependent creatine transporter 1

CSF: cerebrospinal fluid

CTP: cytidylyl triphosphate

cDNA: complementary deoxyribonucleic acid

DNA: deoxyribonucleic acid

DNMT: deoxyribonucleic acid N-methyltransferase

GAA: guanidinoacetic acid

GABA: γ-aminobutyric acid

GAMT: guanidinoacetate N-methyltransferase

GLT1: glutamate transporter 1

GNMT: glycine N-methyltransferase

GS: glutamine synthetase

HA: hepatic artery

Hcy: homocysteine

HF: high fat

HFC: high fat creatine

HPLC: high-performance liquid chromatography

HV: hepatic vein

Ka: association constant

Km: Michaelis constant

LCAD: long chain acyl-CoA dehydrogenase

MAT I/III: methionine adenosyltransferase

MGAT: monoacylglycerol acyltransferase

MTHFR: methylenetetrahydrofolate reductase

MRS: magnetic resonance spectroscopy

MS: methionine synthase

MTP: microsomal triglyceride transfer protein

NAD^+: nicotinamide adenine dinucleotide (oxidized)

NADH: nicotinamide adenine dinucleotide (reduced)

NAFLD: nonalcoholic fatty liver disease

NASH: nonalcoholic steatohepatitis

OAT: ornithine aminotransferase

OCT: optimal cutting temperature compound

PC: phosphatidylcholine

qPCR: quantitative polymerase chain reaction

PCR: polymerase chain reaction

PCr or CrP: creatine phosphate

PE: phosphatidylethanolamine

PEMT: phosphatidylethanolamine *N*-methyltransferase

PPARα: peroxisome proliferator-activated receptor alpha

PPARγ: peroxisome proliferator-activated receptor gamma

PPi: pyrophosphate

PV: hepatic portal vein

mRNA: messenger ribonucleic acid

RNA: ribonucleic acid

RhBG: Rhesus family B glycoprotein, ammonium transporter

SAH: S-adenosylhomocysteine

SAHH: S-adenosylhomocysteine hydrolase

SAM: S-adenosylmethionine

SBDF: ammonium 7-fluoro-2-oxa-1,3-diazole-4- sulphonate

SD: standard deviation

SDS-PAGE: sodium dodecylsulphate-polyacrylamide gel electrophoresis

SHMT: serine hydroxymethyltransferase

SLC1A2: solute carrier 1A2

SLC1A4: solute carrier 1A4

SLC6A8: creatine transporter (solute carrier 6A8)

SREBP-1c: sterol regulatory element binding protein 1c

TBARS: thiobarbituric acid reactive species

TG: triglyceride

UCP2: mitochondrial uncoupling protein

VLCAD: very long chain acyl-CoA dehydrogenase

VLDL: very low density lipoprotein

Chapter 1

General Introduction

1.1.1 A Brief History of Hepatology

The liver is one of the first and largest organs that can be seen upon opening the abdomen of a mammal and its distinct appearance can change dramatically as a result of alterations in physiology. It is likely that the physical attributes of the liver and those changes related to pathological situations led cultures throughout antiquity to use the liver as a tool for mystical and religious explanations of various phenomena. Ancient cultures such as the Babylonians, Assyrians and Etruscans believed that the future could be foretold by examining the livers of sacrificial animals (52). Many of these cultures consulted a mystic who used the excised liver of an animal (sometimes human) to foretell the outcomes of coming battles; history is littered with numerous depictions of this custom. There is also the famous Greek legend of the Titan, Prometheus, who stole fire from Zeus and gave it to humankind. As a punishment Zeus tied Prometheus to a rock where a great eagle came daily to feast on his liver only to have it grow back for the next day's feast. This story could very well have been crafted through some experience with liver regeneration, another striking attribute of the liver. And so the liver has been revered and celebrated by humankind throughout the ages. The basic, gross fundamental attributes of the liver have been identified and used as evidence to rationalize and explain historic events. At present we know many of the finer details related to the crude historic descriptions of the liver, but it is the prominence and early recognition of this organ that has driven philosophers and scientists to ask questions about how the liver works.

Perhaps the most notable early studies on animal physiology were carried out in Greece by Galen of Pergamum 129-199 AD. It was generally accepted in the time of Galen that the liver was responsible for turning digested food into blood, and that the

liver was the origin of all of the veins (125). While we now know this to be inaccurate it is not hard to understand the ideas of the time. The liver does contain a great deal of blood (to which it owes most of its red /brown color) and considering the visible vasculature and position (central and above all other organs in the peritoneal cavity) of the liver these conclusions are understandable. These ancient Greek ideas were held by medical practitioners for centuries and it was not until the 15th century when Paracelsus began to challenge old ideas and introduced the idea that the liver was separating food chemically and not "digesting" the food itself (125). Some time after Paracelsus the famous Italian physician Andreas Vesalius 1514-1564 AD published his work *De Humani Corporis Fabrica* (On the Material of the Human Body). Within this body of work there is an intricate diagram and detailed description of the anatomy of the digestive tract and greater insight into the function of the liver. Even though Vesalius maintained many of the older Galenic ideas about nutrition he was able to identify the vasculature of the liver and had the correct notion that nutrients travel from the intestines to the liver, as seen in the description he gives in his Epitome (167):

"The veins suck out from the intestines (especially the small ones) whatever is suitable for the making of blood, together with the aqueous and thin refuse of the stomach's concoction, and carry it to the workshop of the liver, where the blood is made…..It is the tinder of the natural or nutritive faculty or, as Plato said, of the part of the soul which desires the pleasures of love, food, and drink."

-Andreas Vesalius

Exactly how the machinery of the liver works would not be discovered until much later and it was not until the relatively recent discovery and characterization of enzymes

that the full role of the liver would be clear. The discovery and characterization of the urea cycle and its catalytic mechanism for the removal of nitrogen in the liver by Hans Krebs is profoundly important in our current understanding of the liver. We now know that the liver has a unique vasculature and is responsible for the metabolism of all the macronutrients and many of the micronutrients. It is with a similar fascination to that shared by people throughout the ages that I began, and indeed, continue my study of how the human body works. It is the goal of this thesis to describe some aspects of hepatic metabolism with a particular focus on the metabolism of creatine and the amino acids. I will introduce creatine metabolism and hepatic zonation and then present three research papers that address some questions that have arisen regarding liver creatine and amino acid metabolism.

1.1.2 Scope of Thesis

This thesis deals mainly with creatine metabolism in the liver and pancreas. In addition it addresses a question regarding hepatic zonation in the liver. The first paper examines the methylation of GAA in rat liver and the effect of methionine thereon. The possibility of de novo creatine synthesis from arginine derived from the urea cycle and recycling of the ornithine formed to arginine in the liver is also tested. The second paper deals with the role of the pancreas in total body creatine synthesis in the rat. The third paper examines the effects of dietary creatine supplementation in preventing non-alchoholic fatty liver disease (NAFLD).

Finally, the fourth paper deals with the fate of perivenous hepatocytes in rats that have undergone portacaval anastomosis as a model for the condition of liver cirrhosis. The author appreciated that the study conducted in the following chapter is not directly related

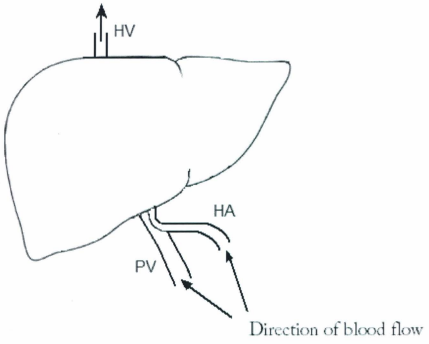
to the topics addressed in the three preceding chapters. Nevertheless, it is intimately related to the field of hepatic amino acid metabolism which encompasses the creatine studies. In fact chapter 5 represents the first study undertaken during my PhD research. However, the focus of the laboratory changed to creatine due to grant funding exigencies. For this reason the remaining studies dealt with creatine metabolism.

1.1.3 Overview of Liver Anatomy and Metabolism

In this section I will briefly address some of the metabolic functions of the liver and some anatomical features that are relevant to the work that I will present. The liver sits high within the peritoneal space in the abdomen, very close to the stomach, pancreas and intestines. It has an unusual vasculature being fed by both arterial and portal venous blood (Figure 1.1). The hepatic artery carries arterial blood; however this only accounts for approximately 25% of total hepatic blood flow (52). The remaining 75% of blood flow is supplied by the hepatic portal vein which carries venous blood from splanchnic organs including the pancreas, stomach, spleen and intestine. The portal blood is somewhat lower in oxygen content than is arterial blood but contains a much larger concentration of nutrients and hormones. In addition, the liver secretes bile which is stored in the gall bladder (except in some species, notably the rat where bile is secreted directly into the intestine) which in turn transfers the bile to the intestine providing bile acids, phospholipids and cholesterol that assist in the digestion of fat.

Figure 1.1

Hepatic blood flow. Direction of blood flow is denoted by arrows and symbols are defined as follows: Hepatic Artery (HA), Portal Vein (PV) and Hepatic Vein (HV). Original art.


HV
HA
PV
Direction of blood flow

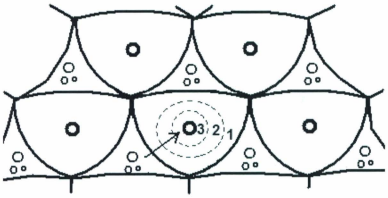
The liver performs numerous, varied and sometimes opposing metabolic processes. These are often facilitated by a unique micro-structural organization that adds a level of complexity to hepatic metabolism (132). Liver cells are grouped into small roughly hexagonal units called acini: coming from the Latin word 'acinus' meaning "berry' (Figure 1.2 and 1.3). The acinus represents the smallest functional unit of the liver above the level of the cell. Both the hepatic portal venous blood and arterial blood feed into the acinus and this blood collects in the central vein after it transits the acinus. As the blood flows along the acinus the contents of the blood change based on what molecules are being taken up and released by the cells (Figure 1.3). As a result the cells that occupy different positions in relation to the blood flow take on different roles and this phenomenon is called hepatic zonation. The separation of metabolic processes plays a crucial role in the regulation of metabolism.

1.1.4 Creatine Metabolism

Creatine is perhaps most widely known as a popular sports supplement taken by athletes and bodybuilders. The role that creatine plays in improving muscle performance is most significant for very short (seconds) bursts of power that consume a large amount of ATP such as resistance exercises (133, 169). In the presence of ATP, creatine can be converted to creatine phosphate through the action of the enzyme creatine kinase which catalyzes the reaction shown in equation 1 below.

Figure 1.2

Cross-section of liver *acini*: blood flows from the portal triad across the acinus and collects in the central vein. Arrow indicates direction of blood flow. Metabolic zones: zone 1 (periportal), zone 2 (intermediate), zone 3 (perivenous). Adapted from Kuntz; Hepatology 2008 (52).



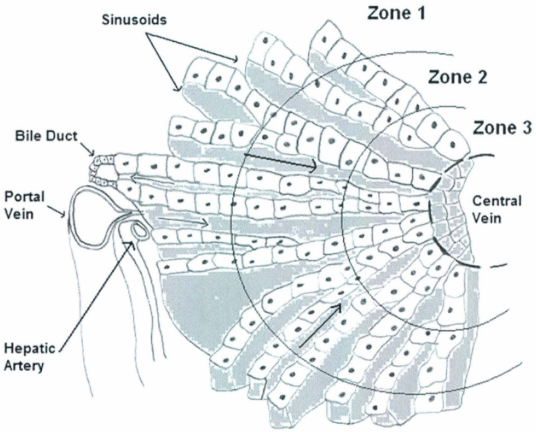
Central Vein

Portal Triad

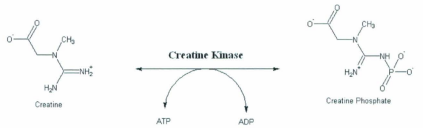
1 Periportal zone
2 Intermediate zone
3 Perivenous zone

Figure 1.3

Diagram of proposed hepatic zonation of metabolism. Taken from (27).


Sinusoids
Zone 1
Zone 2
Zone 3
Bile Duct
Portal Vein
Central Vein
Hepatic Artery

Equation 1:



Physiologically, this reaction is reversible and the substrates creatine phosphate, ATP, creatine and ADP are held in equilibrium. There are four known isoforms of creatine kinase: brain (bCK), muscle (mCK), ubiquitous mitochondrial (UmiCK) and sarcomeric mitochondrial creatine kinase (SmiCK) (176). At the cellular level, bCK and mCK are localized in the cytosol while miCK is found in the mitochondria of cells that have the full creatine kinase "high energy" phosphate shuttle. Mitochondrial creatine kinase normally exists as an octamer and is found in heart, brain, muscle, retina, and sperm; the sarcomeric enzyme is found mainly in the muscle (89). The brain and muscle isoforms of creatine kinase are expressed in their respective tissues where they form functional dimers. The heart is the notable exception which expresses both the brain and muscle isoforms, which form a mixed dimer of the enzyme. The creatine, creatine phosphate system is most active in fast-twitch muscle, heart and brain but is also active in the other tissues that express mitochondrial creatine kinase (186). The creatine, creatine phosphate system buffers ATP in a spatial as well as a temporal fashion. The concentration of creatine phosphate along with the presence of cytosolic creatine kinase enzyme near sites of ATP utilization allows for the quick regeneration of ATP at the expense of creatine phosphate while the localization of mitochondrial creatine kinase at the site of ATP generation allows for the synthesis of creatine phosphate and regeneration of ADP. Thus creatine acts as a carrier of "high-energy" phosphate groups between locations of

utilization and generation within cells buffering ATP in a spatial fashion. Some advantages of this phenomenon are that the diffusion rates of creatine phosphate and creatine are much higher than those of ATP and ADP (82, 191) making their transfer through aqueous cytosol faster. The higher concentrations of creatine and creatine phosphate increase the 'effective' ATP concentration without increasing the actual ATP concentration, the consequence of which would affect enzymes that use ATP as a substrate or allosteric regulator.

1.1.5 Creatine Biosynthesis

Creatine was first isolated in the 1830's by Michel-Eugene Chevreul from muscle extracts and named after the Greek word "Kreas" meaning 'flesh'. Creatine is a naturally occurring, endogenously synthesized metabolite found in most vertebrates (163). Creatine is found in the highest concentrations in tissues that have high activities of creatine kinase such as the muscle and brain. Creatine is not known to be metabolized enzymatically in mammals other than by conversion to creatine phosphate by creatine kinase, but creatine and creatine phosphate spontaneously cyclize to form creatinine which is eventually excreted in the urine at a rate of about 2 percent of total creatine per day (186). Therefore since total body creatine is relatively constant in adults, the lost creatine must be replaced either by dietary intake or by *de novo* biosynthesis. Major dietary sources of creatine are meat and dairy products.

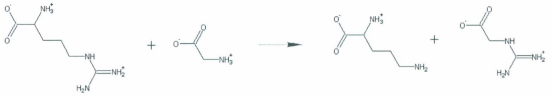
Creatine arises from the metabolism of the amino acids arginine, glycine and methionine and its synthesis involves only two enzymes (Figure 1-4). The first step of creatine synthesis requires the enzyme arginine:glycine amidinotransferase which catalyzes the transfer of the guanidino group of an arginine molecule onto the amine of a

glycine molecule, yielding ornithine and guanidinoacetate. The second enzyme is guanidinoacetate N-methyltransferase which catalyzes the transfer of the methyl group from S-adenosylmethionine (SAM), resulting in a molecule of creatine and S-adenosylhomocysteine (SAH). The process of creatine synthesis is largely an inter-organ pathway involving mainly the kidney and liver. The enzymes of creatine biosynthesis are located in various tissues and are expressed at varying levels. AGAT is located primarily in the pancreas and kidney; however, some activity has been reported in the heart, lung, spleen, muscle, brain, testis and thymus (163). There have also been reports of AGAT activity detected in the livers of cow, pig, monkey and human, meaning that the complete synthesis of creatine might be occurring in this tissue. However there are difficulties with the traditional assay for AGAT in liver due to interference by ornithine produced by the arginase enzyme (186). GAMT is perhaps more widely expressed but the highest activities are present in the pancreas and liver with lower expression in a much broader range of tissues (163).

Figure 1.4

Creatine Biosynthesis. AGAT; Arginine:glycine amidinotransferase, GAMT; Guanidinoacetate N-methyltransferase.

AGAT



Arginine Glycine Ornithine GAA

GAMT

SAM +

GAA Creatine

The primary regulation of creatine synthesis occurs in the kidney. There is a feedback regulation by creatine on the expression of AGAT in the kidney at the pre-translational level (113). Growth hormone also affects the expression of renal AGAT as described in an elegant study by McGuire et al. (114) using hypophysectomized rats. Apart from the regulatory effect of growth hormone and creatine on creatine synthesis the concentration of the substrates for GAMT and AGAT can also affect creatine synthesis. Like the other methyltransferase enzymes, the velocity of GAMT is influenced by the concentrations of SAM and SAH; GAMT activity increases in the presence of higher SAM concentration and decreases in the presence of higher SAH concentration (37). Therefore changes in the concentration of SAM or SAH will have an effect on the rate of methylation of GAA. Finally, renal infusion studies by Edison et al. (49) have shown that infusion of arginine significantly increases the production of GAA in the kidney.

1.1.5.1 Arginine:glycine Amidinotransferase

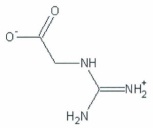
Arginine:glycine amidinotransferase occurs as an 84 kDa dimer and is located in the mitochondria. It is translated on cytoplasmic ribosomes and is translocated into the mitochondria with the cleavage of a 3 kDa section of the protein, resulting in the most active form of the enzyme (112). The protein structure contains five $\beta\beta\alpha\beta$ sections which are arranged in a 'basket' shape with alpha helices forming the sides and beta sheets forming the base (73). The active site involves coordination of helix 9 and a loop called the "300-loop" for binding of the substrates. The mechanism of AGAT is a ping-pong type reaction involving initial binding of arginine, transfer of the amidino group onto the enzyme, release of ornithine, followed by the binding of glycine and subsequent transfer of the amidino group onto the amine group of glycine. The AGAT protein sequence has

been determined and there is a conserved cysteine residue in the active site of the AGAT protein which forms isothiourea in the process of transferring the amidino group (74).

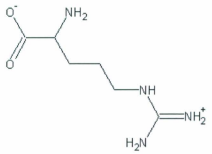
Physiologically the AGAT enzyme runs in favor of guanidinoacetate formation and is inhibited by ornithine. Some older studies have shown that the enzyme can indeed run in the reverse direction forming glycine and arginine (139). This is supported in studies by Walker using radioactive 2-^{14}C-ornithine (171) and 2-^{14}C-glycine as substrates for purified AGAT enzyme (174). It was found that radioactivity was present in arginine and guanidinoacetate. The K_m for glycine, arginine, guanidinoacetate and ornithine have been determined *in vitro* to be 1.8-3.1, 1.3-2.8, 3.9 and 0.1 mM respectively (186). This would suggest that AGAT is responsive to relatively lower amounts of ornithine than any of the other potential substrates. The question of whether the reverse reaction occurs under differing physiological situations or *in vivo* is unanswered. Other substrates may also be used in certain physiological situations. For instance, the compound canavanine (Figure 1.5), which is the guanidinoxy analogue of arginine is found in a number of legumes including alfalfa and jack beans, and is a known substrate of AGAT (172) and arginase (40). Thus if canavanine is present in the diet it might very well serve as a substrate for the AGAT enzyme.

Figure 1.5

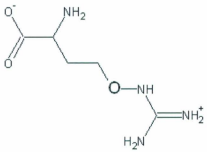
Structures of Guanidinoacetate, Arginine and Canavanine. The oxygen in Canavanine is bolded to illustrate the difference between the guanidine and guanidinoxy compounds.



Guanidinoacetate



Arginine



Canavanine

1.1.5.2 Guanidinoacetate Methyltransferase

GAMT is a member of the large family of methyltransferases responsible for the irreversible transfer of the methyl group of SAM onto an amine. Rat liver GAMT was first purified by Ogawa et al. in 1983; it was determined to have a molecular weight of 26 kDa and a sulphydryl character that was integral for activity (126). Later GAMT was shown to have 4 cysteine residues, one of which was located in the active site (57) and the catalytic mechanism was determined after the crystal structure was elucidated (93). GAMT adopts the α/β sandwich structure common to all SAM dependent methyltransferases, binding SAM and GAA in appropriate proximity for reaction. The reaction is irreversible and is not inhibited by the presence of excess creatine (up to about 5 mM) or any creatine analogues (57). GAMT seems not to be acutely regulated nor does it appear to be regulated by dietary creatine, at least in the liver, and therefore GAMT appears to be a slave to the concentration of GAA that it encounters. There seem to be no accounts of GAMT regulation and only a few accounts of its activity in the pancreas (23, 163). Activity of GAMT in the pancreas is high and even though the tissue is small, it may have an impact on total body creatine synthesis. The second paper attempts to address the question of whether dietary creatine has a regulatory effect on GAMT expression in the pancreas and whether the pancreas can indeed produce creatine *de novo*.

1.1.5.3 Creatine Transport into Cells

Creatine transport is well described in the tissues that use the creatine kinase system such as the muscle and brain. The creatine transporter, SLC6A8, has been shown to have a higher affinity for creatine than for any other guanidino compounds, it is sodium-dependent and is capable of concentrating creatine as high as 100 fold within cells

relative to plasma concentration (64). Expression of SLC6A8 correlates well with the creatine content of tissues as well as the expression of creatine kinase, with the notable exception of the kidneys which have a much higher creatine transporter content and very little creatine kinase activity (186). The function of the SLC6A8 in the kidneys is to reabsorb creatine, as the urine contains very little of this molecule. There have been reports of the creatine transporter protein in mitochondria of liver and kidney (178) but these experiments were carried out using an antibody now known not to be specific; in particular it cross-reacts with the E2 subunit of pyruvate dehydrogenase (151). The SLC6A8 transporter is not found at high levels in any of the tissues known to synthesize creatine; however there must be some mechanism, whether passive or active, that transports creatine out of these tissues.

1.1.6 Creatine Deficiency

Creatine deficiency was first described in 1994 by Stockler et al. (155) and was later characterized as a deficiency of the GAMT enzyme (156). Since then, deficiencies of both enzymes of creatine synthesis as well as the sodium-dependent creatine transporter have been characterized (144). Creatine deficiency syndromes are apparent in very young individuals and interestingly, all of these syndromes show symptoms of neurological impairment, perhaps emphasizing the importance of creatine for normal development and function of the brain. The features of creatine deficiency syndromes are presented in a condensed form in Table 1-1.

AGAT and GAMT deficiencies present with similar symptoms (Table 1-1). GAMT deficiency displays slightly more severe neurological dysfunction, usually attributed to the neurotoxic properties of GAA (118). Inheritance of GAMT deficiency is

autosomal recessive. Reports of this deficiency are relatively recent and thus the number of cases is low but increasing as more research is completed (46). GAMT deficiency results in elevated guanidinoacetate (GAA) and decreased levels of creatine in plasma and in some other tissues. Mutations in the *gamt* sequence have been identified (5, 75) and include deletions and point mutations affecting the active site either directly or indirectly, by changing the structure of the active site itself or the N-terminal section of the protein.

AGAT deficiency was first reported in 2000 by Bianchi et al. (17) and shortly thereafter, it was characterized as a transition mutation resulting in a premature stop codon in the *agat* gene (76). At present, there are only three families with children who have been found to be deficient in AGAT (50) and all have different mutations. AGAT deficient subjects have only trace amounts of guanidinoacetate in their tissues and almost no creatine. Deficiency in the SLC6A8 protein is different from the other two creatine deficiency syndromes in that patients do not have a total lack of creatine; in fact, they have normal or elevated plasma and CSF creatine, but are not able to transport creatine into the muscle and brain (22). There is some evidence that creatine transporter deficiency may have a relatively high frequency of occurrence. One study found that approximately 2% of males with undiagnosed developmental delay were creatine transporter-deficient (123).

Diagnosis of the creatine deficiency syndromes is accomplished by ^{31}P NMR (MRS) in the brain and muscle, determination of plasma GAA and creatine concentrations and enzyme activities in cultured fibroblasts. Both AGAT and GAMT

Table 1-1

Clinical and biochemical characteristics of GAMT, AGAT, CRTR deficiency and diagnostic tests. (159)

Table 1. Clinical and biochemical characteristics of GAMT, AGAT and CRTR deficiency and diagnostic tests

Disorder	Clinical characteristics	Biochemical characteristics	Diagnostic test	Confirmation
GAMT	Mental retardation	Deficiency of brain creatine	Brain MRS	GAMT activity (f,l)
	Speech delay	Accumulation of guac	Guac u, p, csf, dbs	
	Epilepsy (intractable)	Low creatinine and creatine excretion	Creatinine 24 h urine Creatine & creatinine in CSF*	GAMT mutations (b,f,l,dbs)
	(Extra) pyramidal symptoms & signs	High urinary uric acid/ creatinine ratio	Creatine, creatinine, uric acid in urine	
AGAT	Mental retardation	Deficiency of brain creatine Low guac excretion	Brain MRS Guac u, p, csf, dbs	AGAT activity (f,l)
	Speech delay	Low creatine excretion ?	Creatine urine	AGAT mutations (b,f,l,dbs)
		Low creatinine	Creatinine 24 h urine	
	(Epilepsy)	excretion ?		
CRTR	Mental retardation	Deficiency of brain creatine	Brain MRS	CRTR activity (f,l)
	Speech delay	Low creatinine excretion ?	Creatinine 24 h urine	CRTR mutation (b,f,l)
	Epilepsy	High urinary creatine/ creatinine ratio	Creatine and creatinine urine	

Abbrevations: Guac, guanidinoacetate; u, urine; p, plasma; csf, cerebrospinal fluid; dbs, dry blood spot sample; f, fibroblasts; l, lymphoblasts; b, blood; ?, expected but not measured so far in respective patients. *Stöckler *et al.*, 1997; Schulze *et al.*, 1997.

deficiency have been successfully treated with creatine supplementation between 350-2000 mg/kg/day and it appears that earlier intervention leads to a better prognosis (144). Treatment of SLC6A8 deficient subjects with oral creatine has not been reported to be beneficial (102).

1.1.7 Creatine in the Brain

The creatine deficiency syndromes highlight the importance of creatine in the brain. Creatine in the brain is about one-third of the creatine concentration found in muscle. The firing of a neuron relies on sodium inflow to collapse its concentration gradient. This gradient is subsequently re-formed and maintained within the cells by sodium:potassium ATPase and so demands large, acute energy consumption for which creatine phosphate is integral. The creatine kinase system supports many of the ion transporting ATPase proteins by maintaining the concentration of ATP near the functioning proteins (177). But creatine may serve an additional role in the brain. There has been a large scale clinical trial launched by the National Institutes of Health (NIH) to find compounds that may be used as a treatment for Parkinson's disease and creatine has been included as a candidate (38). Parkinson's disease is caused by a gradual loss of dopaminergic neurons mainly in the substantia nigra which leads to movement disorders (185). Creatine is reported to be neuroprotective, preventing mitochondrial dysfunction and oxidative damage and is believed to be therapeutic in a range of brain disorders (3).

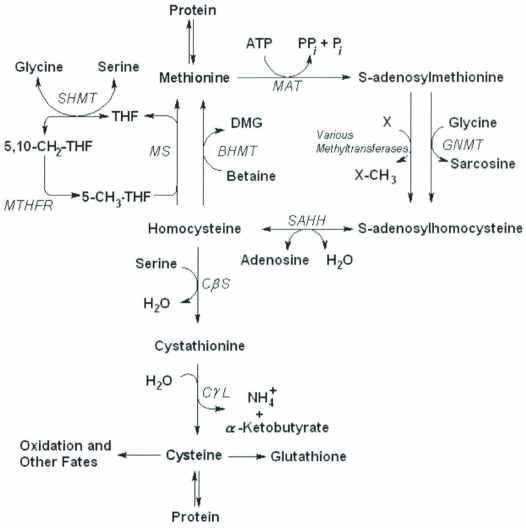
1.1.8 Methionine and Creatine metabolism

Methionine is one of two sulfur-containing amino acids that can be incorporated into proteins. Aside from its role in protein synthesis, methionine is the precursor for the

universal methyl donor SAM. A detailed diagram of methionine metabolism is given in Figure 1.6. SAM is the substrate used by a family of methyltransferase proteins including glycine-N-methyltransferase (GNMT), guanidinoacetate methyltransferase GAMT, phosphatidylethanolamine methyltransferase (PEMT), DNA methyltransferase (DNMT) and others. Biological methyltransferases are mainly regulated by the concentrations of SAM and SAH; a higher SAM/SAH ratio results in an increased rate of methylation and vice versa (37). The availability of SAM in the liver seems to be partially mediated by the most active methyltransferase, GNMT (119). The regulation of many biological methylation reactions also relies in part on the availability of methyl-accepting substrates as outlined by the Stead et al. (26, 152) who introduced the term 'methylation demand'. In addition, a few methylation reactions are compartmentalized within cells and use different SAM/SAH pools, adding a layer of complexity. Two examples of this are PEMT which is located in the ER (148), and DNMT in the nucleus (109). Pools of tetrahydrofolate are also compartmentalized (108), affecting the regeneration of methionine from homocysteine and thus affecting the respective pool of SAM.

Figure 1.6

Methionine metabolism. Symbols are defined as follows: MAT; Methionine adenosyltransferase, GNMT; Glycine N-methyltransferase, SAHH; S-adenosylhomocysteine hydrolase, BHMT; Betaine:homocysteine methyltransferase, MS; Methionine synthase, SHMT; Serine hydroxymethyltransferase, MTHFR; Methyltenetrahydrofolate reductase, CβS; Cystathionine β-synthase and CγL; Cystathionine γ-lyase.


Protein
ATP
PPi + Pi
Glycine
Serine
Methionine
MAT
S-adenosylmethionine
SHMT
THF
DMG
X
Glycine
Various
Methyltransferases
GNMT
5,10-CH2-THF
MS
BHMT
Betaine
X-CH3
Sarcosine
MTHFR
5-CH3-THF
Homocysteine
SAHH
S-adenosylhomocysteine
Adenosine
H2O
Serine
CβS
H2O
Cystathionine
H2O
CγL
NH4+
+
α-Ketobutyrate
Oxidation and
Other Fates
Cysteine
Glutathione
Protein

For every molecule of creatine that is synthesized, one equivalent methyl group is required. It was originally calculated that creatine synthesis was responsible for consuming 70-75% of the labile methyl groups that are metabolized per day (120, 187). However more recently this number has been revised to give a value of 40% of methyl groups being used for synthesis of creatine, still a considerable portion (153). Thus, creatine biosynthesis is a major consumer of methyl groups.

1.1.9 Non-Alcoholic Fatty Liver Disease

Non-Alcoholic Fatty Liver Disease (NAFLD) is the term used to describe a number of conditions (not related to alcohol consumption) that lead to fibrosis and can progress to full blown liver cirrhosis. Fatty liver has been associated with obesity and decreased insulin sensitivity, and is considered the hepatic manifestation of the metabolic syndrome (47). The hallmark of NAFLD is the accumulation of triglycerides in lipid droplets within hepatocytes, often accompanied by inflammation referred to as non-alcoholic steatohepatitis (NASH). A "two-hit" model has been proposed; the first hit is infiltration of fat into hepatocytes increasing sensitivity to the second hit which can include oxidative stress, lipid peroxidation, pro-inflammatory cytokine-mediated cell injury and cell death (6). The pathogenesis of NAFLD is not yet clear. The disorder can arise from three general situations 1) excessive influx of fatty acids that have been released from adipose tissue or from digestion, 2) diminished hepatic export of TG (increased VLDL synthesis/decreased export), 3) impaired beta oxidation, each of which can result from overfeeding, obesity, drugs that inhibit microsomal triglyceride transfer protein (MTP), methionine/choline deficiency, some vitamin deficiencies and some genetic disorders (95).

Several different animal models have been generated in order to study NAFLD; these different models display varying degrees of inflammation, fibrosis and metabolic syndrome. Mice with knock-outs of either fatty acyl-CoA oxidase, methionine adenosyltransferase, SREBP-1c, PEMT, PPARα, leptin/leptin receptor or *pten* all develop steatosis but none shows the full spectrum of NAFLD and only a few show signs of steatohepatitis. Dietary models include methionine-choline deficient, arginine deficient, atherogenic, high-fructose/high-sucrose, and high fat diets (6, 101, 111). From the literature, the animal models that display the most similarity to NAFLD are the PEMT, SREBP-1c, leptin/leptin receptor knock-outs and the atherogenic, methionine-choline deficient and high fat diets. These models all progress to NASH and fibrosis.

1.1.10 Phosphatidylcholine and the Role of Methylation in Lipid Metabolism

Phosphatidylcholine (lecithin) is integral to the structure of biological membranes, the packaging and transport of lipids and the enterohepatic circulation of bile acids. Phosphatidylcholine represents more than 90% of the phospholipids found in mucus and has been used successfully as a therapy in patients with ulcerative colitis (158). Phosphatidylcholine is one of the major phospholipids found in membranes and lipoproteins, together with phosphatidylethanolamine and phosphatidylserine. The various properties and proportions of the phospholipids can affect the behavior of membranes as well as the proteins functioning within and proximal to the membrane. In addition to its structural functions, phosphatidylcholine is a substrate for other enzymes such as phospholipases and lecithin: cholesterol acyltransferase, forming other important molecules such as sphingomyelin, lysophosphatidylcholine and cholesterol esters. Phophatidylcholine can be catabolized, through a number of steps, to choline and

glycerol-3-phosphate and choline itself can be metabolized further into betaine which is used to remethylate homocysteine (Figure 1.7). Choline is considered an essential nutrient; an adequate intake for adolescent and adult humans is between 400 and 500 mg/day (140).

Phosphatidylcholine itself is synthesized in the liver by two separate pathways. In the Kennedy pathway, choline kinase converts a molecule of choline to phosphocholine which phosphocholine cytidylyltransferase converts to cytidine diphosphocholine. Diacylglycerol cholinephosphotransferase transfers the phosphocholine group to a molecule of 1,2-diacylglycerol (105) (Figure 1.8a). The second pathway involves three successive methylations of the nitrogen of phosphatidylethanolamine, resulting in a molecule of phosphatidylcholine, catalyzed by PEMT (Figure 1.8b). In rats, approximately 70% of *de novo* phosphatidylcholine is derived from the Kennedy pathway leaving PEMT to synthesize the remaining 30% (134). The requirement of three methyl groups for every one molecule of phosphatidylcholine synthesized means that even the synthesis of a relatively small amount of this molecule could have a significant impact on methylation. PEMT knock-out mice fed a normal chow diet had 50% less

Figure 1.7

Phosphatidylcholine catabolism.

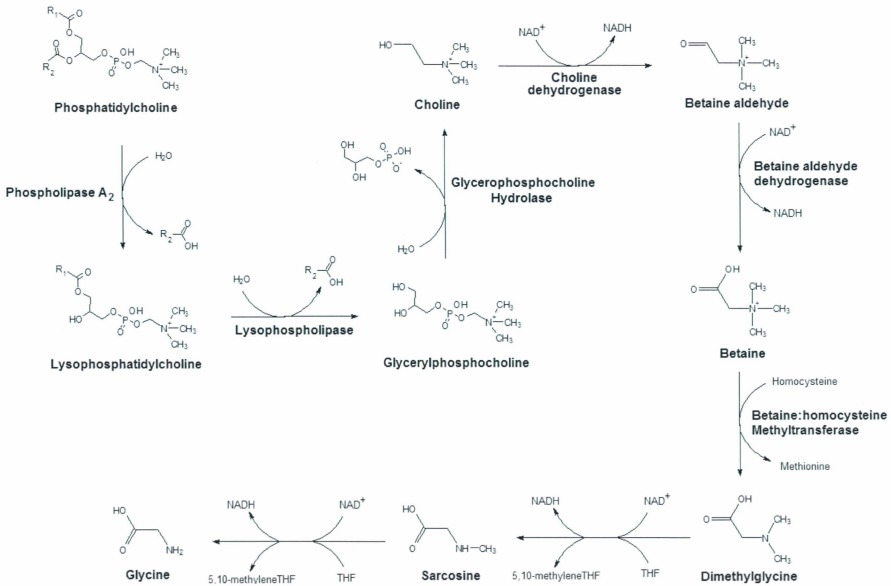
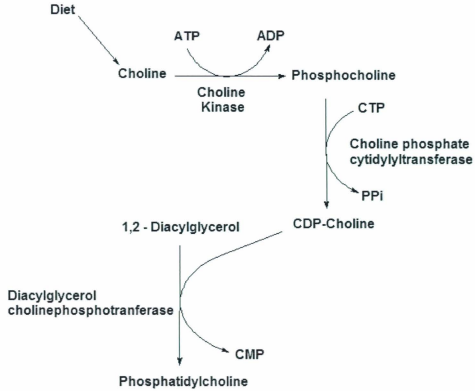

Phosphatidylcholine
Choline
NAD+
NADH
Choline dehydrogenase
Betaine aldehyde
Phospholipase A2
H2O
Glycerophosphocholine Hydrolase
Betaine aldehyde dehydrogenase
NADH
Lysophosphatidylcholine
Lysophospholipase
Glycerylphosphocholine
Betaine
Homocysteine
Betaine:homocysteine Methyltransferase
Methionine
Glycine
NADH
NAD+
5,10-methyleneTHF
THF
Sarcosine
NADH
NAD+
5,10-methyleneTHF
THF
Dimethylglycine

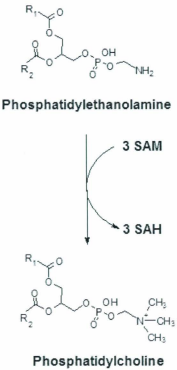
Figure 1.8

Phosphatidylcholine synthesis. The Kennedy pathway (A), PEMT pathway (B). R_1 and R_2 are fatty acid chain; generally R_1 is 14 carbons (palmitic acid) and R_2 is 16 (oleic acid).

A) Kennedy Pathway



B) PEMT Pathway



plasma homocysteine than control mice and isolated liver cells from these animals secreted 50% less homocysteine than cells from control mice (124). This suggests that even under normal conditions PEMT places a heavy demand on the provision of methyl groups. Phosphatidylcholine synthesis seems to be important in hepatic steatosis as steatosis may be brought about by feeding diets deficient in methionine and choline. In addition, another methyl donor, betaine, alleviates symptoms of the closely related alcohol-induced steatohepatitis in mice (87). Thus the main hypothesis of the third paper is that sparing methyl groups by reducing the need for creatine synthesis in the liver would provide more methyl groups for phosphatidylcholine synthesis and hence provide more phosphatidylcholine for VLDL synthesis, thus alleviating fatty liver.

1.2.0 Portacaval Anastomosis

Portacaval anastomosis (also termed 'portacaval shunt') is the diversion of blood flow from the hepatic portal vein away from the liver into the systemic circulation. This phenomenon can arise when there is portal hypertension, such as during conditions of portal thrombosis, chronic fatty liver, liver cirrhosis or another physical blockage (i.e. surgical procedure) (97). In these situations collateral vessels can carry portal blood directly to the hepatic vein. In addition, portacaval anastomosis is a routine surgical treatment for chronic portal hypertension. The physiological impact of portacaval anastomosis is significant. The blood that collects in the portal vein feeding the liver is very different from other venous and arterial blood, as discussed in section 1.1.3. There are higher concentrations of amino acids and ammonia present in plasma after digestion of a meal in humans with transjugular intrahepatic portosystemic shunt (TIPS) (130) (a slightly different means of bypassing the liver). The concentration of ammonia in the

plasma is higher in rats with portacaval anastomosis fed a high protein diet versus a normal protein diet (13). Hepatic encephalopathy may result from hyperammonemia and changes in the levels of plasma amino acids after portacaval anastomosis (52, 54).

1.2.1 Heterogeneity of Liver Nitrogen Metabolism

Liver cells that span the acinus have different metabolic functions. The periportal cells are mostly responsible for net gluconeogenesis, fatty acid oxidation, ureagenesis and albumin synthesis while the perivenous cells are responsible for net glycolysis, fatty acid synthesis, glutamine synthesis and xenobiotic metabolism (131). In addition the majority of blood that flows into the liver has passed through the intestine so that its composition will reflect absorptive processes and intestinal metabolism. The liver is largely responsible for the oxidation and metabolism of most amino acids and so too it must deal with the free ammonia resulting from this metabolism. The liver has two ways of dealing with free ammonia; 1) the urea cycle and 2) glutamine synthesis. The urea cycle catalyzes the synthesis of urea; this metabolic end-product is excreted in the urine. Glutamine synthetase uses ammonia as a substrate in synthesis of the amino acid glutamine so that toxic levels of ammonia do not accumulate. The urea cycle is a low affinity, high capacity system and works best under relatively high concentrations of ammonia while glutamine synthetase is a high affinity, low capacity enzyme (84). The two processes are localized to different cell populations across the acini of the liver (Figure 1.3). The urea cycle enzymes are active in the periportal hepatocytes while glutamine synthetase is active in the last 2 layers of perivenous hepatocytes (83). In general, it is thought that the urea cycle deals with the bulk of ammonia leaving glutamine synthetase to 'scavenge' the slight excess ammonia that has 'escaped'.

The liver contains both glutaminase and glutamine synthetase activities. The balance of glutamine across the liver, however, is often close to zero (179). Glutamine is catabolized in periportal cells by glutaminase and glutamate dehydrogenase to form α-ketoglutarate and two ammonia molecules. Alternatively, the combination of glutaminase and aspartate aminotransferase can produce aspartate and ammonia which are directly used in the urea cycle (115). There are two isoforms of phosphate dependent glutaminase: 1) kidney-type and 2) liver-type. The kidney-type glutaminase is found in extra-hepatic tissues such as the kidney, brain and cells of the immune system (36). The kidney-type glutaminase, like the liver isoform is found in mitochondria but has a much lower Km for glutamine, a much higher Ka for phosphate and is inhibited at lower concentrations of glutamate compared to the liver isoenzyme. The elegant retrograde and anterograde liver perfusion experiments of Häussinger et al. (67-69) show very clearly that glutaminase supplies ammonia to ureagenesis and that glutamine synthetase detoxifies ammonia that has escaped the urea cycle. Thus, glutaminase and glutamine synthetase both function at the same time in different locations along the liver acinus.

1.2.2 Perivenous Hepatocytes in Portacaval Anastomosis

Girard and Butterworth (62) found that there was a marked loss of glutamine synthetase activity in the liver and a small increase in glutamine synthetase in the brain and muscle of rats that had received surgical portacaval-shunts. Later it was found that there was a significant up-regulation of glutamine synthetase expression in the muscle and brain in these animals (45), presumably to deal with excess ammonia that is present in the plasma and extracellular fluid post shunt. Normally, excess ammonia is converted to glutamine by the liver, preventing hyperammonemia. How then would glutamine

synthetase activity in the liver decrease? There are two general scenarios which could result in reduced glutamine synthetase activity: 1) glutamine synthetase expression in perivenous cells is reduced or 2) the perivenous cells are lost. From this arises a key question: Is perivenous cell function lost in portacaval anastomosis? The fourth paper that is presented here addresses this question by measuring the activity and cellular location of ornithine aminotransferase, an enzyme known to be localized in the same perivenous cells as glutamine synthetase (98).

1.3 Specific Objectives

Each of the papers discussed include their respective objectives and rationale in the introduction sections. However, I will briefly summarize the specific objectives in this section.

Chapter two asks whether the liver is capable of synthesizing creatine and specifically whether arginine produce within the liver can be used for creatine synthesis. In addition, we sought to better describe the capacity of the rat liver for creatine synthesis. To address these questions we used both *in vivo* and *in vitro* experiments. Chapter three presents a similar study to chapter two but instead dealing with the role of the pancreas in creatine synthesis. This study asks whether pancreatic acinar cells are capable of synthesizing creatine and GAA. From the data the capacity of the pancreas for creatine synthesis is calculated. In addition, we ask whether the AGAT enzyme in pancreas is regulated by dietary creatine supplementation.

Chapter four sought to determine whether dietary creatine supplementation can improve phosphatidylcholine synthesis in the livers of rats fed high-fat diets. The hypothesis was that sparing methyl groups for the PEMT reaction would increase

phosphatidylcholine synthesis, thereby improving hepatic VLDL packaging and secretion, and would thus alleviate the accumulation of triglycerides in the liver. The specific objectives were to analyze lipid accumulation in the livers of these animals, measure the phospholipids and the expression of some genes involved in lipid metabolism.

Chapter five addresses the fate of perivenous hepatocytes in the livers of rats that have undergone surgical portacaval shunts. It has been suggested previously that the perivenous cells are lost after portacaval shunting based in the observation that glutamine synthetase activity and expression are dramatically reduced in this condition (45, 62). To determine whether the perivenous cells are lost we specifically sought to measure the activity and expression of ornithine aminotransferase and an ammonia transporter (RhBG) respectively; two enzymes that are colocalized in the perivenous cells along with glutamine synthetase.

1.4 Goals and Outlook

The overall goal of this thesis is to expand on the field of amino acid metabolism in the liver. The works that are presented are meant to address some current questions. The goals are to determine the role of the liver and pancreas in total body creatine synthesis in the rat and to examine the effects of dietary creatine in a model of NAFLD. In addition I wished to examine the organization of the liver with respect to perivenous amino acid metabolism in portacaval-shunted rats. The creatine projects have implications in the understanding of inter-organ amino acid metabolism, net amino acid utilization and hepatic methylation reactions. The portacaval shunt project has implications in hepatic zonation, hepatic encephalopathy and the balance between

glutamate, glutamine and ammonia. Before continuing to the next section I will leave you with quotations from two Nobel Laureates who worked in very different fields.

> "It is not enough to say that we cannot know or judge because all the information is not in. The process of gathering knowledge does not lead to knowing. A child's world spreads only a little beyond his understanding while that of a great scientist thrusts outward immeasurably. An answer is invariably the parent of a great family of new questions. So we draw worlds and fit them like tracings against the world about us, and crumple them when we find they do not fit and draw new ones."
>
> - John Steinbeck - Nobel Prize in Literature 1962

> "The important thing is not to stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day."
>
> - Albert Einstein - Nobel Prize in Physics 1921

I think that both of these quotations illustrate the passion about science shared by most and perhaps more importantly the understanding that no matter how great achievements may seem, they are often built on relatively small discoveries.

Chapter 2

Creatine Synthesis: Hepatic Metabolism of Guanidinoacetate and Creatine in the Rat, *in vitro* and *in vivo*

Creatine Synthesis: Hepatic Metabolism of Guanidinoacetate and Creatine in the Rat, *in vitro* and *in vivo*

Robin da Silva[†], Itzhak Nissim[¶], Margaret E. Brosnan[†], John T. Brosnan[†]

[†]Department of Biochemistry, Memorial University of Newfoundland, St. John's, NL, Canada, A1B3X9

[¶]Division of Child Development, Abramson Pediatric Research Center, University of Pennsylvania School of Medicine, Philadelphia, PA, USA, 19104-4318

Running Head: Hepatic Creatine Synthesis

Address for Correspondence: John T. (Sean) Brosnan, Department of Biochemistry, Memorial University of Newfoundland, St. John's, NL, CANADA A1B3X9

email: jbrosnan@mun.ca

Phone: 709-737-8540

FAX: 709-737-2422

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2.1 Abstract

Since creatinine excretion reflects a continuous loss of creatine and creatine phosphate, there is a need for creatine replacement, from the diet and/or by *de novo* synthesis. Creatine synthesis requires three amino acids, methionine, glycine and arginine, and two enzymes, L-arginine:glycine amidinotransferase (AGAT), which produces guanidinoacetate (GAA), and guanidinoacetate methyltransferase (GAMT), which methylates GAA to produce creatine. In the rat, high activities of AGAT are found in the kidney, whereas high activities of GAMT occur in the liver. Rat hepatocytes readily convert GAA to creatine; this synthesis is stimulated by the addition of methionine, which increases cellular S-adenosylmethionine concentrations. These same hepatocytes are unable to produce creatine from methionine, arginine and glycine. ^{15}N from $^{15}NH_4Cl$ is readily incorporated into urea but not into creatine. Hepatic uptake of GAA is evident *in vivo* by livers of rats fed a creatine-free diet but not when rats were fed a creatine-supplemented diet. Rats fed the creatine-supplemented diet had greatly decreased renal AGAT activity and greatly decreased plasma [GAA] but no decrease in hepatic GAMT or in the capacity of hepatocytes to produce creatine from GAA. These studies indicate that hepatocytes are incapable of the entire synthesis of creatine but are capable of producing it from GAA. They also illustrate the interplay between the dietary provision of creatine and its *de novo* synthesis and point to the crucial role of renal AGAT expression in regulating creatine synthesis in the rat.

Keywords: inter-organ metabolism, in vivo hepatic fluxes, S-adenosylhomocysteine, methylation demand

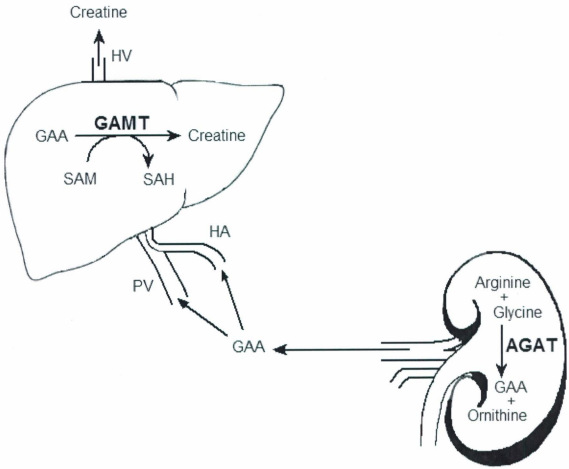
2.2 Introduction

In vertebrates, creatine and creatine phosphate function as a "buffer" for ATP in tissues, maintaining ATP levels when energy demand is transiently greater than the rate of ATP synthesis. They also serve in an energy shuttle between the sites of ATP synthesis and utilization (16). Creatine can be obtained from the diet and/or synthesized *de novo* in the body from the amino acids arginine, glycine and methionine. The entire pathway of creatine synthesis consists of just two enzyme-catalyzed reactions (Figure 2.1). First the amidino group from arginine is transferred to the amino group of glycine, yielding ornithine and guanidinoacetic acid (GAA). This is catalyzed by the enzyme arginine:glycine amidinotransferase (AGAT). GAA can then be methylated on the original glycine nitrogen using S-adenosylmethionine (SAM) as the methyl donor. This reaction yields creatine and S-adenosylhomocysteine (SAH) and is catalyzed by the enzyme guanidinoacetate N-methyltransferase (GAMT) (186).

The need to synthesize creatine arises from the fact that under physiological conditions creatine and creatine phosphate are spontaneously converted to creatinine which is subsequently lost to the urine. Daily creatinine loss is estimated to be on the order of 1.7% of the total body creatine pool or around 2 grams per day in a young 70 kg human male (186). Since the total body pool of creatine is constant, the lost creatine must be replaced either from the diet or from *de novo* synthesis. Humans on a typical western diet obtain about half of their creatine by synthesis and half from the diet. Vegetarians obtain very little dietary creatine so that endogenous synthesis is their major source (153).

Figure 2.1

Creatine biosynthetic pathway. HA; Hepatic Artery, PV; Portal Vein, HV; Hepatic Vein.


Creatine
HV
GAMT
GAA
Creatine
SAM
SAH
HA
PV
GAA
Arginine
+
Glycine
AGAT
GAA
+
Ornithine

In the rat, AGAT is highly active in the kidneys while GAMT is highly active in the liver. This has given rise to the idea that GAA is synthesized primarily in the kidney and then transported to the liver where it is methylated to form creatine (186). Evidence to date strongly suggests that AGAT is a critical control step in creatine synthesis and it has been shown that creatine supplementation down-regulates, while growth hormone up-regulates, AGAT expression (65, 113). The mechanisms by which creatine and growth hormone affect the regulation of AGAT expression have not been identified but it is known that they affect AGAT synthesis at the pre-translational level (65, 113). On the other hand, GAMT isolated from pig or rat liver is not inhibited by creatine but is competitively inhibited by SAH, as are the other methyltransferases (37, 57). In addition to its role in creatine synthesis GAMT should maintain low [GAA], as it has been shown that high GAA levels result in neurotoxicity (118). There is evidence that the regulation of creatine biosynthesis in humans is similar to that in the rat. Dietary supplementation with 4 g/kg diet (similar to the creatine content of red meat) in rats, results in a reduction of renal AGAT activity by 86% and plasma GAA levels by 70% compared to control rats fed a creatine-free diet (49). Creatine ingestion by humans lowers plasma GAA levels which is consistent with down-regulation of AGAT activity (44).

Recent work from our laboratory has shown that the renal output of GAA in rats fed a creatine-free diet is equal to the renal loss of creatinine, indicating that renal GAA production is sufficient to replace creatine and creatine phosphate lost to creatinine formation (49). This implies that in this species extra-renal tissue(s) must convert the GAA released by the kidney to creatine. In humans however, the renal production of GAA appears to represent only 20% of the daily loss of creatinine (49), suggesting that

GAA must be synthesized in other tissues. It has been proposed by Brosnan and Brosnan that the entire creatine synthetic pathway may occur in the liver (28). They coined the term "Arginine Bicycle" to illustrate a situation where arginine could be acted upon by either arginase to form urea or by AGAT to form GAA; in either case ornithine would be formed and metabolized via the urea cycle enzyme ornithine transcarbamoylase. There is also evidence for appreciable expression of AGAT mRNA in human liver (189). Despite past failures to measure AGAT activity under standard assay conditions in rat liver, there is immunohistochemical evidence for the AGAT protein in the cytosol of rat hepatocytes (112). The goal of this paper is to examine creatine synthesis by the rat liver both *in vivo* and *in vitro*. We also sought to establish whether AGAT is functionally active in this tissue. Our results show a brisk production of creatine from GAA by hepatocytes *in vitro*, as well as an hepatic uptake of GAA *in vivo*. We could find no evidence for a functional AGAT in the liver despite using very sensitive methods

2.3 Materials and Methods

Reagents: HPLC-grade methanol was obtained from Fisher Scientific Ontario, Canada. $^{15}NH_4Cl$ was obtained from Cambridge Isotope Laboratories Inc. All other chemicals and reagents were obtained from Sigma.

Animals: Male Sprague-Dawley rats 250-350 grams were used for all experiments. Animals were housed on a 12:12 hour light:dark cycle and fed Purina Rodent Chow 5001 *ad libitum*. Rats used for the determination of hepatic fluxes of GAA and creatine were fed a purified AIN-93G diet either free of creatine or supplemented with 0.4% creatine monohydrate, by weight, in place of an equivalent amount of corn starch. Rats were anesthetized with sodium pentobarbital, i.p., at a dose of 60 mg/100 grams body weight. All procedures were approved by Memorial University of Newfoundland Institutional Animal Care Committee and were in accordance with the Guidelines of the Canadian Council of Animal Care.

Isolated Hepatocytes: Rat hepatocytes were prepared as described by Seglen (147). Viability of hepatocytes was measured by trypan blue exclusion. Average viability was 95%. All hepatocyte incubations were carried out at 37°C in a shaking water bath using 25 mL glass Erlenmeyer flasks with rubber stoppers. Substrates were prepared in Krebs-Henseleit medium (Containing 1.3 mM calcium) and gassed with 95% O_2/5% CO_2. Incubations were begun by the addition of aliquots of hepatocyte suspension to the Krebs-Henseleit medium containing the substrates. Each flask was gassed with 95% O_2/5% CO_2 for 20 seconds after the hepatocytes were added and then stoppered. Incubations were carried out in triplicate. Incubations were stopped with the addition of 100 μL of ice-cold 30% (w/v) perchloric acid. Precipitated protein was pelleted at 10,000

x g for 10 minutes at room temperature. 700 μL of the supernatants were removed and neutralized with 20 μL of universal indicator, 40 μL of 50% K_2CO_3 and 20% KOH until the pH was between 6 and 8. After standing in ice for 15 minutes, precipitated salts were removed by centrifugation at 10,000 x g for 5 minutes at room temperature. Supernatants were then assayed for creatine as described below. Hepatocytes incubated for SAM and SAH measurement were pelleted at 3000 x g for 2 minutes at room temperature and flash-frozen in liquid nitrogen after removal of the supernatant.

Hepatic Blood Sampling: Blood sampling was performed while rats were under anesthesia. Blood was drawn into chilled heparinized syringes, and centrifuged for 15 minutes at 4,000 x g at room temperature and plasma was removed and stored at -20^{o}C until analysis. Samples were drawn from the hepatic vein, the hepatic portal vein and the abdominal aorta. The hepatic vein was accessed via cannulation of the right jugular vein with PE 50 tubing. The position of the catheter tip was visually confirmed before and after blood was withdrawn. Hepatic uptake and output was calculated from the concentrations in these three vessels, by assuming that the portal vein accounted for 75% of hepatic blood flow and the hepatic artery accounted for 25% (135).

Creatine, GAA, SAM and SAH measurement: Creatine was assayed enzymatically and by HPLC. The enzymatic method was performed as previously described (107) except that the assay was scaled down for use in 96 well microplates. The absorbance was measured in a Molecular Devices SpectraMAX 190 spectrophotometer, using Softmax Pro software, version 3.1.1. The creatine concentration was determined by reference to a standard curve. Both GAA and creatine were assayed by the HPLC method of Buchberger and Ferdig (29). GAA and creatine were derivatized with ninhydrin and the

product was separated on a C18 reverse phase YMC column, (3 μm particle size and 12 nm pore size, Waters) employing a Waters 600E solvent delivery system and a Waters 717 autosampler. The derivatized products were detected by means of a Shimadzu RF-535 fluorescence detector using an excitation wavelength at 390 nm and an emission wavelength at 470 nm. SAM and SAH were assayed by HPLC using a previously described method (80). SAM and SAH were extracted by adding 3 volumes of 8% TCA to cell pellets, which was then centrifuged at 12,000 x g for 10 min at room temperature; supernatants were filtered through a 0.45 μm nylon membrane and injected onto the column.

GAMT and AGAT Assays: GAMT was assayed using a modification of the previously described method of Ogawa et al. (126). Fresh rat livers were homogenized in 4 volumes of a buffer containing 0.25 M sucrose, 10 mM HEPES and 1 mM EDTA. Liver homogenates were centrifuged at 100,000 x g at 4°C for 1 hr and the supernatants were assayed for GAMT activity. The assay contained 35 mM Tris buffer (pH 7.4), 7 mM 2-mercaptoethanol, 50 μM S-adenosylmethionine and 0.2 mM GAA. After a ten minute pre-incubation of all the constituents of the assay except GAA, the assay was started by the addition of GAA. The blanks did not contain GAA. Homogenates were incubated at 37°C and were stopped with 75 μL of 15% (w/v) TCA. This was followed by immediate neutralization with 72 μL of 1 M Tris pH 7.4. Precipitated protein was pelleted at 10,000 x g for 5 min at room temperature and creatine was assayed via the HPLC method described above. AGAT was assayed as previously described (164). Total protein was assayed using the Biuret method.

GC Mass Spectrometry: Approximately 500 μl of the neutralized PCA extract of

hepatocytes were added into vials containing: saturated aqueous sodium bicarbonate (50 μL); toluene (600 μL); and hexafluoroacetylacetone (50 μL). The mixture was incubated for 2 hour at 80°C under continuous stirring, then allowed to cool, centrifuged at 3,000 x g for 4 min, and approximately 500 μL of the upper toluene phase was transferred to another vial and dried under nitrogen flow. This solid was suspended in 50 μL of t-butyldimethylsilyl (t-BDMS) and incubated for 30 min at 60°C to form the t-BDMS derivatives. The m/z 344, 345, 346 and 347 ions of the guanidinoacetic acid t-BDMS derivative, and the m/z 360, 361, 362 and 363 ions of the creatine t-BDMS derivative were monitored. Sample preparation for ^{15}N-labeled urea and amino acids was carried out as described (25). GC-MS measurements of ^{15}N isotopic enrichment were performed on either a Hewlett Packard 5970 Mass Selective Detector (MSD) or a 5971 MSD, coupled with a 5890 HP-GC, GC-MS Agilent System (6890 GC- 5973 MSD) or Hewlett-Packard (HP-5970 MSD), using electron impact ionization with an ionizing voltage of -70 eV and an electron multiplier set to 2,000V.

Data and Statistical Analysis: The linearity of creatine and urea production by isolated hepatocytes was tested using linear regression. Creatine production by hepatocytes as a function of [GAA] was fitted to a curve using non-linear regression. Plasma values for GAA and creatine were analyzed using paired t-tests, pairing different plasma samples from the same animal. SAM and SAH data were analyzed using a two-way ANOVA with a Newman-Keuls post-test. All other data were analyzed using unpaired Students t-tests. All statistical analyses employed Prism Graph Pad software version 3.02.

2.4 Results

Creatine Production by Isolated Rat Hepatocytes

Creatine sysnthesis in hepatocytes was linear with time, for up to 90 minutes, and with tissue dry mass, up to 13 mg dry wt/mL of incubation medium. All subsequent studies were carried out within these linear ranges. The effect of GAA concentration on the rate of creatine synthesis was also examined (Figure 2.2). The data fitted well to a rectangular hyperbola and half-maximal GAA concentrations (13.3 μM) and maximal rates of creatine synthesis (2.1 nmol/hr/mg dry wt) were calculated. The addition of 0.5 mM methionine in these rats nearly doubled the maximal rate (3.6 nmol/hr/mg dry wt) without affecting the half-maximal concentration of GAA. The stimulation of creatine synthesis by methionine is likely due to increased provision of S-adenosylmethionine. Incubation of hepatocytes with methionine resulted in 2.9 and 2.6-fold increases in SAM over controls in the presence and absence of GAA, respectively (Table 2-1). In these same incubations SAH increased by 3.4-fold and 3.8-fold, respectively. The addition of methionine or GAA significantly lowered the ratio of SAM to SAH compared to the control; however incubations with both methionine and GAA did not result in a further lowering of this ratio.

Figure 2.2

Effect of [GAA] on creatine synthesis. Hepatocytes were incubated with varying [GAA], with (▲) or without (■) 0.5 mM methionine. Each flask contained an average of 6.5 mg dry mass of cells and was incubated at 37°C for 60 minutes. n=4. Data are expressed as mean ± S.D. and fit to a curve using non-linear regression. $P < 0.05$ taken as significant.

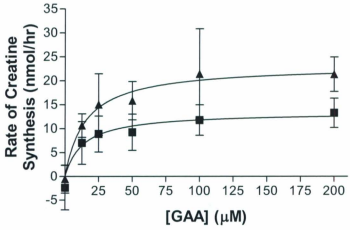

Rate of Creatine Synthesis (nmol/hr)
35
30
25
20
15
10
5
0
-5
25
50
75
100
125
150
175
200
[GAA] (μM)

Table 2-1

Effect of added methionine and GAA on hepatocyte SAM and SAH levels and on the SAM/SAH ratio.

	GAA	Control	Methionine
SAM	-	63.4 ± 13.1	167.9 ± 24.4*
SAH	-	10.2 ± 2.4	38.5 ± 3.7*
SAM/SAH	-	6.3 ± 1.1†	4.4 ± 0.8*
SAM	+	54.7 ± 6.7	156.5 ± 17.4*
SAH	+	12.6 ± 2.7	42.6 ± 5.4*
SAM/SAH	+	4.5 ± 0.8	3.7 ± 0.6

Hepatocytes were incubated for 60 min at 37°C and were incubated 0.5 mM methionine and 125 μM GAA. Data are expressed in nmoles/g wet mass of liver cells as the mean ± SD. $p<0.05$. $n=4$

* Indicates significant difference from the control. † Indicates significant difference from corresponding incubations with GAA.

Creatine Synthesis in the Intact Rat

We next examined the fluxes of GAA and of creatine across the liver in rats fed both creatine-free and creatine-supplemented diets (Figure 2.3). Creatine-supplementation had a marked effect on circulating GAA concentrations which were decreased by about 60% compared to the rats fed the creatine-free diets. There was no difference between the GAA concentrations of any of the vessels serving the liver in rats fed the creatine-supplemented diet. Hence, in these animals there was no significant hepatic uptake or output of GAA (Figure 2.3A). In contrast, there was a significant hepatic uptake of GAA in the rats fed the creatine-free diet (Figure 2.3B). The net concentration difference across the liver, around 3.7 μM, was quite appreciable, amounting to more than 70% of the GAA available in the portal vein and hepatic artery.

We also measured creatine levels in the plasma samples (Figure 2.4). Circulating creatine levels were about four-fold higher in the creatine-supplemented rats than in those fed creatine-free diet. There was no difference between the creatine levels in any of the three vessels in the rats fed either the creatine-free or creatine-supplemented diet (Figure 2.4).

Figure 2.3

Differences in plasma GAA concentration across the liver of rats fed a diet supplemented with 0.4% creatine A, and rats fed a diet free of creatine B. Plasma values are represented as follows: arterial (clear), portal vein (light grey), hepatic vein (dark grey), concentration difference across liver (hatched). Different letters indicate a statistically significant difference ($p<0.05$) between the different blood vessels. The asterisk denotes a significant difference ($p<0.05$) between the vascular inflow and outflow values. The hepatic inflow was calculated as described in the text. Data are expressed as mean ± S.D. n=6.

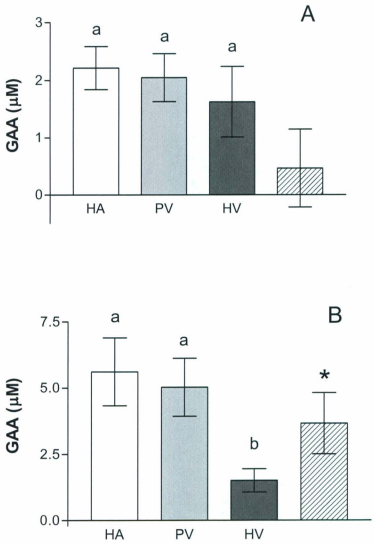
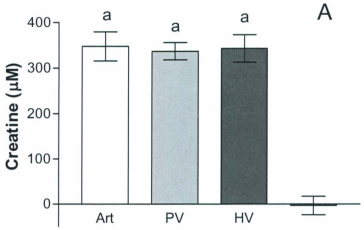
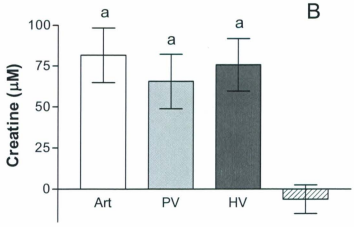

A
GAA (µM)
3
2
1
0
a
a
a
HA
PV
HV
B
GAA (µM)
7.5
5.0
2.5
0.0
a
a
b
*
HA
PV
HV

Figure 2.4

Differences in plasma creatine concentration across the liver of rats fed a diet supplemented with 0.4% creatine A, and rats fed a diet free of creatine B. Plasma values are represented as follows: arterial (clear), portal vein (light grey), hepatic vein (dark grey), concentration difference across liver (hatched). There was no significant difference ($p>0.05$) between the creatine concentrations in the different vessels. The data are expressed as mean ± S.D. n=6.


A
a
a
a
400
300
200
100
0
Creatine (µM)
Art
PV
HV


B
a
a
a
100
75
50
25
0
Creatine (µM)
Art
PV
HV

Enzyme Activities for the Enzymes of Creatine Synthesis

The activities of the enzymes involved in creatine synthesis were measured in rats that had been fed creatine-supplemented and creatine-free diets (Figure 2.5). Renal AGAT activity was depressed by 84% in rats fed a diet supplemented with creatine. Hepatic GAMT activity did not differ between the rats fed creatine-free or creatine-supplemented diets. There was no difference in the rate of creatine synthesis from GAA between hepatocytes isolated from rats fed a creatine-free or creatine-supplemented diet.

Stable Isotope Analysis of Creatine Synthesis in Rat Hepatocytes

Hepatocytes incubated with ^{15}N-ammonium chloride as well as methionine and glycine, but without GAA, produced urea at a linear rate, under all substrate conditions. Incubations were performed both in the presence and absence of ornithine as it has been shown to inhibit the activity of AGAT (173). We were unable to detect creatine in these incubations using the enzymatic method and we were only able to detect trace amounts of creatine, which were present at time zero, using the HPLC method. However, there was no increase in creatine after incubation. Isotopic analysis showed significant enrichment of both M+1 and M+2 isotopomers of urea; however, only trace enrichment was found in creatine (Table 2-2).

Figure 2.5

Enzyme activities of AGAT and GAMT, and creatine production by hepatocytes isolated from rats fed different diets. Renal AGAT activity, A; hepatic GAMT activity, B; and creatine synthesis in hepatocytes, C, the clear bars represent rats fed the creatine-free diets and the shaded bars represent rats fed the creatine-supplemented diets. Hepatocytes were incubated with 0.5 mM methionine and 125 μM GAA at 37^{o}C for 60 min. All values are expressed as mean ± S.D. The asterisk indicates statistical significance $p<0.05$. n=4.

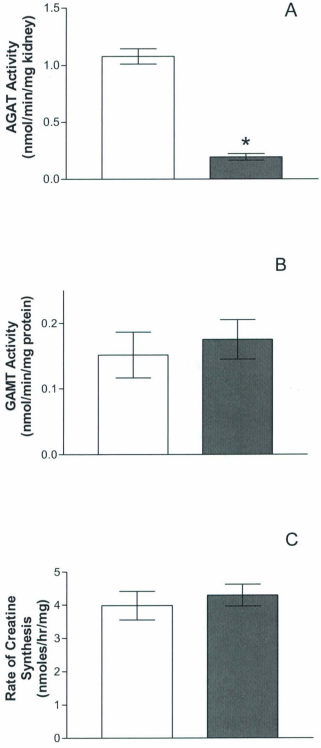

A
AGAT Activity
(nmol/min/mg kidney)
1.5
1.0
0.5
0.0
*
B
GAMT Activity
(nmol/min/mg protein)
0.2
0.1
0.0
C
Rate of Creatine
Synthesis
(nmoles/hr/mg)
5
4
3
2
1
0

Table 2-2

Isotopic enrichment of urea and creatine in hepatocyte incubations with ^{15}N-ammonium chloride.

	Urea (M+1)	Urea (M+2)	Creatine (M+1)	Creatine (M+2)
- ornithine	43.0 ± 10.6	55.3 ± 6.1	2.9 ± 3.5	4.1 ± 4.7
+ ornithine	38.3 ± 7.9	61.3 ± 6.0	1.9 ± 1.8	5.9 ± 5.9

Incubations included 5 mg dry mass hepatocytes for 30 minutes at 37°C. Hepatocytes were incubated with 1 mM ^{15}N-ammonium chloride, 2 mM pyruvate, 0.5 mM methionine, 1 mM glycine and 1 mM ornithine where indicated. Values are expressed as atom percent excess. n=4

2.5 Discussion

We have clearly demonstrated that rat hepatocytes can convert GAA to creatine. This is consistent with our previous finding that isolated hepatocytes released 50% more homocysteine when incubated with methionine and GAA compared to cells incubated with methionine alone (152). The average maximal rate for creatine synthesis of 3.08 $nmol \cdot min^{-1} \cdot mg\ dry\ wt^{-1}$ in hepatocytes found in this study corresponds well with the increase in the rate of homocysteine export reported by Stead et al. (152). Our data agree with the notion of methylation demand suggested by Stead et al. (152), i.e. that methylation is sensitive to increases in substrate concentration. In this regard it should be noted that Stead et al. (152) found that feeding GAA to rats increased plasma homocysteine, while feeding creatine reduced plasma homocysteine.

How well can the synthesis of creatine by isolated hepatocytes account for creatine synthesis *in vivo*? The liver of a 250 g rat weighs about 10 g, of which 70% is water. Using our maximal rates of creatine synthesis by isolated hepatocytes (3.08 $nmol \cdot min^{-1} \cdot mg\ dry\ wt^{-1}$) we can calculate a maximum *in vivo* rate of hepatic creatine synthesis of 221 $\mu moles \cdot 24\ h^{-1} \cdot 250\ g\ rat^{-1}$. However, a more physiological estimate requires that we take into account the substrate levels that occur *in vivo*. The K_m of GAMT for SAM has been reported to be 49 μM (37) and hepatic SAM levels are almost 200 nmoles/g (9), therefore, assuming that SAM is uniformly distributed in the different cell compartments, it is likely that *in vivo* SAM levels are not normally limiting. However, *in vivo* levels of GAA are certain to be a limiting factor. It is therefore necessary to take into account the [GAA] that is presented to the liver (about 5 μM) and the [GAA] required for half-maximal rates of creatine synthesis by isolated hepatocytes (about 13.3 μM). Using these

data we can predict an *in vivo* rate of hepatic creatine synthesis of about 60 μmol/24 h/250 g rat. We can compare this to the rate of hepatic GAA uptake *in vivo*, by multiplying our [GAA] difference across the liver (3.7 μM) by the hepatic venous plasma flow rate (4.32 mL/min/100 g body wt) reported by Welbourne et al. (183). Such a calculation yields a rate of hepatic GAA uptake of 57 μmoles/24 h/250 g rat. The close agreement between these two estimates reflects the physiological relevance of our *in vitro* experiments. We may also compare these data to the rate of urinary creatinine excretion from rats on creatine-free diets, about 55 μmoles/24 hr/250 g rat (data not shown). It is probably somewhat fortuitous that these numbers are in such close agreement. Nevertheless, allowing for the assumptions required for these calculations, it is evident that the liver is the major producer of creatine in the rat and that our *in vitro* studies with hepatocytes are a good reflection of the situation *in vivo*. We do not, however, imply that the liver is the sole site of creatine synthesis. Both AGAT and GAMT occur in tissues other than kidney and liver. In particular, it is known that the brain can synthesize creatine (186). The CNS may even be considered as autonomous in creatine synthesis as there is evidence that the blood brain barrier is poorly permeable to creatine (22).

Our results also show that rat liver *in vivo* can clear over half of the GAA presented to it; to our knowledge this is the first time this has been shown. Creatine output by livers of animals fed creatine-free diets *in vivo* did not attain statistical significance. However, given that plasma creatine in these animals is about 70 μM, the production of an additional 3.7 μM by the liver would be too small for us to reliably demonstrate. However the clear uptake of GAA by the liver *in vivo* and the demonstration of creatine synthesis from GAA by isolated hepatocytes indicates that the liver produces creatine *in*

vivo and thus would require a means by which creatine exits the liver. Creatine transporters have been described in muscle (121), brain, kidney and intestine (24). These are Na^+ - and Cl^--dependent active transporters which concentrate creatine intracellularly. Intracellular to extracellular creatine gradients of greater than 100 to 1 are known in skeletal muscle (24). However such transporters seem inappropriate for the liver. We have found creatine levels to be approximately 0.25 μmole$\cdot g^{-1}$ in freeze-clamped rat liver (data not shown). Therefore the concentration gradient in liver is outward. We suggest that hepatocytes may express a novel creatine transporter as well as a GAA transporter. These issues have not yet been explored.

With respect to the question of whether the entire pathway of creatine synthesis occurs in rat hepatocytes, we must conclude that it does not. Using a highly sensitive HPLC method (limits of detection, 25 nM), we were unable to detect creatine production by isolated hepatocytes incubated with pyruvate (as a source of energy as well as a source of oxaloacetate for aspartate production) and ^{15}N-ammonium chloride, methionine and glycine. This was confirmed by the absence of significant isotopic enrichment of creatine from ^{15}N-ammonium. In these same incubations, there was synthesis of urea, together with substantial labeling of urea. A significant flux of arginine derived from the urea cycle into the creatine synthetic pathway would yield similarly labeled creatine. Thus we conclude that the entire creatine synthetic pathway does not occur in rat hepatocytes. We caution however that this may be a species-specific observation and that in other mammals the liver may contain functional AGAT activity and thus the entire creatine synthetic pathway. There is considerable variability in the tissue distribution of AGAT and GAMT activities among different species (162). As discussed in the introduction,

renal production of GAA in humans is only 20% of creatinine loss and thus humans are likely candidates to have the entire creatine synthetic pathway in the liver. To our knowledge, there has been only one study by Sandberg et al. (139) that reported GAA uptake by human liver. However, we do not consider this result to be reliable because the reported plasma [GAA], of about 20 μM, is some 4 to 5-fold than reported by modern techniques (49, 145, 156). This discrepancy is likely due to the use of the older Sakaguchi colorimetric method which is not specific for GAA; in particular, arginine interferes in this assay.

Finally, our results shed light on the regulation of creatine synthesis in the rat. The feeding of creatine decreased neither the hepatic GAMT activity nor the capacity for creatine synthesis by isolated hepatocytes. This contrasts vividly with the massive down-regulation of renal AGAT in such animals (49, 113). We also confirmed the finding of Edison et al. (49) that creatine feeding markedly reduces circulating GAA levels, from about 5 μM to about 2 μM. Since the [GAA] for half-maximal rates of creatine synthesis in hepatocytes is about 13 μM, hepatic creatine synthesis should be almost directly proportional to circulating [GAA]. This, in turn, indicates that creatine synthesis in rats is primarily regulated in the kidney rather than the liver.

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Disclosures

None of the authors have a conflict of interest with regard to this study.

Chapter 3

Rat Pancreatic Acini Synthesize Guanidinoacetate and Creatine from Amino Acids

Rat Pancreatic Acini Synthesize Guanidinoacetate and Creatine from Amino Acids

Robin P. da Silva, Kathy Clow, John T. Brosnan[1], Margaret E. Brosnan

Department of Biochemistry, Memorial University of Newfoundland, St. John's NL, A1B3X9

RUNNING TITLE: Creatine synthesis in the Pancreas

WORD COUNT: 3087, Number of Figures: 3, Number of Tables 3.

AUTHOR LISTING FOR INDEXING: da Silva, Clow, Brosnan, Brosnan

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[2] Author Disclosure: RP da Silva, K Clow, ME Brosnan and JT Brosnan have no conflicts of interest to disclose.

[3] Abbreviations used: AGAT, arginine:glycine amidinotransferase; GAMT, guanidinoacetate methyltransferase; GAA, guanidinoacetate; CRT1, sodium-dependent creatine transporter; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; AIN-

93G; American Institute of Nutrition diet 93 for growth and lactation; ANOVA, analysis of variance.

3.1 Abstract

Creatine is an important molecule involved in cellular energy metabolism. Creatine is spontaneously converted to creatinine at a rate of 1.7% per day; creatinine is lost in the urine. Creatine can be consumed in the diet or synthesized from endogenous amino acids through the action of the enzymes arginine:glycine amidinotransferase (AGAT) and guanidinoacetate N-methyltransferase (GAMT). The immediate precursor of creatine is guanidinoacetatic acid (GAA) which is synthesized from arginine and glycine. The liver contains the GAMT enzyme and the kidney contains AGAT activity. Although the pancreas contains both AGAT and GAMT, its role in creatine synthesis has not been fully characterized. We examined the enzymes of creatine synthesis in the pancreas as well as the synthesis of GAA and creatine by isolated pancreatic acini. We also examined whether these pancreatic enzymes were regulated by dietary creatine. We found that the pancreatic acini have activities of both AGAT and GAMT and have the capacity to synthesize guanidinoacetate and a small amount of creatine. Finally we found that dietary creatine supplementation led to a decrease in AGAT activity in the pancreas though it did not affect its mRNA or protein abundance. This contrasts with the reduction of AGAT enzyme activity, mRNA and protein abundance in the kidney suggesting that the regulatory mechanisms that control the expression of this enzyme in the pancreas are different from those in the kidney.

3.2 Introduction

Creatine is a key molecule for the maintenance of cellular ATP in cells such as those in muscle and brain which contain high activities of creatine kinase. Creatine and creatine phosphate together with the enzyme creatine kinase act as a buffer for ATP in cells. In addition, creatine phosphate is a carrier of "high energy" phosphate between sites of ATP synthesis and utilization (188). Creatine and creatine phosphate are spontaneously converted to creatinine at a rate of about 1.7 % per day and this is lost in the urine (186). Therefore the maintenance of constant levels of creatine in the body means that this quantity of creatine must be replaced. Creatine can be obtained in the diet by ingestion of meat and dairy products and through endogenous synthesis from amino acids.

Creatine is synthesized *de novo* from the metabolism of the amino acids arginine, glycine and methionine. Two enzymes are involved in its synthesis; arginine:glycine amidinotransferase (AGAT) and guanidinoacetate N-methyltransferase (GAMT). AGAT catalyzes the transfer of the amidino group of arginine onto the nitrogen of glycine, to give ornithine and guanidinoacetate (GAA). GAMT is then responsible for the transfer of a methyl group from S-adenosylmethionine (SAM) onto the alpha amine of GAA which gives creatine. In vertebrates the highest activities of GAMT are found in the liver, pancreas and brain while the highest activities of AGAT have been measured in the kidney, pancreas and brain (162).

We have recently shown that the rat kidney can produce GAA in sufficient quantities to replace creatinine lost in the urine (49). In addition, we have shown that the liver has a maximum capacity to methylate GAA that is far greater than that required to

replace the daily loss of creatine (39). However, the actual hepatic methylation of GAA is limited by the delivery of GAA. What then is the function of the creatine synthetic enzymes in the other tissues, such as brain and pancreas? Creatine synthesis in the brain has been thoroughly examined by Braissant and Henry (22) who suggest that the adult rat brain may be able to produce enough creatine to satisfy its own requirements. Although the activities of the enzymes responsible for creatine synthesis have been reported to be quite high in the pancreas, the role of this organ in creatine synthesis is not yet clear.

In the present study we ask whether the pancreas is capable of synthesizing creatine from amino acids. Sorensen et al. (150) have found that the catalytic activity and immunoreactivity of AGAT is primarily found in the pancreatic acini rather than the islet cells. Therefore we use isolated pancreatic acini to examine the effects of various substrates on the synthesis of GAA and creatine. Also, since there is a known down-regulation of AGAT in the kidney by dietary creatine supplementation, we examine whether creatine exerts a regulatory action on the enzymes of creatine synthesis in the pancreas.

3.3 Materials and Methods

Animals: Male Sprague-Dawley rats, between 250 and 350 grams body weight, were used for the experiments. All animals were fed *ad libitum* and kept on a 12:12 hr light:dark cycle in a climate-controlled room. Control animals were fed the AIN-93G diet. For the creatine supplementation study the experimental group was fed an AIN-93G diet that contained 0.4% creatine, by weight, in place of an equivalent amount of corn starch. Both diet groups were fed for a period of 14 days. Surguries were performed in the morning when the rats were still in the absorptive state. All procedures were approved by the Memorial University of Newfoundland Institutional Animal Care Committee and were in accordance with the Guidelines of the Canadian Council of Animal Care.

Tissue Collection: Rats were anesthetized with sodium pentobarbital, i.p., at a dose of 65 mg/kg body weight. The pancreas was removed for the isolation of acini, and measurement of GAMT activity as described in detail below. The kidneys and a portion of the pancreas were freeze-clamped in aluminum tongs pre-cooled in liquid nitrogen and stored at -80°C for analysis of metabolites, mRNA and AGAT activity.

Isolation of Pancreatic Acini: Pancreatic acini were isolated according to a modification of a previously published method (18). Immediately after removal from the rat, the pancreas was placed in a Petri dish where any visible adipose tissue was dissected and separated from the pancreatic tissue. The pancreas was then injected with 5 mL of Solution A; Krebs-Henseleit solution containing 1.3 mM calcium chloride, 10 mM glucose, 2.1 mM lactate, 0.15 mM pyruvate, 0.2% w/v fatty acid free bovine serum albumin (BSA) and 135 U/mL collagenase (type 4). It was then placed in a 50 mL Erlenmeyer flask, together with 25 mL of Solution A. The atmosphere in the flask was

replaced with 95% O_2/5% CO_2 by gassing for 30 seconds and the flask was stoppered and placed in a shaking water bath at 37° C for 15 minutes. A continuous stream of 95% O_2/5% CO_2 gas was fed through the stopper throughout the isolation procedure. After 15 minutes, the collagenase (Type 4) solution was removed, fresh Solution A (without collagenase) was added and the flask shaken for another 15 mintues. This wash was repeated once more. Solution A was then removed and 10 mL of solution B was added to the flask. Solution B had the same composition as Solution A but with 4% BSA and without collagenase. The pancreas was gently teased apart by repetitive pipetting through plastic pipettes with decreasing bore sizes. The suspension was passed through a 250 μm filter and centrifuged at 200 x g for 2 minutes. The supernatant was removed and the acini were resuspended in fresh solution B. This wash was repeated three times. Finally the acini were re-suspended in Krebs-Henseleit medium containing 0.2% BSA. Viability was assessed by means of trypan blue exclusion. Because acini are isolated in their multi-cell ultrastructure quantitative determination of viability was impossible. Therefore a qualitative estimation of viability was performed for each isolation procedure.

Incubations were initiated by the addition of aliquots of the pancreatic acini suspension into pre-warmed (37°C) 25 mL Elenmyer flasks containing Krebs-Henseleit medium and substrates. The substrates were prepared in Krebs-Henseleit medium. The atmosphere inside each Erlenmeyer flask was replaced with 95% O_2/5% CO_2 by gassing for 20 seconds and sealing with a rubber stopper. Incubations were carried out in triplicate. Incubations were stopped by the addition of 100 μL of ice-cold 30% (w/v) perchloric acid. Precipitated protein was pelleted at 10,000 x g for 10 minutes at room

temperature. 700 μL of the supernatants were removed and neutralized with 20 μL of universal indicator, 40 μL of 50% K_2CO_3 and 20% KOH until the pH was between 6 and 8. After standing in ice for 15 minutes, precipitated salts were removed by centrifugation at 10,000 x g for 5 minutes at room temperature. Supernatants were then assayed for creatine as described below. Aliquots of cells that were not subjected to de-proteinization were assayed for GAMT and AGAT activity as described below.

Enzyme Activities: GAMT was assayed on fresh tissue according to a previously described method (39). AGAT was assayed on fresh or frozen tissue as previously described (164). Total protein for all tissue homogenates was assayed using the Biuret method.

Real-time PCR: Frozen pancreatic and kidney tissue (-80°C) were ground to a fine powder in a ceramic mortar and pestle pre-cooled with liquid N_2. Approximately 100 mg of powdered tissue was placed in a sterilized microcentrifuge tube containing 500 μL of ice-cold Trizol reagent and immediately homogenized with a hand-held electronic homogenizer. Total RNA was extracted using the standard Trizol extraction procedure provided by Invitrogen (Burlington, Ontario). RNA quality was assessed by separation on an agarose gel and also by measuring absorbance at 260 nm and 280 nm using a nano-drop spectrophotometer system (Thermo Fisher Limited, Ottawa, Ontario). Primers were designed using Primer3Plus online software and obtained from Integrated DNA Technologies (Coralville, Iowa). PCR was conducted using a Roche Lightcycler (Laval, Quebec) using Lightcycler3 software. The AGAT forward primer was "CTGTGCAGCTGAAGACAAGG" and the reverse primer was "CTGTGAATGGTGGGACACAG". GAMT forward primer was

"ACTCATGCTTTCCGTTTGCT" and reverse primer was "AGGCACCTGAGTCTCCTCAA". Target RNAs were normalized to 18S ribosomal RNA with forward primer "GTGATCCCCGAGAAGTTTCA" and reverse primer "CTGCTTTCCTCAACACCACA".

Creatine, GAA, SAM and SAH measurement: GAA and creatine were assayed by the HPLC method of Buchberger and Ferdig (29). HPLC was conducted on a Waters 600E solvent delivery system and a Waters 717 autosampler. SAM and SAH were assayed as described previously (80).

Western Blots: AGAT protein was detected using an affinity-purified anti-AGAT rabbit polyclonal antibody raised against the sequence RPDPIDWSLKYKTPDFE, amino acids 142-159 of rat AGAT (accession number P50442-1), (Open Biosystems Huntsville, Alabama). The secondary antibody was a horseradish peroxidase–conjugated anti-rabbit IgG (Bio-Rad Mississauga, Ontario), visualized using the Immunstar chemiluminescence system (Bio-Rad Mississauga, Ontario). Band detection was performed using an enhanced luminol system (Immunn-Star, Bio-Rad inc.) and analysed with an Alpha Innotech Chemilmager 4400 (Alpha Innotech, San Leandro, California, USA).

Reagents: All reagents were purchased from Sigma Aldrich with the exception of collagenase, from Worthington (Lakewood, New Jersey), and perchloric acid, from Fisher Scientific (Ottawa, Ontario). All reagents used for PCR were purchased from Invitrogen (Burlington, Ontario).

Statistical Analysis: Statistical analyses were performed on Prism Graph Pad software version 3.02. Data were compared using unpaired Students t-tests with the exception of the data from the isolated pancreatic acini incubations which were analyzed by one-way

ANOVA. P-values less than 0.05 were taken as significant.

3.4 Results

Average pancreas weights were 1.38 ± 0.21 g and 1.47 ± 0.12 g for the control and creatine-supplemented groups, respectively; these were not significantly different from each other.

Rates of GAA production by pancreatic acini isolated from rats fed a creatine-free diet are reported in Table 3-1. Pancreatic acini produced significant quantities of GAA in the presence of methionine, glycine and arginine. In the presence of high concentrations of arginine and glycine (2 mM each), GAA production in pancreatic acini was approximately 8 fold higher than in the presence of near physiological concentrations of these amino acids (0.2 mM each). Pancreatic acini were capable of synthesizing creatine under all substrate conditions, although, the rates were much lower than the rates of GAA synthesis. This was consistent with the activities of AGAT and GAMT measured in the isolated acini; the activity of GAMT was only 2.3% of the AGAT activity. The capacity for GAA production in pancreatic acini therefore greatly outstrips the capacity for GAA methylation (creatine production).

We examined the pancreatic content of key metabolites, as well as activities, mRNA and protein abundance of key enzymes required for creatine synthesis in pancreas from rats fed creatine-free or creatine-supplemented diets. The concentrations of selected metabolites are shown in Table 3-2. Pancreatic content of GAA, creatine and creatine phosphate was 5-fold, 3-fold and 2-fold higher, respectively, in the creatine-supplemented animals than in the controls. The elevated concentrations of creatine

Table 3-1

Activities of AGAT and GAMT in, and rates of GAA and creatine accumulation by, isolated pancreatic acini.

Incubation Conditions / GAA and Creatine Production	Zero Time	5 μM GAA 0.5 mM Methionine	100 μM GAA 0.5 mM Methionine	0.2 mM Arginine 0.2 mM Glycine 0.5 mM Methionine	2 mM Arginine 2 mM Glycine 0.5 mM Methionine
GAA	0.39 ± 0.14	-	-	3.07 ± 0.49*	21.65 ± 9.01†
Creatine	0.29 ± 0.12	0.95 ± 0.61*	1.14 ± 0.82*	1.64 ± 1.45*	1.85 ± 1.46*
AGAT Activity	3.41 ± 1.36 nmole/min/ mg protein				
GAMT Activity	0.08 ± 0.06 nmole/min/mg protein				

GAA and creatine were measured at zero time and after 40 minute incubations in pancreatic acini isolated from rats fed creatine-free diets. GAA and creatine accumulation are given in nmoles per mg protein per 40 minutes and expressed as means ± standard deviations. An asterisk indicates a significant difference from the zero time incubations. † indicates a significant difference from all other incubation conditions. AGAT and GAMT were assayed in isolated acini as described in the methods section (n=6)

Table 3-2

Metabolite concentrations in the pancreas of rats fed creatine-free and creatine-supplemented diets.

Diet / Metabolite	Creatine-free Diet	0.4% Creatine Diet
GAA (μmoles/g wet tissue)	0.05 ± 0.04	0.22 ± 0.06*
Creatine (μmoles/g wet tissue)	0.16 ± 0.05	0.44 ± 0.12*
Creatine Phosphate (μmoles/g wet tissue)	0.20 ± 0.08	0.37 ± 0.10*
ATP (μmole/g wet tissue)	0.98 ± 0.33	1.07 ± 0.32
SAM (nmoles/g wet tissue)	26.57 ± 2.84	17.40 ± 2.77*
SAH (nmoles/g wet tissue)	1.06 ± 0.39	0.84 ± 0.04
SAM/SAH	27.2 ± 7.5	20.7 ± 3.7

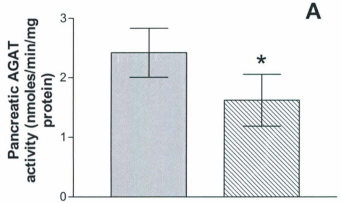
Data are presented as means ± standard deviations. An asterisk indicates a significant difference between diet groups, (n=5).

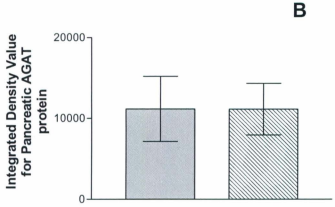
phosphate in the creatine-supplemented animals are probably a consequence of the increased pancreatic creatine levels. The concentration of SAM in the pancreas of creatine-supplemented rats was only 65% of the level observed in the control animals. The level of SAH as well as the SAM-to-SAH ratio in the pancreas were not statistically different between diet treatments.

The enzyme activity and relative mRNA expression for AGAT and GAMT in the pancreas are given in Figure 3.1 along with the protein abundance of AGAT. Creatine supplementation reduced pancreatic AGAT activity by 34% while the protein and mRNA were unchanged. Pancreatic GAMT activity and mRNA were not affected by dietary creatine. We were unable to measure GAMT protein by western blot due to the lack of a suitable antibody. For comparison, renal AGAT activity, protein and mRNA abundance are presented in Figure 3.2. There was a much greater effect of dietary creatine on renal AGAT activity which was reduced by 83% in rats fed the creatine-supplemented diet. Relative expression of AGAT mRNA and protein in the kidneys of creatine-supplemented animals was also reduced by 47% and 60%. The failure of pancreatic AGAT mRNA to be decreased by dietary creatine supplementation suggests that AGAT in pancreas is not regulated at the pre-translational level as is the case with renal AGAT.

Figure 3.1

The effect of dietary creatine on pancreatic AGAT and GAMT. AGAT enzyme activity (A), integrated density values from AGAT Western blot (B), relative expression of AGAT mRNA in pancreas (C), GAMT enzyme activity (D), and relative expression of GAMT mRNA in pancreas (E). Creatine-free diets (grey bars) and creatine-supplemented diets (hatched bars). Data for mRNA target genes were normalized to 18S ribosomal RNA. Values are given as means ± standard deviations. An asterisk signifies statistical significance (n=5).


A
Pancreatic AGAT activity (nmoles/min/mg protein)
3
2
1
0
*


B
Integrated Density Value for Pancreatic AGAT protein
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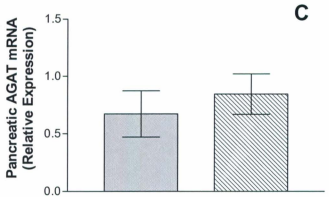
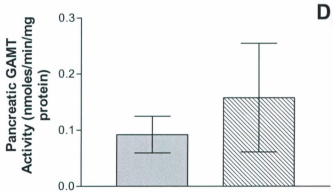
10000
0


C
Pancreatic AGAT mRNA (Relative Expression)
1.5
1.0
0.5
0.0


D
Pancreatic GAMT Activity (nmoles/min/mg protein)
0.3
0.2
0.1
0.0

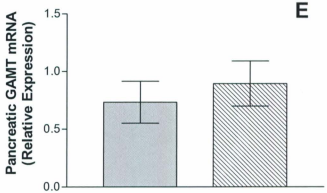
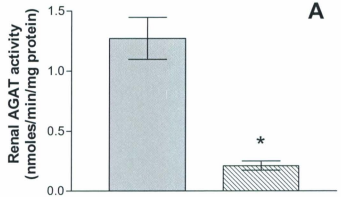
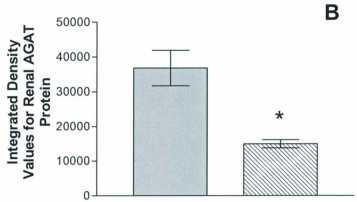
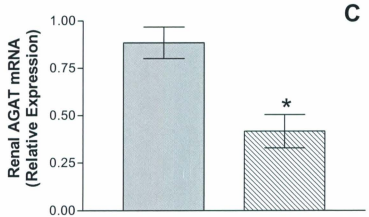

E
Pancreatic GAMT mRNA (Relative Expression)
1.5
1.0
0.5
0.0

Figure 3.2

The effect of dietary creatine on renal AGAT. Enzyme activity (A), integrated density values from AGAT Western blot (B) and relative expression of AGAT mRNA in kidney (C). Creatine-free diets (grey bars) and creatine-supplemented diets (hatched bars). Data for mRNA target genes were normalized to 18S ribosomal RNA. Values are given as means ± standard deviations. An asterisk signifies statistical significance (n=5).


A
Renal AGAT activity
(nmoles/min/mg protein)
1.5
1.0
0.5
0.0
*


B
Integrated Density
Values for Renal AGAT
Protein
50000
40000
30000
20000
10000
0
*


C
Renal AGAT mRNA
(Relative Expression)
1.00
0.75
0.50
0.25
0.00
*

3.5 Discussion

Significant AGAT activity and GAA production were observed in pancreatic acini. This agrees with the immunohistochemical localization of AGAT protein exclusively in the pancreatic acini (150) and the determination of AGAT enzyme activity (163) in the pancreas. AGAT has high K_m values for arginine (~1-3 mM) and glycine (~2-3 mM) (186). This is consistent with the increase in GAA production when the concentrations of these amino acids are increased from 0.2 mM to 2 mM. The sensitivity to arginine and glycine levels suggests that the transport of these substrates into pancreatic acinar cells is not limiting to the synthesis of GAA in the pancreas and hence that plasma substrate concentration influences GAA synthesis. However, creatine production in pancreatic acini was not different among any of the various substrate concentrations. The low activity of GAMT relative to the AGAT activity may account for this observation. Thus, from the rates of GAA and creatine production and the enzyme activities measured in pancreatic acini, we must conclude that the capacity for GAA synthesis in the pancreas is much higher than the capacity for creatine synthesis.

In order to gain a better insight into the potential impact that the pancreas might have on total body creatine synthesis in the rat, we have made some calculations of pancreatic creatine and GAA production. We calculate a rate of creatine synthesis in the presence of 0.2 mM arginine, 0.2 mM glycine and 0.5 mM methionine of 4.4 μmoles of creatine/day per 250 g rat. This compares with the measured loss of creatinine of 55 μmoles/day/250 gram rat which is equivalent to the daily renal GAA production (49). Thus the pancreas could produce as much as 8 % of the creatine that is lost per day. A similar calculation of GAA production yields a rate of pancreatic GAA production of 8.3

μmoles of GAA/day/250 gram rat (15 % renal GAA production). Given that pancreatic venous blood drains into the hepatic portal vein and flows through the liver, it is possible that the pancreas releases GAA into the plasma to be used for creatine synthesis in the liver.

Dietary creatine significantly elevated creatine and creatine phosphate levels in the pancreas. The increase in creatine phosphate probably reflects the action of the reversible creatine kinase enzyme. Although the function of creatine in the pancreas is not fully understood, there is some suggestion that the creatine kinase system may have an effect on ATP levels in the pancreatic islets and thus the secretion of hormones from the endocrine pancreas (175). A substantial creatine kinase activity has been found in islets isolated from mouse pancreas (128) and phosphocreatine has been shown to increase response of the K-ATP channels in rat pancreatic islets (96). K-ATP channels are integral to the mechanism of release of insulin from the β-cells of the pancreas. A recent study by Rocić et al. (136) supports the role of the creatine kinase system in β-cells, showing that the provision of creatine in the presence of various concentrations of glucose significantly increased ATP levels. In addition, Rocić et al. showed that insulin secretion was increased by the provision of creatine, independent of the presence of added glucose (136) which suggests that changes in pancreatic creatine levels alone may impact insulin secretion in the β-cell. Marco et al. (110) have shown that creatine, GAA and arginine (at quite high concentrations) can significantly increase the secretion of glucagon and insulin in isolated pancreatic mouse islets. The mechanism through which guanidino compounds affect hormone release from pancreatic islets remains to be elucidated. However, there is good evidence from studies using inside-out patch clamps

of mouse pancreatic β-cells that the addition of PCr prevented the opening of KATP channels similar to the effect of added ATP suggesting that PCr is involved in the regulation of insulin secretion through modulation of ATP and ADP levels (146).

An interesting aspect of creatine metabolism in the pancreas is that it appears GAA is synthesized exclusively in the acini (exocrine pancreas) (150), while the known pancreatic functions of creatine are located in the islets (endocrine pancreas) (110, 128). It is tempting to speculate that the acinar synthesis of GAA and creatine may function to provide islets with creatine, rather than supplying the rest of the body. It should be noted that creatine is synthesized by some cell types in the brain and concentrated in others (21). This might similarly be the case among different cell populations in the pancreas.

We observed a high ratio of SAM-to-SAH in the pancreas indicating that there is a high methylation potential in this tissue. The pancreatic SAM-to-SAH ratio is 6-fold higher than that reported by us for the liver (79). There is also evidence that the pancreas has a very active methionine cycle. Recently Wilson et al. (184) have shown, in a study using metabolic tracers, that the pancreas has the highest production of homocysteine from methionine per gram among a comprehensive list of tissues. Creatine supplementation appeared to have an effect on pancreatic methylation potential as SAM levels were significantly lower in the pancreas of creatine-supplemented rats. Since the activities of the methyltransferases are increased in the presence of higher SAM (37) these data could suggest that dietary creatine supplementation may have an impact on methylation in the pancreas.

The effect of dietary creatine on AGAT expression and activity appears to be different in the pancreas than in the kidney. We found that dietary creatine reduces the

activity of AGAT in the pancreas by about 34%. In addition, we found that AGAT mRNA and protein expression in the pancreas were unchanged by dietary creatine supplementation. This is quite different from what we observed in the kidney. Renal AGAT activity, protein and mRNA expression were all significantly reduced in the presence of dietary creatine supplementation. This confirms the finding of McGuire et al. (113) who have shown that both renal AGAT activity and mRNA expression are significantly reduced by 74% and 63%, respectively, in the presence of dietary creatine supplementation. Our data indicate that the pre-translational mechanism of regulation that responds to dietary creatine in the kidney may not occur in the pancreas.

In summary, we show that pancreatic acini contain activities of both AGAT and GAMT enzymes and are capable of producing significant GAA as well as a small amount of creatine *de novo*. The capacity for GAA synthesis is greater than the capacity for creatine synthesis. In addition we show different effects of dietary creatine-supplementation on AGAT activity and expression in the pancreas and kidney which implies that the mechanisms of AGAT regulation present in the kidney may not be present in the pancreas.

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Chapter 4

Creatine supplementation prevents the accumulation of fat in the livers of rats fed a high-fat diet.

Creatine supplementation prevents the accumulation of fat in the livers of rats fed a high-fat diet.

Rafael Deminice[1,2], Robin P. da Silva[1], Simon G. Lamarre[1], Colin Brown[1], George N Furey[1], Shannon A McCarter[1], Alceu Afonso Jordao[2], Karen B Kelly[3], Krist King-Jones[3], René L. Jacobs[3], Margaret E. Brosnan[1], John T. Brosnan[1]*.

[1] Department of Biochemistry, Memorial University of Newfoundland, St. John's, Canada.

[2] Laboratory of Nutrition and Metabolism, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil;

[3] Department of Agricultural, Food and Nutritional Science and the Group on the Molecular and Cell Biology of Lipids, University of Alberta, Edmonton, Alberta T6G 2P5,

Canada,

Deminice R, da Silva RP, Lamarre SG, Brown C, Furey G, McCarter S, Jordao AA, Kelly K, King-Jones K, Jacobs RL, Brosnan ME, Brosnan JT*.

* Corresponding Author: John T. Brosnan, Department of Biochemistry, Memorial University of Newfoundland, St. John's, NL, CANADA A1B3X9.

Phone: 709-864-8540, FAX: 709-864-2422, e-mail: jbrosnan@mun.ca

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Number of figures: 3

Running title: creatine supplementation and fatty liver

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4.1 Abstract

The aim of the present study was to examine the effects of creatine supplementation on liver fat accumulation induced by a high-fat diet in rats. Rats were fed one of three different diets: control liquid diet, high-fat liquid diet, or high-fat liquid diet supplemented with creatine. The control and high-fat liquid diets contained, respectively, 35 and 71% of energy derived from fat. Creatine supplementation involved the addition of 1% (wt/vol) of creatine monohydrate to the liquid diet. The high-fat diet increased total liver fat concentration, liver triglycerides, liver TBARS and decreased the hepatic S-adenosylmethionine (SAM) concentration. Creatine supplementation normalized all of these perturbations. Creatine supplementation significantly decreased the renal activity of L-arginine:glycine amidinotransferase and plasma guanidinoacetate and prevented the decrease in hepatic SAM concentration seen in rats fed the high-fat diet. However there was no change in either the phosphatidylcholine/phosphatidylethanolamine ratio or phosphatidylethanolamine *N*-methyltransferase (PEMT) activity. The high-fat diet decreased mRNA for PPARα, as well as two of its targets, CPT1a and LCAD. Creatine supplementation normalized these mRNA levels. In conclusion, creatine supplementation prevented the fatty liver induced by feeding rats a high-fat diet probably by normalization of the expression of key genes of beta-oxidation.

Key-words: high-fat diet, fatty liver, creatine supplementation, PPARα, fatty acid oxidation.

4.2 Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases throughout the world (2). NAFLD is a clinical pathological state that develops in the absence of alcohol abuse and is characterized by the accumulation of triglyceride in hepatocytes. It can progress to nonalcoholic steatohepatitis (NASH) and in some cases, to fibrosis and cirrhosis (48). However, the mechanisms involved in nonalcoholic fatty liver disease are uncertain (48, 99). The two-hit model proposes that the first hit involves a simple accumulation of fat in the liver, increasing the susceptibility of liver to more severe damage by the second hit that involves mitochondrial dysfunction, lipid peroxidation and inflammation (41).

Fat accumulation and NASH progression have been associated with impairment of methionine metabolism in liver (91), resulting in decreased availability of S-adenosylmethionine (SAM) as well as an increase in homocysteine levels and oxidative stress (91, 92). In support of this mechanism, it is known that supplementation with betaine, an important methyl donor for the remethylation of homocysteine to methionine, may protect the liver from fat accumulation and lipid peroxidation. This has been observed in both rodent models of alcoholic (51) and non-alcoholic (99) fatty liver. Kwon et al. (99) suggested that the elevation of SAM may play a critical role in the protective effect of betaine.

Creatine occurs naturally in food, especially in meat and fish. Creatine, in the form of creatine monohydrate, is taken by many athletes for its ergogenic properties. In humans, approximately 1-2 g of creatine is required to replace that lost by irreversible conversion to creatinine. In human omnivores, half of this is provided in the diet while

the remainder is endogenously synthesized (24). The first step in creatine synthesis involves the reversible transfer of the amidino group of arginine to glycine to form guanidinoacetic acid (GAA) and ornithine in a reaction catalyzed by the enzyme arginine:glycine amidinotransferase (AGAT); this enzyme is very active in kidneys. Next, the irreversible transfer of a methyl group from SAM to GAA is catalyzed by the enzyme S-adenosylmethionine:guanidinoacetate *N*-methyltransferase (GAMT) which is most active in the liver. The products of this reaction are creatine and S-adenosylhomocysteine (SAH). Creatine synthesis is responsible for a considerable consumption of SAM in the liver (153), as much as 40% of the total body SAM requirement. Previous studies have shown that creatine supplementation down regulates renal AGAT activity (49) and therefore the endogenous formation of creatine. It also reduces homocysteine production (43, 152). Since both phosphatidylethanolamine *N*-methyltransferase (PEMT) and GAMT use the same hepatic SAM pool, creatine supplementation may increase SAM availability to phosphatidylcholine formation via PEMT, thus increasing VLDL secretion and diminishing fat accumulation in liver.

The aim of the present study was to examine the effects of creatine supplementation of rats fed a high-fat diet on liver fat accumulation, methionine metabolism and lipid peroxidation. Creatine supplementation does indeed prevent development of fatty liver in response to a high fat diet but by a mechanism other than increased SAM availability.

4.3 Material and Methods

Animals and treatment

Male Sprague-Dawley rats (initial weight ~120 g) were obtained from the Memorial University of Newfoundland Animal Care Unit. All procedures were approved by the Animal Care Committee of the same institution and were in accordance with the Guidelines of the Canadian Council on Animal Care. The rats were kept in individual cages on a 12/12 hour light/dark cycle at a mean temperature of 22ºC and were randomly assigned to 3 groups of 6 rats each: control (C); high-fat (HF); and high-fat with creatine (HFC). Group C was fed with a standard liquid diet with 35% of energy from fat, 18% from protein and 47% from carbohydrates (Dyets catalogue # 710027).

The high-fat groups received a high-fat liquid diet with 71% of energy derived from fat, 18% from protein and 11% from carbohydrates (Dyets catalogue # 712031). The diets were purchased from Dyets Inc (Bethlehem, PA, USA). The overall compositions of both the control and the HF diet, were identical to those described by Lieber et al. (106) Creatine supplementation was performed by adding 1% (wt/vol) creatine monohydrate to the high-fat liquid diet. The rats had free access to food throughout the 3 weeks. Food intake was measured daily to assess total energy, total fat and creatine consumption. Body weight was measured twice a week to determine the weight gain.

Tissue preparation

After the experimental period, the animals were anesthetized with an intra-peritoneal injection of sodium pentobarbital (65 mg/kg i.p.). The fed animals were sacrificed between 8 and 10 am. Blood was collected into heparinized tubes, centrifuged

and the plasma stored at -80ºC. A portion of the liver was freeze-clamped with aluminum tongs pre-cooled in liquid nitrogen, weighed and stored at -80ºC. A portion of fresh liver tissue was weighed and cut in small cubes of approximately 5 × 5 × 5 mm and embedded using the Optimal Cutting Temperature (OCT) Compound (Leica. Germany) for Oil-Red-O histopathologic evaluation. One kidney was removed and immediately homogenized in ice-cold 50 mmol/L potassium phosphate buffer (pH 7.4) with a Polytron (Brinkmann Instruments, Toronto, ON, Canada) for 25 s at 50% output. This homogenate was used for the analysis of AGAT activity.

Hepatic histology and lipid analysis

For histopathologic evaluation, 7 μm sections of OCT-blocked liver tissue were immersed in propylene glycol for seven minutes and stained with Oil-Red-O for 10 minutes. The sections were washed 3 times with distilled water, counterstained in haematoxylin for 20 min and washed again in distilled water before microscopic analysis. Adobe Photoshop® CS3 software (Adobe, San Jose, California, USA) was used to estimate the percentage of red pixels in each image.

Liver total fat was determined by homogenizing 1 g of liver in 1.5 mL of distilled water; 5 mL of chloroform-methanol (2:1) was added and the tubes were thoroughly mixed. After centrifugation, the chloroform phase was transferred to a pre-weighed tube, the extraction repeated twice and the chloroform phases combined, evaporated to dryness and re-weighed. The fat was re-suspended in 1 mL 1-propanol for the measurement of total liver triglycerides and cholesterol, using commercially available kits from Diagnostic Chemicals (Charlottetown, PEI, Canada, catalogue # 236-17 and #234-60,

respectively, for triacylglycerol and cholesterol). Hepatic phosphatidylcholine and phosphatidylethanolamine were measured by a phosphorus assay after separation by thin-layer chromatography, as described by Jacobs et al. (78).

Creatine, GAA, and sulfur-containing metabolites.

Both GAA and creatine were assayed by the HPLC method of Buchberger and Ferdig (29). For SAM and S-adenosylhomocysteine (SAH) determinations, freeze clamped liver samples were homogenized in ice-cold 8% (wt/vol) trichloroacetic acid and the homogenates centrifuged at 13,000 g for 5 min at 4°C. The supernatants were analyzed by HPLC using a Vydac C_{18} column (model 2187P54) equilibrated with 96% of buffer A (50 mmol/L NaH_2PO_4 containing 10 mmol/L heptanesulfonic acid at pH 3.2) and 4% acetonitrile. SAM and SAH were separated by means of a gradient of 96–80% of *buffer A* and 4–20% of acetonitrile for 15 min. SAM and SAH peaks were detected at 258 nm and quantified using Millennium32 (version 2) software (Waters, Milford, MA). Total plasma homocysteine and cysteine concentrations were determined by reverse phase HPLC and fluorescence detection of ammonium 7-fluoro-2-oxa-1,3-diazole-4-sulphonate (SBDF) thiol adducts by the method of Vester and Rasmussen (168).

AGAT activity assay and Immunoblot

Protein was assayed using the Biuret method. Kidney AGAT activity was determined as described by Van Pilsum et al. (164). For the Western blotting of kidney AGAT, proteins were separated by SDS-PAGE (12% polyacrylamide gel) and transferred by electroblotting to nitrocellulose membranes. AGAT-protein was detected using an

affinity-purified anti-AGAT rabbit polyclonal antibody raised against the sequence RPDPIDWSLKYKTPDFE, amino acids 142-159 of rat AGAT (accession number P50442-1) (Open Biosystems, Inc.). The blots were incubated at 4ºC overnight with the primary antibodies diluted in 5% non-fat dried-milk, followed by 45 min incubation with horseradish peroxidase–conjugated anti-rabbit IgG (Bio-Rad), and visualized by chemiluminescence. Band detection was performed using an enhanced luminol system (Immunn-Star, 144 Bio-Rad) and analysed with an Alpha Innotech Chemilmager 4400 (Alpha Innotech, San Leandro, California, USA).

Gene Expression

Real-time reactions were carried out to analyze the gene expression of PEMT, BHMT1, GNMT, MGAT, PPARα, CD36, CPT1a, LCAD, VLCAD and UCP2. Total RNA was isolated from frozen liver using Trizol® (Invitrogen). RNA quality was assessed with an Agilent 2100 bioanalyzer, using an RNA 6000 Nano kit. Samples were treated with DNAse I (Invitrogen) to digest genomic DNA; RNA was then reverse transcribed using Superscript II (Invitrogen). Primer sets and a corresponding probe for each gene of interest were designed using the Universal Probe Library (Roche) based on the NCBI reference nucleotide sequences for rattus norvegicus. Each primer pair and probe combination was tested by qPCR (StepOnePlus, Applied Biosystems). Primer mixes for each gene were combined in a single assay which was used to pre-amplify the cDNA of the genes of interest in each sample. Pre-amplification was tested using a probe for cyclophilin by qPCR. Forty-eight gene assays and cDNA samples were loaded into separate wells on a 48-by-48 gene expression chip (Fluidigm). qPCR was run on the

Biomark™ system (Fluidigm) for 40 cycles. Relative RNA expression for each gene in a sample was standardized to the endogenous housekeeping gene cyclophilin (Ppia), and calculated using the comparative threshold ($\Delta\Delta C_T$) method. All assays were performed in triplicate.

Insulin, glucose and hepatic TBARS

Plasma glucose was measured using hexokinase and glucose-6-phosphate dehydrogenase, as described by Bergmeyer et al. (15). Plasma insulin was measured using a commercially available kit from Crystal Chem Inc (catalogue # INSKR020) employing a rat insulin standard. Liver thiobarbituric acid reactive species (TBARS) was determined using commercially available kits from ZeptoMetrix Corporation (catalogue #081192).

Statistical analysis

Data were reported as mean ± standard deviation (SD). Groups were compared by analysis of variance (ANOVA) and orthogonal contrasts were used to identify specific differences between pairs of treatments using the SAS statistical package (version 8.2). In all analyses, the level of significance was set at $P < 0.05$.

4.4 Results

There were no differences in body weight gain or food intake for the 3 groups during the 3 week experimental period. As expected, fat intake was higher ($P < 0.05$) in HF and HFC than in the control group. The liver weight was elevated in the HF group and this was prevented by creatine supplementation (Table 4-1).

Three weeks of ingesting the high-fat diet resulted in a significant increase in hepatic total fat (55%), triglycerides (87%) and total cholesterol (25%) compared to rats fed the control diet. Supplementation of the high-fat diet with creatine prevented the hepatic accumulation of these lipids. Increased hepatic TBARS was evident after three weeks of feeding the high-fat diet. This increase was prevented by creatine supplementation (Table 4-2). The effects of creatine supplementation on liver fat were clearly evident in the Oil-Red-O-stained histological sections (Figure 4.1). The difference in fat accumulation between HF and HFC sections was confirmed by image analysis. Ingestion of the HF diet increased plasma glucose which was partially reversed by creatine supplementation. Plasma insulin concentration did not differ between the groups.

The plasma creatine concentration increased significantly in the HFC group. The HF diet decreased the liver SAM concentration by 18% when compared with C group. Creatine supplementation prevented the decrease in liver SAM concentration in HFC rats, maintaining it at the same level as the control group. No changes were found in plasma Hcy concentration between the groups. Creatine supplementation increased plasma cysteine; the reason for this is not known (Table 4-3).

Table 4-1

Weight gain, liver weight, and intake of energy, fat, and creatine in rats fed Control (C), high-fat (HF), or HF liquid diet with creatine (HFC) for 3 wk.

	C	HF	HFC
Weight gain (g)	184 ± 21	193 ± 16	174 ± 13
Liver weight (% body weight)	4.2 ± 0.3[a]	4.8 ± 0.4[b]	4.1 ± 0.2[a]
Energy intake (kJ/d)	502 ± 46	509 ± 24	480 ± 24
Fat energy Intake (kJ/d)	176 ± 16[a]	361 ± 16[b]	341 ± 17[b]
Creatine intake (g/kg • d)	-	-	3.1 ± 0.1

Values are means ± SD, n=6. Means in a row with superscripts without a common letter differ $P < 0.05$.

Table 4-2

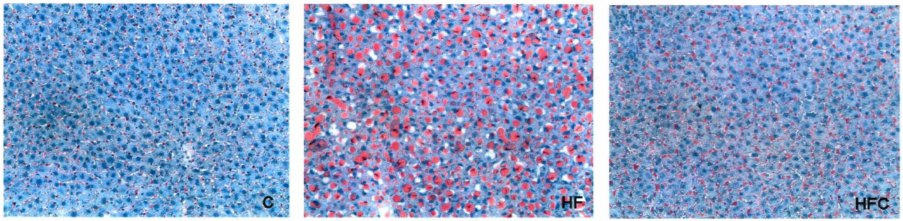
Liver total fat, triglycerides, cholesterol and TBARS, and plasma glucose and insulin in rats fed Control (C), high-fat (HF) or HF liquid diet with creatine (HFC) for 3 wk.

	C	HF	HFC
Liver Total Fat (mg/g tissue)	66.1 ± 14.1^{a}	102.4 ± 23.3^{b}	52.4 ± 12.0^{a}
Liver Triglycerides (μmol/g tissue)	22.5 ± 6.7^{a}	42.2 ± 9.2^{b}	16.6 ± 4.5^{a}
Liver Cholesterol (μmol/g tissue)	4.9 ± 0.7^{a}	6.1 ± 0.8^{b}	4.9 ± 0.6^{a}
Plasma Glucose (mmol/L)	7.2 ± 1.4^{a}	9.6 ± 1.7^{b}	7.8 ± 1.2^{ab}
Plasma Insulin (pmol/L)	82.8 ± 24.1	97.4 ± 14.3	79.4 ± 20.7
Liver TBARS (nmol/g tissue)	136 ± 2^{a}	155 ± 16^{b}	134 ± 10^{a}

Values are means ± SD, n=6. Means in a row with superscripts without a common letter differ $P < 0.05$.

Figure 4.1

Oil-red-O stained liver sections of rats fed Control (C), high-fat (HF), or HF liquid diet with creatine (HFC) for 3 wk. Values are means ± SD, n=3. Means without a common letter differ, $P < 0.05$.



	C	HF	HFC
Image quantification (% of red pixel)	23.8±7.3[a]	37.5 ±10.5[b]	21.1 ±5.6[a]

Table 4-3

Plasma levels of creatine, GAA, homocysteine, and cysteine, and liver levels of SAM, SAH, and SAM:SAH ratio in rats fed Control (C), high-fat (HF), or HF liquid diet with creatine (HFC) for 3 wk.

	C	HF	HFC
Plasma			
Creatine (mmol/L)	0.085 ± 0.019^{a}	0.0934 ± 0.015^{a}	0.995 ± 0.152^{b}
GAA (μmol/L)	5.1 ± 0.6^{b}	6.1 ± 0.4^{b}	1.7 ± 0.1^{a}
Hcy (μmol/L)	6.4 ± 1.0	6.2 ± 1.0	5.0 ± 0.8
Cysteine (mmol/L)	0.196 ± 0.015^{a}	0.219 ± 0.012^{a}	0.300 ± 0.026^{b}
Liver			
SAM (nmol/g tissue)	67.2 ± 6.3^{b}	55.2 ± 5.4^{a}	66.4 ± 7.1^{b}
SAH (nmol/g tissue)	7.1 ± 1.1	7.9 ± 0.8	7.5 ± 0.5
SAM:SAH	9.6 ± 0.8^{b}	7.1 ± 0.8^{a}	8.8 ± 0.7^{b}

Values are means ± SD, n=6. Means in a row with superscripts without a common letter differ $P < 0.05$.

As has been reported (49, 113), creatine supplementation led to decreased renal AGAT activity and concentration (Figure 4.2) and plasma GAA concentration in rats given creatine, compared to control rats and those on high fat diet (Table 4-3). Creatine supplementation increased liver PE and decreased the PC/PE ratio compared to rats fed the other diets. There were no differences in PEMT mRNA expression among any of the groups (Table 4-4).

We examined the abundance of genes involved in the metabolism of S-containing amino acids, fatty acids and phospholipids. HF increased BHMT1, GNMT and MGAT1 expression. Creatine supplementation normalized to control levels the abundance of these genes. With regard to fatty acid metabolism, rats fed the HF diet had reduced PPARα mRNA levels as well as those of its downstream targets CPT1a and LCAD, genes of mitochondrial ß-oxidation when compared to rats fed the other diets (Table 4-4). Creatine supplementation normalized the expression of these genes. Previously, Koonen et al. (94) showed that the hepatic expression of CD36, a fatty acid transporter, alters the *in vivo* rate of fatty acid uptake and triglycerides storage in mice. In our study, CD36 was not affected by the high fat diet, but it was reduced in livers of creatine- supplemented rats when compared to control rats. The level of mRNA for VLCAD and UCP2 was not affected by these diets (data not shown)

Figure 4.2

Renal AGAT protein and activity (A) , and hepatic content of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylcholine/phosphatidylethanolamine ratio(B) in rats fed Control (C), high-fat (HF), or HF liquid diet with creatine (HFC) for 3 wk. Values are means ± SD, n=6. Means in a row with superscripts without a common letter differ $P < 0.05$.

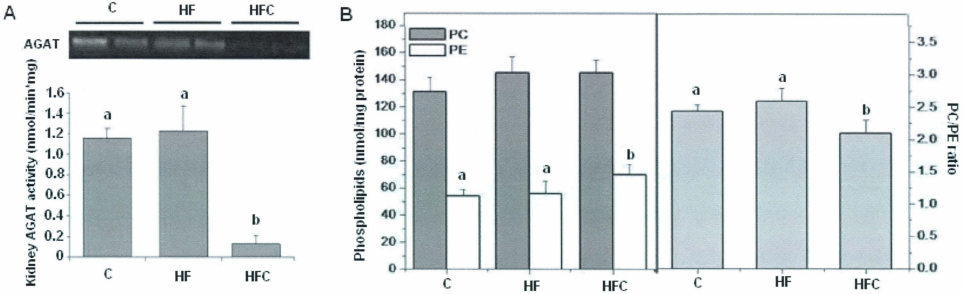

A
C
HF
HFC
AGAT
Kidney AGAT activity (nmol/min*mg)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
a
a
b
C
HF
HFC
B
PC
PE
Phospholipids (nmol/mg protein)
180
160
140
120
100
80
60
40
20
0
a
a
b
C
HF
HFC
PC/PE ratio
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
C
HF
HFC

Table 4-4

PEMT, PPARα, CD36, CPT1a, LCAD, BHMT, GNMT, and *MGAT* mRNA levels in rats fed Control (C), high-fat (HF), or HF liquid diet with creatine (HFC) for 3 wk.

	C	HF	HFC
mRNA			
PEMT	1.00 ± 0.14	1.00 ± 0.09	0.98 ± 0.17
PPARα	1.10 ± 029^{b}	0.55 ± 022^{a}	1.08 ± 0.29^{b}
CD36	1.01 ± 0.016^{b}	0.85 ± 0.27^{b}	0.52 ± 0.19^{a}
CPT1a	0.82 ± 0.25^{b}	0.44 ± 0.22^{a}	0.83 ± 0.20^{b}
LCAD	1.04 ± 022^{b}	0.55 ± 0.08^{a}	0.88 ± 0.10^{b}
BHMT	0.64 ± 0.12^{a}	1.20 ± 0.52^{b}	0.54 ± 0.22^{a}
GNMT	1.00 ± 0.05^{a}	1.73 ± 0.2^{b}	0.88 ± 0.25^{a}
MGAT	1.01 ± 0.23^{a}	1.64 ± 0.29^{b}	0.96 ± 0.36^{a}

Values are relative mRNA expression, standardized to cyclophilin, given as means ± SD, n=6. Means in a row with superscripts without a common letter differ $P < 0.05$.

4.5 Discussion

The principal finding of the present study is that creatine supplementation prevented the hepatic fat accumulation that occurs upon feeding a high-fat diet to rats for three weeks. The effects of betaine supplementation on SAM availability and the prevention of fatty liver are well known (88, 90, 99). Previous studies have shown that betaine supplementation regulates phosphatidylcholine synthesis and normalizes VLDL production rates (88), thus preventing either high fat diet- or ethanol-induced fatty liver (10, 51, 88, 90, 99). PC biosynthesis is required for the normal secretion of VLDL by hepatocytes. Both the PEMT (81) and liver-specific CTα knockout mice (77) have impaired PC biosynthesis which leads to fatty liver. Furthermore, it has been shown that a dramatic reduction in the PC/PE ratio results in steatohepatitis and liver failure (104). To our knowledge, this is the first study that shows that creatine can exert such a protective effect.

Creatine supplementation is known to modulate methylation demand (49) and decrease the plasma homocysteine concentration (43, 49), brought about by a remarkable (90%) down-regulation of renal AGAT enzyme activity. In the present study, creatine supplementation also caused a significant reduction in the plasma GAA concentration (Table 4-3) and, consequently, a reduction in the endogenous formation of creatine. Creatine supplementation also prevented the decrease in liver SAM concentration seen in HF-fed rats. SAM is required to form several methylated compounds through transmethylation reactions (153), of which phosphatidylcholine synthesis and creatine synthesis are quantitatively the most prominent. Phospholipid methylation is thought to be the major SAM-consuming pathway, since hepatic phosphatidylcholine (PC) synthesis

by PEMT is responsible for approximately 50% of hepatic Hcy formation (124). Creatine synthesis is also responsible for a very considerable consumption of hepatic SAM (153), perhaps as much as 40%. Because both PEMT and GAMT share the same hepatic SAM pool, we originally hypothesized that creatine supplementation could lead to a sparing effect on SAM utilization and result in increased PC formation. The increased hepatic SAM levels are consistent with such a sparing effect. However, PC levels and PEMT mRNA were unaltered by dietary treatment. Although creatine supplementation did increase PE and, consequently, resulted in a small decrease in the PC/PE ratio, it is unlikely that altered phospholipid metabolism plays an important role in either the development of steatosis in rats fed the HF diet or in its prevention by creatine. Nevertheless, we cannot rule out an increase in a key hepatic pool of PC that is not reflected in our measurements of total hepatic PC. We therefore examined the abundance of other key hepatic mRNA levels to see if they could shed light on how creatine supplementation might prevent fat accumulation.

The HF diet reduced mRNA levels for PPARα as well as those of its downstream targets CPT1a and LCAD. PPARα is essential in the modulation of lipid transport and metabolism, mainly through activating mitochondrial and peroxisomal fatty acid beta-oxidation pathways (1, 19). Carnitine palmitoyltransferase 1a (CPT1a) is a regulatory enzyme in mitochondrial ß-oxidation, and a target of PPARα (19, 53). LCAD, which is also a target of PPARα, catalyzes a key reaction in beta-oxidation (143). Creatine supplementation normalized these changes in mRNA levels. Creatine also reduced CD36 mRNA levels (a fatty acid transporter). Together, these changes could provide a mechanism by which creatine reduces fat accumulation in the liver. Previous studies have

shown impaired fatty acid metabolism induced in rats fed a high-fat diet (1, 56, 154) as well as in NASH patients (12). In this regard, the findings of Ayoma et al. (7), that PPARα-null mice have both hepatic steatosis and decreased mRNA levels for proteins involved in fatty acid oxidation, are of particular relevance. Abdelmegeed et al. (1) also found severe steatosis, inflammation and increased parameters of oxidative stress in PPARα-null mice who were fed a high-fat diet for 3 weeks. These authors concluded that PPARα plays a critical role in preventing steatosis and fat-related oxidative stress and inflammation via effects on fatty acid catabolism (154). To the best of our knowledge, our study is the first to demonstrate the regulation of PPARα expression and its downstream targets by creatine.

Feeding a high fat diet increased the hepatic abundance of mRNA for both BHMT and GNMT. These RNA abundances were normalized in creatine-supplemented rats. These results suggest that feeding high-fat diet may induce BHMT to promote remethylation of homocysteine, and GNMT to normalize the hepatic SAM:SAH ratio. BHMT transfers a methyl group from betaine to homocysteine, to regenerate methionine. GNMT uses SAM to methylate glycine, producing sarcosine (N-methylglycine) which, via sarcosine dehydrogenase, is reconverted to glycine with the transfer of a one-carbon group to the mitochondrial folate pool.

Creatine supplementation was at 3.1 g/kg/day, which is appreciably higher than the 0.3 g/kg/day classically proposed for humans (66, 72). However, the 1% creatine supplemented diet was chosen because it has been previously shown to downregulate the renal L-arginine:glycine amidinotransferase (49) and decrease plasma Hcy concentration (43, 152) in rats. It should be recognized that allometric scaling factors must be taken into

account when comparing doses between animals of different sizes. In general, mass-specific metabolic rate scales as the 0.75 exponent of mass. Therefore, a 300 g rat has approximately four times the mass-specific metabolic rate of a 70 kg human. When expressed on this physiological basis, the rats received approximately 2.5-fold the creatine supplementation recommended for humans.

In conclusion, creatine supplementation prevented fatty liver induced by 3 weeks of a high-fat diet in rats. This is the first study that shows this protective effect of creatine. This effect is not totally explained by modulation of methyl balance and consequent increased SAM availability, the explanation for the known effect of betaine on fatty liver. Our results do suggest that creatine supplementation also normalized some enzymes of methionine metabolism. In addition, and critically, creatine supplementation normalized the expression of PPARα as well as its downstream targets, CPT1 and LCAD, genes that code for key mitochondrial enzymes of beta oxidation. Since the expression of these genes were decreased by the high-fat diet, their normalization suggests a possible mechanism by which creatine may prevent liver fat accumulation. Further studies are necessary to elucidate the exact mechanisms by which creatine supplementation modulates the expression of PPARα.

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Chapter 5

The Effect of Portacaval Anastomosis on the Expression of Glutamine Synthetase and Ornithine Aminotransferase in Perivenous Hepatocytes

The author appreciates that the study conducted in the following chapter is not directly related to the topics addressed in the three preceding chapters. Nevertheless, it is intimately related to the field of hepatic amino acid metabolism which encompasses the creatine studies. In fact chapter 5 represents the first study undertaken during my PhD research. However, the focus of the laboratory changed to creatine due to grant funding exigencies. For this reason the remaining studies dealt with creatine metabolism.

The Effect of Portacaval Anastomosis on the Expression of Glutamine Synthetase and Ornithine Aminotransferase in Perivenous Hepatocytes

Running Title: Perivenous hepatocytes after portacaval anastomosis.

Authors: Robin da Silva, Oliver Levillain[†], John T. Brosnan, Silvia Araneda [†], Margaret E. Brosnan

Institutes: Memorial University of Newfoundland, University Claude Bernard Lyon†

Corresponding Author: Margaret E. Brosnan, email: mbrosnan@mun.ca, Tel. 864-2511 Fax. 864-2422

Keywords: Portacaval shunt, Hepatic Zonation, Amino acids, RhBG

5.1 Abstract

There is functional zonation of metabolism across the liver acinus, with glutamine synthetase restricted to a narrow band of cells around the terminal hepatic venules. Portacaval anastomosis, where there is a major rerouting of portal blood flow from the portal vein directly to the vena cava bypassing the liver, has been reported to result in a marked decrease in the activity of glutamine synthetase. It is not known whether this represents a loss of perivenous hepatocytes or whether there is a specific loss of glutamine synthetase. To answer this question, we have determined the activity of glutamine synthetase and of another enzyme from the perivenous compartment, ornithine aminotransferase, as well as the immunochemical localization of both glutamine synthetase and ornithine aminotransferase in rats with a portacaval shunt. We have also determined the expression of a perivenous ammonium transporter, RhBG, in these animals. Portacaval shunt caused a marked decrease in glutamine synthetase activity and an increase in ornithine aminotransferase activity. Immunohistochemical analysis showed that the glutamine synthetase and ornithine aminotransferase proteins maintained their location in the perivenous cells; the mRNA concentration for RhBG remained unchanged. These results indicate that there is no generalized loss of perivenous hepatocytes but rather a significant rearrangement in expression of these proteins and hence metabolism in this cell population.

5.2 Introduction

The functional zonation of metabolism across the hepatic acinus is well established (61, 86, 100). This phenomenon is attributed both to differences in the composition of blood as it travels from the portal triad to the central vein and to the differential expression of proteins in different regions of the acini. Acini are microscopic structures containing cells that are provided with blood from the branches of the two afferent vessels (the portal vein and the hepatic artery) and drained by the efferent vessel (hepatic vein). Cells nearest the portal triad (periportal) have the first opportunity to take up molecules and hormones from portal venous and arterial plasma while cells nearest the hepatic vein (perivenous) are presented with blood that is relatively depleted in oxygen and some hormones and may be enriched in metabolites produced in periportal cells. The differential expression of such functional molecules as enzymes, transporters and receptors across the acinus has been well documented (85). Several metabolic processes have been localized to discrete regions of the acinus; it is known that periportal cells primarily oxidize fatty acids and some amino acids while performing gluconeogenesis and ureagenesis whereas perivenous cells primarily oxidize glucose, perform lipogenesis and synthesize glutamine (84).

The zonation of nitrogen metabolism across the hepatic acinus is particularly striking. Ammonia arising from amino acid catabolism in enterocytes and that provided by the metabolism of intestinal microflora is largely converted to urea in the periportal region of the acini; ammonia that escapes conversion to urea is largely converted to glutamine in the perivenous hepatocytes (84). Studies using immunohistochemical and *in situ* hybridization techniques have indicated that glutaminase and the enzymes of urea

synthesis are localized exclusively to periportal cells (117), while glutamine synthetase (GS) has been localized to a 1-3 cell layer of perivenous hepatocytes (60, 61, 149, 165, 166). The differential localization of glutaminase and glutamine synthetase supports the idea of simultaneous glutamine synthesis and catabolism in the liver (180). Glutaminase, in the periportal cells (117) provides ammonia from glutamine to the urea cycle, while the synthesis of glutamine in the perivenous cells functions as a 'scavenging' system for excess ammonia (179). Consistent with this idea is the finding that the system N transporter that is partially responsible for glutamine uptake is found in periportal cells (30) while the SLC1A2 and SLC1A4 proteins (20) and system G transporter activity responsible for glutamate uptake are found primarily in perivenous cells (157). The RhBG transporter, necessary for providing ammonium for glutamine synthesis, has also been localized to perivenous cells in the liver (181, 182).

In liver diseases that result in fibrosis and portal hypertension, angiogenesis can result in the shunting of blood from the portal vein to the systemic circulation; this is referred to as portacaval anastomosis. In human liver cirrhosis, the surgical shunting of portal blood relieves backpressure and prevents further organ and vascular damage (137, 138). Among the major complications of cirrhosis and portacaval anastomosis are hyperammonemia and hepatic encephalopathy (127). Shunting of portal blood around the liver will result in marked changes in the composition of arterial blood; in addition, portacaval anastomosis results in altered expression of some proteins, presumably because of alterations in metabolite and hormone concentrations. Rats with surgical portacaval anastomosis show up to 90% reduction in glutamine synthetase activity (62). In addition, the GS protein is only faintly detected in humans with fibrotic liver disease

(131). However, it is not known whether there is generalized loss of perivenous cell function in portacaval anastomosis or there is simply a down-regulation of glutamine synthetase expression. Other enzymes such as ornithine aminotransferase (OAT), which are localized to the same perivenous cell population as glutamine synthetase (98), have not been examined under such conditions. The goal of this study was to determine whether perivenous cell function is lost in portacaval anastomosis. We demonstrate that there is a marked loss of glutamine synthetase together with an increased expression of hepatic OAT in rats with portacaval anastomosis and that these activities remain restricted to a small band of cells at the central venous terminus of the acinus. This indicates that there is a specific loss of glutamine synthetase, rather than a generalized loss of perivenous cell function in portacaval anastomosis.

5.3 Materials and Methods

Reagents: All reagents were obtained from Sigma-Aldrich (Missouri, USA) unless otherwise indicated. Ferric chloride was obtained from Fisher Scientific (New Jersey, USA). Sodium pentobarbital was obtained from Ceva Sante Animale (Libourne Cedex France).

Animals: Male Sprague-Dawley rats were obtained from Charles River, Montreal. All portacaval shunt and sham surgical procedures were performed by Charles River, about 2 weeks before the animals were shipped to Memorial University of Newfoundland. All procedures were approved by Memorial University of Newfoundland Institutional Animal Care Committee (IACC) and were in accordance with the guidelines set by the Canadian Council of Animal Care (CCAC).

Experimental Model: Animals were maintained in metabolic cages for two weeks on a 12:12 hour light:dark cycle in a temperature-controlled environment (20°C). They were fed a rodent chow diet containing 20% crude protein (Purina 5001 Rodent Chow). Sham-operated rats were pair-fed to the shunted rats who were allowed to eat *ad libitum*. Body weight was measured every three days. On the morning of the experiment, rats were anaesthetized with sodium pentobarbital (65 mg/kg body weight, i.p.). Samples of blood were taken from the abdominal aorta into heparinized syringes and placed on ice for a few minutes while liver tissue was taken. A piece of liver for RNA analysis was immediately freeze-clamped with aluminum tongs, pre-cooled in liquid nitrogen. Other pieces of liver were quickly taken for enzyme assays.

Enzyme Assays: Glutamine synthetase (GS) was measured by the method of Vorhaben

et al. (170). Ornithine aminotransferase (OAT) was assayed using the method of Knox and Herzfeld (71). Arginase was assayed by measuring the urea produced by reaction with thiosemicarbazide, using the method previously described by Schimke (142). All assays were carried out at 37°C and were shown to be linear with time and protein under the conditions employed.

Plasma Amino Acid Analysis: Arterial blood was centrifuged at 3,000 x g for 15 min and plasma was removed with a Pasteur pipette. 0.4 mL aliquots of plasma were added to 0.1 mL of 10% sulfosalicylic acid and allowed to sit on ice for 30 mins, at which time 0.3 mL of Lithium Citrate Sample Dilution Buffer pH 2.2 (Beckman Coulter) was added. Samples were mixed thoroughly and centrifuged at 10,000 x g for 5 mins. The supernatant was removed and amino acids were analyzed using a Beckman 121 MB amino acid analyzer with a Benson D-X 0.25 cation exchange column and a single-column three buffer lithium method.

Immunohistochemistry: Liver sections were prepared as follows. A small piece of liver tissue was rinsed in saline and placed in 4% phosphate-buffered formaldehyde and shaken gently. Samples were allowed to fix at 4°C for 24 hours at which point they were embedded in paraffin blocks. Four 5 μm-thick sections were cut using a cryostat (Microm, Francheville, France) and placed on glass slides (ChemMate, Dako). Slices were deparaffinized with two 15 min washes with xylene at room temperature, step-rehydrated with two 5 min washes in 100% ethanol and two 5 min washes in 95% ethanol, followed by one 10 min wash in 70% ethanol, and, finally, two 5 min washes in doubly-distilled water. Non-specific sites were coated by incubating for 2 hours in

phosphate-buffered saline (PBS: 10 mM phosphate buffer, 150 mM NaCl, 10 mM KCl pH 7.4) supplemented with 0.05% NaN_3, 0.3% Triton X-100, 1% bovine serum albumin (BSA) (Sigma) and 1% goat serum (DAKO). Slides were rinsed by means of three 15 min washes in PBS and were incubated at 4-8 °C with rabbit polyclonal OAT antibody (1:75 dilution (103) mouse monoclonal glutamine synthetase antibody (dilution 1:75) (Chemicon International, Temecula, CA) in PBS supplemented with 0.05% NaN_3, 0.3% Triton X-100, 1% BSA. Sections underwent three 15 min washes with the same buffer (without antibody). Alexa-conjugated antibodies were centrifuged at 10,000 x g for 2 min before use. For confocal microscopy, sections were incubated for 60 min at room temperature in the dark with PBS containing 0.5% gelatin and Alexa 488-conjugated affinity-purified goat anti-rabbit (dilution 1:300) and Alexa 633-conjugated affinity-purified goat anti-mouse (dilution 1:300, Jackson Laboratories Inc, USA). Sections were washed for two hours in the same buffer. Slices were counterstained for 3 mins with 2 μg/mL Hoechst stain (Polysciences Inc. USA). Sections were washed for 2-6 hrs in PBS and mounted with Fluoprep (Biomerieux, France). Images were taken with a Leica confocal system TCS-SP2 (oil immersion objective: x 63 magnification), using a filter set at 505-559 nm for Alexa 488 (OAT protein), 678-768 nm for Alexa 633 (glutamine synthetase protein) and 403-496 nm (Hoechst). Photographs were analyzed using a Leica (DMR, Germany) microscope equipped with a color video camera (Kodak DC290 digital zoom) coupled to a computer (MacOS 9.2). All data were analyzed using the Image J software. Pictures from confocal microscopy were analyzed and merged using Image J software and transferred to Adobe Photoshop CS3 extended version.

Semi-quantitive PCR: Total RNA was prepared from freeze-clamped livers by a rapid

guanidinium thiocyanate method (35). 2 μg of RNA was reverse transcribed using a one step reverse transcription kit (Qiagen; Mississauga, ON, Canada) and amplified by 30 cycles. An upstream primer (5'-CTCCTCTTTGCGATCTTTG-3') and a downstream primer (5'-CCATGGAAAGAGTGGAGGAA-3') were designed for rat ammonia transporter RhBG sequence (Genbank Accession NM_183054). Primers were designed to amplify a 267-bp fragment (Invitrogen; Burlington, ON, Canada). A 768-bp fragment of rat β-actin gene was co-amplified using amplimer set primers. PCR products were separated on a 2% agarose gel. Ethidium bromide (Biorad; Mississauga, ON, Canada) stained gels were visualized by UV illumination (using ChemiImager 4000) and quantified by densitometry, using Alphaease software (Alpha Innotech, San Leandro, CA). The integrated density values for RhBG were normalized to those of β-actin to give the relative expression.

Statistical Analysis: Enzyme activity, plasma amino acid and Integrated Density Values from the RNA analysis were all analyzed using a Student t-test in Prism Graph Pad software version 3.02. All values are reported as the mean $\pm$ standard deviation.

5.4 Results

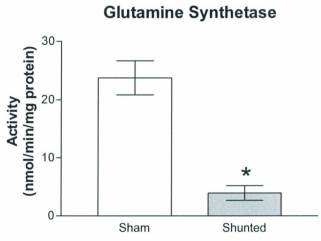
The body weights of the shunted rats were significantly lower when they were received approximately two weeks post surgery, than were the sham-operated animals. This is probably because both groups were fed *ad libitum* until the rats had arrived at Memorial University, at which point the sham-operated rats were pair-fed according to the shunted rats' intake. When all rats were receiving the same amount of food, the shunted rats gained weight more quickly than the sham-operated groups so that their weights were not different from the sham-operated rats after 14 days of pair-feeding. Sham-operated control and Shunted animals weighed 354.5 ± 24.2 g and 345.0 ± 37.4 g, respectively on the day of tissue sampling.

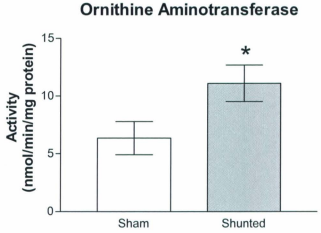
Glutamine synthetase activity was significantly lower in the shunted rats, being only 16% of that found in sham operated rats (Figure 5.1a). However, ornithine aminotransferase activity was significantly higher in the shunted rats, being 170% of that found in the sham-operated animals (Figure 5.1b). We also assayed arginase, which is expressed in the periportal region of hepatic acini. There was no significant difference in arginase activity between the groups (Figure 5.1c).

The relative integrated density values for the mRNA of the ammonium transporter RhBG were not significantly different between the groups (Figure 5.2). Since this transporter is exclusively restricted to the perivenous hepatocytes, its unchanged expression is also consistent with the notion that perivenous hepatocytes are not lost upon portacaval anastomosis.

Figure 5.1

Enzyme activities for glutamine synthetase, ornithine aminotransferase and arginase in liver. Activity is given as nmol/min/mg protein and values are expressed as means ± standard deviations. $P < 0.05$ was taken as significant, $n = 6$


Glutamine Synthetase
Activity
(nmol/min/mg protein)
30
20
10
0
*
Sham
Shunted


Ornithine Aminotransferase
Activity
(nmol/min/mg protein)
15
10
5
0
*
Sham
Shunted

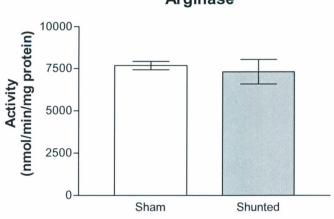
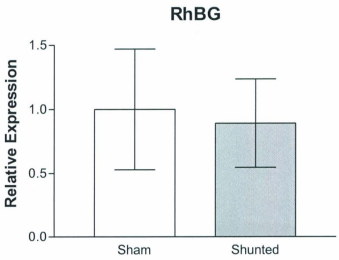
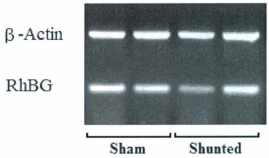

Arginase
Activity
(nmol/min/mg protein)
10000
7500
5000
2500
0
Sham
Shunted

Figure 5.2

Expression of RhBG mRNA in liver. Values are expressed as means ± standard deviation in arbitrary units and all groups were normalized to sham-operated control values. $P < 0.05$ is taken as significant, $n = 3$


RhBG
Relative Expression
1.5
1.0
0.5
0.0
Sham
Shunted


β -Actin
RhBG
Sham
Shunted

Confocal microscopy (Figures 5.3) confirmed the perivenous co-localization of glutamine synthetase and ornithine aminotransferase proteins. Shunted animals showed a marked drop in glutamine synthetase fluorescence and a marked increase in OAT fluorescence compared to the sham-operated rats.

Plasma amino acid analysis, given in Table 5-1, showed significant increases in glutamine, asparagine, histidine, phenylalanine and tyrosine and a significant decrease in threonine in the shunted rats compared to the sham-operated rats.

Figure 5.3

Confocal fluorescence microscopy of liver sections. Pictures a-c correspond to the sham-operated rats while d-f correspond to shunted rats. a and d show GS (red), b and e show OAT (green), and c and f show the overlay with nuclei stained (blue). White bar is 100 μm in length

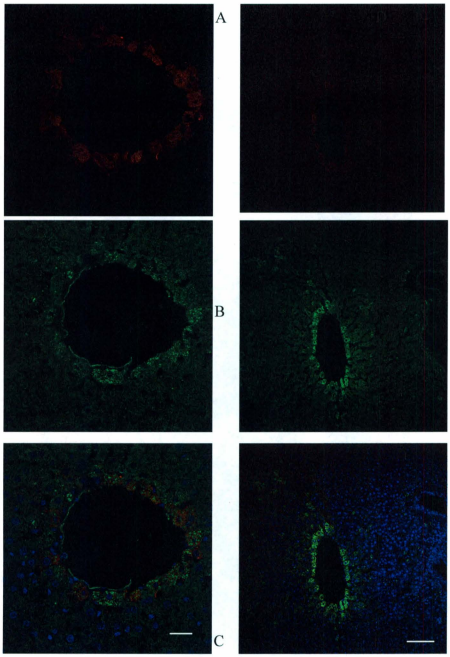

A
B
C

Table 5-1

Plasma amino acids from sham-operated control rats and rats with portacaval shunts.

	Sham-operated	Shunted
Amino Acid	nmol/mL	nmol/mL
Proline	177.3 ± 41.9	209.5 ± 43.0
Ornithine	59.7 ± 11.2	81.5 ± 33.5
Arginine	150.6 ± 26.5	175.5 ± 51.8
Citruline	79.4 ± 17.4	86.6 ± 12.6
Glutamine	495.1 ± 44.7	691.5 ± 114.7*
Glutamic acid	88.5 ± 13.4	82.5 ± 12.7
Aspartic acid	12.2 ± 2.1	16.0 ± 2.5
Asparagine	41.4 ± 8.2	84.6 ± 16.7*
Alanine	373.8 ± 36.4	469.4 ± 93.0
Glycine	237.9 ± 34.5	279.7 ± 38.9
Serine	200.1 ± 7.1	215.3 ± 31.7
Methionine	51.3 ± 6.5	59.9 ± 12.9
Taurine	187.7 ± 37.4	152.2 ± 4.5
Histidine	63.3 ± 10.2	100.6 ± 17.2*
Threonine	194.2 ± 22.2	127.3 ± 26.5*
Lysine	362.0 ± 63.6	363.9 ± 52.5
Leucine	124.1 ± 12.4	103.1 ± 14.3
Isoleucine	73.1 ± 9.9	58.3 ± 5.5
Valine	174.4 ± 17.6	150.4 ± 14.9
Phenylalanine	61.5 ± 3.6	131.0 ± 19.1*
Tyrosine	83.3 ± 4.7	137.6 ± 24.7*
Tryptophan	58.8 ± 15.5	71.7 ± 10.9

Note: All values are expressed as the mean ± standard deviation. $P < 0.05$ was taken as significant and indicated by an asterisk, $n = 6$.

5.5 Discussion

The key question addressed in this study is whether or not perivenous cells are destroyed when animals undergo a portacaval anastomosis. As previously demonstrated by Girard and Butterworth (62) animals with portacaval anastomosis lose up to 90% of glutamine synthetase activity. Although normal hepatic zonation, with perivenous glutamine synthetase, is retained in human hepatic fibrosis, glutamine synthetase was undetectable in cirrhotic nodules (131). There are two possible explanations for the loss of glutamine synthetase in rats with portacaval anastomosis: as a result of the selective loss of perivenous hepatocytes, as suggested by Butterworth's group (45, 62), or a specific loss of expression of glutamine synthetase by these cells. In order to address this question we examined the expression of two other genes known to be selectively expressed in perivenous hepatocytes.

Hepatic ornithine aminotransferase activity was not decreased by the portacaval shunt. In fact it increased in activity. More importantly, its expression remained limited to a small rim of hepatocytes around the centrilobular veins, exactly as seen in the sham-operated control animals. The same population of perivenous hepatocytes also expresses the ammonium transporter, RhBG, in perivenous hepatocytes. Although the lack of suitable antibody prevented us from carrying out an immunohistochemical study of its location, its mRNA expression remained unchanged in the livers of portacaval shunted rats. It is clear, therefore, that portacaval shunting brings about a specific loss of glutamine synthetase, and not of the perivenous hepatocytes.

It is of interest to compare the hepatic expression of these two enzymes of amino acid metabolism, glutamine synthetase and ornithine aminotransferase, with the relevant

amino acid levels. Plasma glutamine and asparagine levels were significantly increased in the shunted rats. It has been established that there is an induction of glutamine synthetase in the muscles of shunted rats (62) and we consider it likely that the increase in plasma glutamine that we found in the shunted rats is, at least in part, due to increased muscle glutamine synthesis. Synthesis of glutamine and asparagine in muscle would also be facilitated by elevated circulating ammonia concentrations seen in animals with portacaval shunt.

There was no significant difference between ornithine levels in the shunted rats and the sham-operated controls, despite an elevated activity of ornithine aminotransferase per gram liver in the shunted rats. Since the hepatic mass is markedly less in the portacaval shunted rats (approximately 50% of control), the ornithine aminotransferase, expressed per rat is not increased. In addition, total hepatic blood flow is decreased by approximately 40% in such animals (14). Therefore the greater OAT expressed per gram of liver tissue may simply offset the loss in total hepatic mass and reduced blood flow.

Immunohistochemistry of both enzymes showed that total protein amount reflected the changes in enzyme activity and confirmed the exclusive localization of these proteins to the perivenous cells of liver acini. RhBG expression did not show the same down-regulation as glutamine synthetase which suggests that ammonia is still capable of uptake by the perivenous cells. We therefore conclude that there is not a generalized loss of perivenous cell function; rather, there are significant changes in the expression of these two enzymes in this cell population.

The primary focus of this study was whether or not perivenous hepatocytes are lost in rats with portacaval shunts. We did not address the mechanism whereby the

expression of glutamine synthetase is decreased. Nevertheless, some comment on this issue is appropriate. Desjardins et al. (45) have found that the decrease in hepatic glutamine synthetase activity and protein content in the livers of portacaval shunted rats is accompanied by a comparable decrease in its mRNA level, suggesting an effect at the level of gene expression. Cadoret et al. (33) have shown that β-catenin signaling in the absence of APC action induces the expression of a number of perivenous-expressed genes, such as glutamine synthetase, ornithine aminotransferase and the glutamate transporter GLT-1. However, since ornithine aminotransferase activity is increased, rather than decreased, in the portacaval shunted rats, impaired β-catenin signaling cannot be invoked to explain the decreased expression of glutamine synthetase. It has been shown that normal hepatic architecture, rather than the supply of portal blood, is critical for glutamine synthetase induction in liver cells (58, 59). These studies suggest a cell-cell interaction hypothesis, in which the proximity of the perivenous cells to the endothelium is a critical factor in the expression of glutamine synthetase.

A number of physiological and pathological parameters are known to affect hepatic glutamine synthetase activity. There is some evidence that the expression of glutamine synthetase in the liver is controlled by oxygen tension. Glutamine synthetase is up-regulated under low oxygen tension and is returned to normal levels when oxygen tension is improved (129). This is not likely to play a role in our rats four weeks after portacaval anastomosis. It has been shown that hepatic oxygen tension and effective blood flow are significantly lower in surgical portacaval anastomosis immediately after surgery but oxygen tension is returned to normal after one week while blood flow remains low (141). In addition, studies by Haüssinger et al. have shown that glutamine

synthetase is sensitive to inhibition by nitric oxide by virtue of nitration of a key tyrosyl residue at its active site (63). In this situation, glutamine synthetase activity is decreased by 50% but glutamine synthetase protein is only decreased by 20%. This mechanism, however, cannot account for the loss of glutamine synthetase activity in the shunted animals since this appears to be brought about by decreased expression of glutamine synthetase messenger mRNA (45).

Hormonal regulation of glutamine synthetase may be at play in rats with portacaval anastomosis. Plasma glucagon levels are markedly elevated in rats with portacaval anastomosis (11). There is very good evidence that glucagon increases the activity of ornithine aminotransferase in vivo (122) and some evidence that glucagon may decrease glutamine synthetase activity in the liver (70).

In conclusion, our experiments clearly point to a specific loss of glutamine synthetase expression, rather than a generalized loss of perivenous hepatocytes in portacaval anastomosis. This appears to be brought about by a relatively specific alteration in the regulation of glutamine synthetase expression. Further studies that address this mechanism are warranted. In addition, it will be of interest to determine whether a similar phenomenon occurs in humans.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

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Chapter 6

General Discussion

and Concluding Remarks

6.1.0 Creatine Biosynthesis in the Liver and Pancreas

6.1.1 Hepatic Creatine Synthesis

In the first paper we set out to investigate creatine synthesis in the liver. We wished to characterize the activity of GAMT in hepatocytes and determine if it is regulated by dietary creatine. In addition we had postulated that there may be AGAT activity present in the liver; this was based on a number of pieces of information and a consideration of whether arginine from the urea cycle could be used to synthesize creatine. Three pieces of evidence supported our hypothesis regarding hepatic AGAT activity in the rat. Firstly, AGAT activity is cited in the livers of other mammals such as human, monkey and pig in a review by Wyss and Kaddurah-Daouk (186). Secondly, AGAT protein was detected in rat liver using immunohistochemistry (112). Lastly, there is a complication with the traditional enzyme assay for the AGAT enzyme. The assay is based on measuring ornithine that is produced in a one-to-one mole ratio with GAA by the AGAT enzyme. The problem arises from the large activity of arginase in the liver. Arginase converts arginine provided in the assay medium into urea and ornithine and so the background is extraordinarily high making it impossible to measure AGAT in this manner. Therefore the activity of AGAT in rat liver may have escaped detection in the past. Taken together these pieces of evidence suggested a potential for GAA synthesis in the liver.

We were not able to detect AGAT activity in rat liver using HPLC to quantify GAA directly. We also incubated hepatocytes with ^{15}N-ammonium (M+1) chloride and were unable to measure either M+1 or M+2 creatine and therefore we must conclude that

arginine derived from the urea cycle is not used for creatine synthesis in rat liver. On the other hand, significant activity of GAMT was detected in rat liver and there was significant methylation of GAA in isolated hepatocyte incubations. The methylation of GAA by hepatocytes is dependent on the external concentrations of GAA provided in the incubation medium consistent with the known kinetics of the GAMT enzyme (126). Our findings are consistent with the notion that creatine synthesis in rat liver is limited to the methylation of GAA that is synthesized in extra-hepatic tissue. This work supports the idea of methylation demand in which the rate of methylation is dictated by the concentration of methyl-accepting substrates.

Isolated hepatocytes incubated in the presence of GAA and methionine showed an increased production of creatine compared to isolated hepatocytes incubated with GAA alone. This finding suggests that methylation in the liver can be influenced by the concentration of methionine that is presented to this organ. The presence of methionine in the incubations of isolated hepatocytes resulted in an elevation of the SAM concentration in these cells. The liver expresses the methionine adenosyltransferase (MATI/III) enzyme which converts methionine and ATP into SAM and pyrophosphate. The MATI/III enzyme normally occurs as a tetramer of subnunits and responds to increased concentrations of SAM by splitting into a dimer that is more active than the tetramer (32) and so is regulated by feed-back activation. The influence of methionine on MAT activity and SAM production suggests that dietary methionine may influence methylation in the liver.

Provision of dietary creatine supplementation did not affect GAMT activity in the liver. Our data point to GAMT in the liver as being a slave to GAA concentrations in the

plasma. We know from previous studies that AGAT activity in the kidney is dramatically down-regulated by dietary creatine supplementation (113, 161) and the plasma GAA concentration is also dramatically lower in creatine supplemented rats (49, 152). We have also determined that there was a significant uptake of GAA across the liver *in vivo* in rats fed diets free of creatine. Using the data collected we were able to make calculations relating to the capacity of the liver for creatine synthesis (methylation of GAA only) and we have found that the liver is capable of synthesizing a quantity of creatine sufficient to replace the amount of creatine that is lost per day. From this we infer that the regulation of creatine synthesis by creatine itself does not occur in the liver but via a reduction of AGAT activity in the kidney which in turn causes a reduction in plasma GAA and thus less GAA is taken up by the liver for methylation.

6.1.2 Pancreatic Creatine Synthesis

In chapter 3, I present a paper on creatine synthesis by pancreatic acini and the regulation of creatine synthesis in the pancreas. The enzymes for creatine synthesis had been detected in the pancreas many years ago (162), but the role that these enzymes serve in this tissue is yet to be determined. AGAT activity has been immunohistochemically localized to the acini in the pancreas (150) and therefore we isolated these cells in order to examine their ability to produce creatine. Since renal AGAT activity is dramatically down-regulated when creatine is supplemented in the diet (113), the regulation of pancreatic AGAT by dietary supplementation with creatine was also examined. The data show that the pancreas can indeed make modest amounts of both GAA and creatine. In addition, the regulation of pancreatic AGAT is not achieved in the same way as renal AGAT.

Pancreatic acini are capable of producing both GAA and creatine from arginine, glycine and methionine. However, AGAT activity and the capacity for GAA synthesis were considerably higher in the pancreatic acini than the GAMT activity and the capacity for GAA methylation. Enzyme activies for the creatine synthetic enzymes in pancreatic acini were in line with previously reported activities in this tissue (162), with significantly less GAMT than AGAT activity. In addition, the synthesis of GAA under supraphysiological substrate concentrations was considerably higher than was the synthesis of creatine. So the fact that the capacity for GAA synthesis outstrips that of creatine synthesis in the pancreatic acini leaves the possibility that the pancreas is producing more GAA than creatine. GAA released by the pancreas could be taken up and methylated in the liver to form creatine. Based on incubations of pancreatic acini with physiological concentrations of the amino acids, I have calculated that the whole rat pancreas could synthesize approximately 4% of the creatine required per day while GAA synthesis (when converted to creatine) would amount to an additional 8% of the creatine required per day. Thus, all together the rat pancreas could contribute up to 12% to total body creatine synthesis. However, the rat kidney is capable of synthesizing all of the GAA required per day (49) and the rat liver can methylate enough GAA to replace the creatine that is required per day (39), possibly making pancreatic creatine synthesis redundant. Observing this from a purely Darwinian perspective, one might ask whether the expression of the creatine synthetic enzymes in pancreatic cells has some benefit or whether they were useful at some earlier stage of evolution and have since become superfluous. Then again, from a developmental perspective, the enzymes for creatine synthesis may be important in early development and later become unnecessary. Indeed,

the expression of AGAT, GAMT and CRT1 all change in rats throughout embryogenesis with AGAT not being expressed in the kidney until 18.5 days while pancreatic AGAT expression is present at 15 days of embryogenesis (23). One need only look at the results of deficiencies of the enzymes and transporter involved in creatine synthesis to understand the important role that creatine plays in brain and neural development (144) as all of the afflicted subjects display serious neuromuscular symptoms and mental retardation. Creatine may indeed play an important role in the secretion of hormones from the endocrine pancreas (96) and there is good evidence that the creatine kinase system affects insulin secretion from the β-cells (146).

Both GAA and creatine could have a functional role in the pancreas. There has been a study in which relatively high concentrations of GAA and creatine stimulate the release of glucagon and insulin from pancreatic islets (110). It is not known how these compounds bring about the increased release of hormones from the pancreatic islets. Activity of creatine kinase has been detected in mouse pancreatic islets (128) and in our study we show that creatine supplementation significantly increases creatine phosphate levels in the pancreas (Table 3-2). An increase in the ATP:ADP ratio in pancreatic β-cells leads to increased insulin release (42, 146).

The paper presented in chapter 3 also provides evidence that the regulation of pancreatic and renal AGAT by dietary creatine does not involve the same mechanism. McGuire et al. showed quite convincingly that renal AGAT is regulated by dietary creatine at the pre-translational level (113). The data from McGuire et al. and from figure 3.2 revealed reduced levels of mRNA and protein for the AGAT enzyme in kidneys of

rats fed creatine-supplemented diets. Figure 3.1 clearly shows that dietary creatine does not affect AGAT mRNA or protein levels in the pancreas. There was however a modest reduction in pancreatic AGAT activity, which implies that there are two different types of regulation that affect AGAT. Creatine itself is not known to have a regulatory effect on the AGAT enzyme (186) and it is impossible to determine what mechanisms are at play in the pancreas. Nevertheless this is an interesting observation and the retention of pancreatic AGAT expression may indicate the significance of GAA and creatine synthesis in the pancreas.

6.1.3 NAFLD: Hepatic Fat Accumulation and Creatine

Consumption of a high fat diet leads to the accumulation of triglycerides in the liver and to insulin resistance (34, 190). Accumulation of excess fat in lipid droplets in the liver is referred to as the "first hit" in the "Two-hit" model of NAFLD progression. The "second hit" includes increased lipid peroxidation and increased production of cytokines resulting in increased inflammation (41, 160). Impaired methionine metabolism has been observed in a number of different inflammatory liver diseases including NAFLD, NASH and alcoholic steatohepatitis (ASH) (8). Methyl-donors such as SAM and betaine have been used successfully to treat NAFLD (34). Betaine supplementation has been shown to dramatically elevate hepatic SAM levels in animals with NAFLD caused by consumption of a high fat diet (99). Through the action of BHMT, betaine is used for the remethylation of homocysteine. It has been postulated that increased availability of SAM allows for increased methylation of PE via the enzyme PEMT. The product of PE methylation is PC which is a phospholipid involved in the packaging and export of VLDL. $PEMT^{-/-}$ mice have been shown to have impaired export of triglycerides

from the liver and so it appears that PC made via PEMT is important for normal packaging of VLDL (105). Thus increased PC facilitates the removal of lipids from the liver.

The paper in Chapter 4 provides evidence that the accumulation of fat in the liver of rats fed high-fat diets is ameliorated by provision of creatine in the diet. Creatine is not a methyl donor; however the conversion of guanidinoacetate to creatine is a methylation reaction catalyzed by GAMT. We observe that the enzyme responsible for GAA synthesis as well as GAA itself is reduced when creatine is supplemented in the diet. It is also known that the methylation of GAA in the liver is proportional to the amount of substrate present in the circulation (Chapters 2 and 3) (39). Thus one might postulate that the down-regulation of creatine synthesis might spare SAM making it available for other methylation reactions. The data did show that creatine supplementation normalized SAM levels in rats fed the high-fat diet and so this is consistent with the idea that SAM is spared in the liver. However, there was no measurable increase in hepatic PC content nor was there a difference in the PE/PC ratio in the creatine-supplemented high-fat group, suggesting that the creatine supplementation did not alter PC metabolism in this case. It is important to point out that we did not measure flux of PC in the liver or the rate of oxidation of lipids in the liver, nor was VLDL secretion determined and so it is impossible to determine if creatine truly affects these parameters in the rats fed high fat diets using the present data.

Creatine appears to have beneficial effects in NAFLD in that it normalized the expression of genes that are altered by the consumption of high-fat diets. The expression of PPARα and its downstream targets CPT1a and LCAD are down-regulated in the livers

of rats fed high-fat diets. This suggests that in the high-fat fed animals triglyceride synthesis would be favored over β-oxidation. Creatine supplementation prevented this fall in PPARα, CPT1a and LCAD. Creatine supplementation also reduced CD36 expression in the liver of rats fed the high-fat diets. CD36 is a key protein involved in the uptake of free fatty acids from circulation and has been associated with increased steatosis, hyperinsulinemia, and insulin resistance in NAFLD, NASH and ASH (116). In fact, induction of CD36 in the liver via activation of the nuclear receptors liver X receptor (LXR), pregnane X receptor (PXR) and peroxisome proliferator agonist receptor gamma (PPARγ) results in hepatic steatosis which is absent in CD36 null mice (192). So it appears that creatine supplementation has two beneficial effects in NAFLD brought about by feeding high-fat diets; reducing the amount of fat accumulation as well as increasing β-oxidation in the liver. Of course we cannot eliminate the possibility of other beneficial effects.

Creatine-supplemention to high-fat fed animals also normalized other parameters. In particular, elevated plasma glucose and elevated hepatic cholesterol and TBARS were normalized. The plasma insulin was not significantly higher in the animals on high fat diets but the values tended to be higher. However the plasma glucose values indicate that insulin sensitivity was improved by creatine supplementation. In addition to lowering hepatic triglycerides dietary creatine also decreased hepatic cholesterol levels which is what would be expected if VLDL-type particles were accumulating in the liver. Normal levels of hepatic TBARS indicate that there are less reactive oxygen species in rats fed the creatine-supplemented high-fat diet and suggest that there is less lipid peroxidation in

this tissue. Thus these side effects of increased accumulation of triglycerides in the liver are not seen when creatine is present in the diet.

6.1.4 Hepatic Zonation in Portacaval Anastomosis

The paper presented in chapter 5 addresses the question of the fate of perivenous hepatocytes when the blood flow from the portal vein is diverted from the liver to systemic circulation. Previous work by Butterworth and colleagues (45, 62) suggested that there was a selective loss of perivenous hepatocytes based on the dramatic reduction of GS activity observed in this cell population after portacaval anastomosis. The effect on GS was also observed in chapter 5 figure 5.1; however a significant increase in OAT activity was also observed. The OAT protein is known to be colocalized with GS in the perivenous cells of hepatic acini (98). Moreover, confocal microscopy confirmed the colocalization of GS and OAT as well as the relative fluorescence intensity of the antibody stains (figure 5.3) in the rats with portacaval shunts. In addition, the ammonia transporter RhBG (figure 5.2), a protein known to be localized in perivenous hepatocytes, was unchanged in the livers of rats with portacaval shunts. The findings in chapter five are strong evidence against the selective loss of perivenous hepatocytes in favor of alterations in gene expression.

Perhaps the most serious consequence of portacaval anastomosis is encephalopathy, with ammonia escaping the high-capacity, low-affinity urea cycle. Appreciable reduction in the normal "ammonia scavenging" GS causes ammonia levels in the plasma to increase. GS is upregulated in the muscle and brain of rats with portacaval shunts (45) and levels of ammonia and glutamine accumulate in the brain. Glutamine has been found to accumulate in the mitochondria of astrocytes causing

mitochondrial impairment and leading to ultimate dysfunction of cells (4). Glutamine in the brain also acts as a precursor for the neurotransmitters glutamate and γ-aminobutyric acid (GABA) which are agonists of the NMDA and GABA receptors respectively. GABA-ergic tone is greater in the brains of patients with hepatic encephalopathy due to elevated pregnenolone-derived neurosteroids brought about by elevated ammonia (31). NMDA hyperexcitability and increased GABA-ergic tone have both been implicated in the progression of neuronal dysfunction in cases of hepatic encephalopathy (55).

The regulatory mechanisms responsible for the reduction in hepatic GS in portacaval shunted rats are unknown. Reduction of hepatic GS activity runs counter to the increase in plasma ammonia, exacerbating the hyperammonemia. There are several possible reasons for the down-regulation of hepatic GS in portacaval shunted rats. It has been postulated that the proximity of perivenous hepatocytes to the endothelial cells has some regulatory effect on GS expression (58). This has not been proven to be the case in the portacaval shunted animals but there is some evidence supporting the notion.

Another possible factor affecting the expression of GS may be the action of glucagon. Pancreatic hormones are found in relatively high concentrations in the portal blood. In portacaval anastomosis these hormones will enter the systemic circulation and become diluted and therefore the effect on the liver is muted. However, circulating concentrations of glucagon are considerably higher in the plasma of rats with portacaval anastomosis (11). There is some evidence that glucagon reduces the expression of GS in the liver (70) and there is convincing evidence that glucagon action increases the expression of OAT in hepatocytes (122). Elevated glucagon would fit very well with the observations in chapter 5; however it may not be the only factor at play in terms of GS

expression.

6.2 Limitations and Future Directions

All scientific techniques have their inherent limitations. Considerations include sampling of tissues, post-sampling treatment, assay variability and sensitivity. The types of statistical analysis used in analyzing data and the number of subjects (statistical power) sampled can also affect the outcome of observed differences and thus can be a limiting. In addition, the conclusions that can be drawn from data collected are dictated by what questions the techniques are capable of addressing. Thus, I will provide a brief explaination of some of the relavant limitations in chapters 2, 3, 4 and 5 in this section.

The studies on creatine synthesis in the liver and pancreas both involve the isolation of cells or groups of cells (acini) from a living tissue. The preparations are extensively washed with synthetic medium and incubated under various substrate conditions. These studies constitute *in vitro* experiments which do not precisely match *in vivo* conditions. Therefore we combine *in vitro* and *in vivo* data to gain a better understanding of the process of creatine synthesis in these two tissues. *In vivo* techniques are not without their limitations as well. The technique of measuring A-V differences in metabolite concentrations across the liver for instance are potentially affected by changes in blood flow that occur throughout the day. Perhaps a definitive technique for measuring creatine synthesis would be to use isotopic tracers to label the substrates of creatine synthesis injected into live animals followed by serial blood sampling. However, in order to determine tissue specific synthesis of creatine the choice of substrate and isotope would have to be tailored to the tissue and the label would have to be injected directly into the afferent vessel with collection of efferent blood after first-pass preventing the

label from travelling to other tissues where it could be metabolized. Future studies in this direction would be beneficial to support or refute the findings in chapters two and three.

There some complications with dietary supplementation studies as well. Dietary creatine supplementation changes the concentration of creatine in the tissues of rats. This in itself may cause cell swelling effects and may result in difficulty interpreting metabolite concentrations in various tissues. Following this, increased creatine in the smooth muscle may have an effect on the tone of blood vessels and thus may vary blood flow values. One limitation of the calculations in chapter two is that we did not measure blood flow through the liver but instead used average values from the available literature. Studies on the effects of creatine on vascular tone and blood flows would be an interesting future direction in this area.

Creatine supplementation in high-fat diet fed rats prevented NAFLD but the study was limited in addressing the mechanism underlying the effect. It is impossible to determine whether the lower lipid levels in the creatine-supplemented rats was due to increased export of fat, decreased import of fat, increased oxidation of fat or a combination of the either of the three individual possibilities. The phospholipid data is limited in that it is impossible to infer flux through PC given a concentration in the liver. In addition the observed changes in the expression of mRNA that were observed may not necessarily translate to differences in flux under physiological conditions. Rather they are used as evidence to support further research. Further studies in the livers of creatine-supplemented high-fat fed rats and their controls should be aimed at measuring oxidation of fatty acids, VLDL secretion, and lipid import to determine which factors are responsible for the observations in chapter 4. Also, future studies to determine the

signaling mechanisms responsible for the changes brought about by creatine supplementation are required.

The study on hepatic zonation addresses the fate of the perivenous hepatocyes but fails to address the underlying cellular mechanism controlling GS and OAT. The changes to arterial plasma after portacaval shunting are dramatic and widespread. Concentrations of many regulatory hormones and metabolites change. Further research directed specifically on the regulatory factors affecting GS and OAT are required. Following this, intervention with the known regulatory molecules may shed light on what changes are taking place in such a complex situation.

6.4 Closing Remarks

The quotes given in section 1.3 are descriptive of the nature of scientific research. I believe that good research often reveals more questions than answers and so I think it is fitting to close this thesis by reviewing some of the interesting questions that this work has highlighted. We have put forth evidence that dietary creatine does not govern the expression of GAMT in rat liver. What are the factors regulating GAMT and is there some benefit to the constitutive expression of this protein? We were unable to measure AGAT activity in the rat liver despite some previous evidence for its expression in this tissue. AGAT activity has been measured in other species as well so we may inquire at which point this activity was lost in rat liver.

The effect that creatine supplementation had on lipid accumulation in rat liver of rats fed high fat diets was quite dramatic. Creatine supplementation certainly prevented many of the negative effects brought about by consuming a high-fat diet. However, the mechanism behind the effect remains to be determined. Does the reduction in

methylation demand spare SAM and make it available for methylation of PE? If so, does this increase PC synthesis and affect VLDL packaging? In addition, the expression of CD36 is altered in the presence of dietary creatine. It remains to be determined if creatine directly affects the expression of this gene or whether there is an indirect mechanism at play.

Finally we deal with the fate of perivenous cells and the zonation of metabolism in the liver of rats with portacaval shunts. The opposing regulation of OAT and GS in the same population of cells is remarkable. It is clear that the constituents of portal blood have major regulatory influence on gene expression in the liver. The portal constituents serve to maintain appropriate activities of GS and OAT and hence the character of zonation of nitrogen metabolism. The precise regulatory factor(s) affecting hepatic GS and OAT expression in portacaval shunted rats remains to be determined. Finding an agent to increase the expression of GS in the liver may be beneficial to patients who have portacaval shunts.

Each of the papers presented provides a modest contribution to the field of amino acid metabolism in the liver and pancreas. The data and ideas help to advance our understanding of creatine biosynthesis and nitrogen metabolism. I hope the questions that arise from this work help to stimulate future research in these areas. To paraphrase the quotes given in section 1.3: Science is constantly changing and it is important that our ideas change in step, as should the questions which fuel the change. This is the nature of the study of the physical world: the beautiful, impossible puzzle.

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