METABOLIC ALTERATIONS OF FREE AND PROTEIN-BOUND BIOTIN IN RATS DURING DIETARY BIOTIN MANIPULATION AND ENDOTOXIN EXPOSURE

By

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The water-soluble B-vitamin biotin is an essential cofactor for the biotin-dependent carboxylases involved in the metabolism of carbohydrate, protein, and fatty acids. Although a frank biotin deficiency is thought to be rare, more recent evidence suggests that a marginal biotin deficiency may be more prevalent than previously appreciated. Despite substantial advances in molecular and analytical techniques, the biotin requirement of healthy and physiologically stressed animals remains poorly understood.

To address these gaps in the understanding of biotin nutriture, the effect of dietary biotin intake on plasma, urine, and tissue levels of free and protein-bound biotin was examined. Rats were fed an egg-white based AIN 76A diet containing 0, 0.06, 0.6, or 100 mg biotin/kg diet for three weeks. Indicators of biotin status (urinary biotin excretion, liver free biotin, and liver protein-bound biotin) in rats fed a diet supplemented to 0.06 mg biotin/kg diet were equal to that of rats fed a 0.6 mg biotin/kg diet, a level

used in previous studies indicating adequate biotin status. This study also demonstrated that distinct differences in these pools occurred as early as 3 weeks on a biotin-deficient diet with no outward signs of biotin deficiency, thus potentially modeling a marginal biotin deficiency in humans.

To determine the changes in biotin metabolism during an acute inflammatory response, bacterial lipopolysaccharide (endotoxin) was administered to biotin-adequate (0.06 mg biotin/kg diet) and biotin-deficient rats (0 mg biotin/kg diet). Endotoxin administration modulated both free and protein-bound biotin pools in adequate rats. Free biotin increased in the serum and liver. Protein bound biotin, represented by the relative abundance of biotinylated carboxylases, also changed; however, increases or decreases were specific for the individual carboxylases. Of note, a novel biotinylated protein with an apparent molecular weight of 61 kDa appeared in endotoxin treated animals. These changes provide plausible mechanisms to account for the observed changes in carbohydrate, protein, and fatty acid metabolism associated with acute inflammation. Biotin deficiency prior to endotoxin challenge delayed or lessened the changes in carboxylase metabolism seen in adequate rats and may indicate a reduced ability to respond to acute inflammation.

CHAPTER 1
LITERATURE REVIEW

Biotin

Structure and Function

Biotin is a bicyclic compound consisting of a tetrahydrothiophene ring, ureido group and a valeric acid side chain. d-(+)-Biotin is the only stereoisomer found in nature and is the only one that is thought to be enzymatically active (1). In living organisms, several states of biotin exist. Free biotin is found in the soluble fraction of cells as well as the serum/plasma of animals. The free vitamin can also be conjugated to a carrier. This carrier can be a simple amino acid as in biocytin (biotin conjugated to lysine) or complex higher molecular weight compounds such as proteins or peptides (2). Finally, catabolism of the vitamin yields the inactive biotin metabolites, of which bisnorbiotin (BNB) and biotin sulfoxide (BSO) are the most abundant (Figure 1).

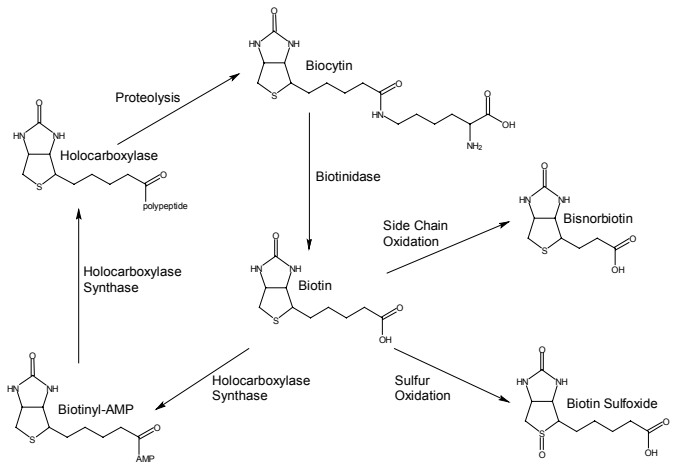

Figure 1-1. Forms of biotin.

Biotin is the cofactor for five biotin-dependent carboxylases: the two isoforms of acetyl-CoA carboxylase (ACC1, 265 kDa (3); ACC2, 280 kDa (4)), pyruvate carboxylase (PC, 129 kDa (5)), methylcrotonyl-CoA carboxylase (MCC, α-chain-79.8 kDa (genbank)), and propionyl-CoA carboxylase (PCC, α-chain-79.3 kDa (6)). Holocarboxylase synthase, found in both the mitochondria and cytosol, catalyzes the condensation reaction of biotin and the apocarboxylases to generate the holocarboxylases. Holocarboxylase synthase recognizes the biotin carrier domain of the apocarboxylase and in a two-step reaction covalently binds biotin to a specific lysine within the polypeptide (Figure 1). Biotin is first converted to biotinyl-AMP and then, through the removal of AMP, biotin is attached to the apocarboxylase to form the holocarboxylase. The site at which biotin is attached is a highly conserved tetrapeptide (AMKM) that resides 35 amino acids from the C-terminal end of the apocarboxylase (7).

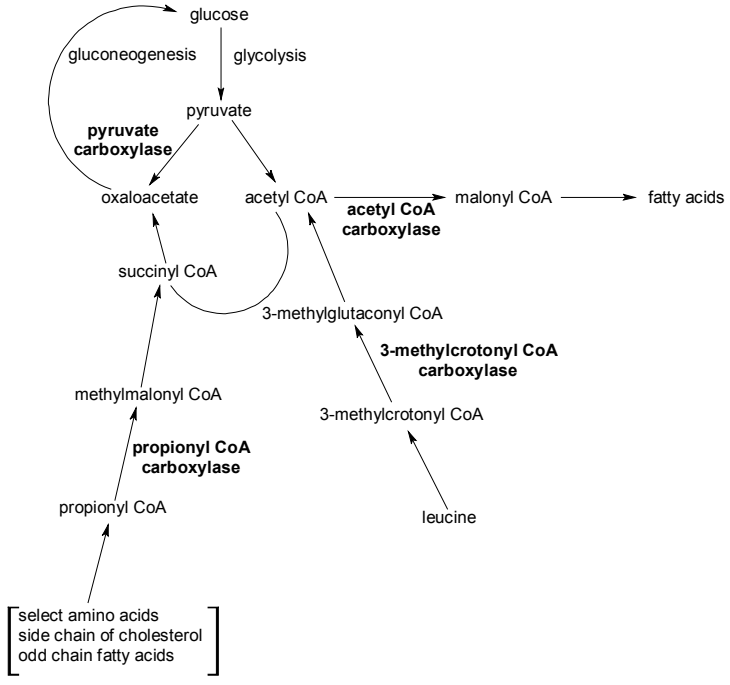

Figure 1-2. Role of biotin-dependent carboxylases in metabolism.

Once biotin is attached, each of these holocarboxylases can then catalyze the incorporation of bicarbonate into a specific substrate (Figure 1-2) (8). This occurs through the formation of a carboxybiotin intermediate at the expense of the hydrolysis of one molecule of adenosine triphosphate (ATP). Carbon dioxide can then be transferred to the substrate to form the carboxylated product.

There is now evidence that two isoforms of ACC exist: ACC1 and ACC2. ACC1 is found in the cytosol of the cell, while ACC2 is inserted into the mitochondrial membrane (9). Both isoforms of ACC catalyze the addition of bicarbonate to acetyl-CoA to form malonyl-CoA. When first discovered, the mitochondrial form of ACC (ACC2) was thought to serve as a storage form of ACC that was transformed to active ACC during times of biotin deficiency. However, it is now known that ACC1, the original active form, is a cytosolic protein, whereas ACC2 is an integral membrane protein associated with the mitochondria by an additional 136 amino acids. The first twenty amino acids of ACC2 are hydrophobic and are thought to target the protein for insertion into the mitochondrial membrane, while the remainder of the sequence is highly homologous with ACC1 (9). In the case of ACC1, the malonyl-CoA produced serves as the substrate for fatty acid synthase during fatty acid elongation. However, in the case of ACC2, the malonyl-CoA produced is thought to regulate mitochondrial fatty acid β-oxidation through its ability to inhibit carnitine palmitoyl-CoA transferase 1, the shuttle responsible for fatty acid transport into the mitochondria (9).

Further evidence for the function of ACC2 in the regulation of fatty acid β-oxidation includes its distribution in various tissues throughout the rat. Both isoforms of ACC are expressed in liver, mammary gland, and brown adipose tissue where both fatty

acid synthesis and β-oxidation occur. In tissue such as white adipose tissue in which fatty acid synthesis primarily occurs, only ACC1 is present. In tissues such as heart and skeletal muscle where rates of fatty acid synthesis are low and rates of fatty acid β-oxidation are high, only ACC2 is present (4).

PC, MCC, and PCC are localized to the mitochondria. PC catalyzes the formation of oxaloacetate from pyruvate and bicarbonate. Oxaloacetate can then be used either as an intermediate in the tricarboxylic acid cycle or in gluconeogenesis. MCC catalyzes the formation of methylglutaconyl-CoA from methylcrotonyl-CoA and bicarbonate during the degradation of the branched-chain amino acid leucine, and PCC catalyses the formation of methylmalonyl-CoA from propionyl-CoA and bicarbonate. Methylmalonyl-CoA is isomerized to succinyl-CoA and can then enter the tricarboxylic acid cycle.

During protein turnover and digestion, carboxylases are degraded to biotinyl-lysine (biocytin). Cellular and secreted proteases cannot hydrolyze the amide bond between biotin and lysine so cleavage by biotinidase is required for the release of biotin (10). Once released from lysine by biotinidase, biotin is available to be absorbed in the intestine or used to biotinylate proteins in the cell.

Functions other than biotin's role as a cofactor for the carboxylases, such as regulation of gene transcription, have been proposed. As early as 1968, biotin deficiency in rats was shown to decrease glucokinase activity (11). Repletion of biotin restored glucokinase activity to normal levels. With advances in molecular biology, it is now known that the repletion of biotin increased the amount of mRNA coding for glucokinase in rat liver (12). Biotin deficiency is also known to decrease mRNA levels of holocarboxylase synthase (13). Posttranscriptionally, biotin deficiency has been shown

to reduce the expression of asialoglycoprotein receptor even though mRNA levels coding for asialoglycoprotein receptor are comparable to biotin adequate controls (14). Biotin may also have a role in DNA packaging, transcription, and replication as indicated by the discovery of biotinylated histones in the nucleus (15). This may be linked to the impaired interaction between histones and DNA observed during biotin deficiency (16); however, it remains to be determined if deficiency reduces nuclear biotin concentration and histone biotinylation. New evidence supporting a role of biotinylated histones in DNA replication was recently published by Stanley et al. (17). The level of biotinylated histones from peripheral blood mononuclear cells (PBMC) was determined to increase four fold following stimulation of cell proliferation with the mitogen concanavalin A.

Sources

Biotin rich foods include egg yolk, liver, soybeans and yeast. Moderate sources include human milk, fish, nuts, and oatmeal. Fruits and meats have generally lower biotin contents. The majority of biotin in meats and grains is protein bound, conjugated via a lysine residue to the protein (18). The bioavailability of protein-bound biotin varies depending on the food source. Biotin from soy and corn is approximately 100% bioavailable. In contrast, biotin from barley and oats is 20-30% bioavailable, and biotin from wheat is virtually unavailable (19). The reason for this variability is unknown but may reflect the susceptibility of these foods to digestive breakdown (20).

Absorption

The mechanism of intestinal hydrolysis of protein bound biotin has not been clearly defined. Biotinidase is thought to play a critical role in releasing biotin from its covalent bond to protein due to the inability of proteases to cleave this bond. It is possible that biotinidase secreted by the pancreas is responsible for releasing the

covalently bound biotin from protein for absorption during the luminal phase of proteolysis (18). Mucosal biotinidase is thought to release biotin from biotinyl oligopeptides, the presumed products of intestinal proteolysis (10).

Absorption of free biotin occurs by both simple diffusion and by a biotin transporter present in the intestinal brush-border membrane (21). This carrier-mediated transport is structurally specific, temperature dependent, and electroneutral. Biotin transport is sodium dependent, coupled 1:1 with Na^+. Both simple diffusion and carrier mediated transport of biotin are thought to occur; however, due to the small amount of biotin in foods, carrier mediated transport is thought to predominate unless pharmacologic doses are consumed.

In rats, the site at which biotin is maximally absorbed is the jejunum (22). While most of the absorption is in the jejunum, significant absorption occurs in the proximal colon. Blood concentrations of biotin increase after administration of biotin into the colon (23). This implies that biotin synthesized by the normal flora in the gut may contribute to biotin levels. It is also equally possible that biotin produced by the bacteria is reintroduced for absorption in the small intestine through reflux or caprophagy (23).

The process of biotin exiting the rat intestine is by electroneutral, carrier-mediated transport across the basolateral membrane. However, it is not Na^+ dependent and does not accumulate biotin against a concentration gradient (24). This carrier-mediated transport out of intestinal cells has been duplicated in the human intestinal epithelial cell line CaCo-2. However, in humans biotin transport out of the intestinal cell is sensitive to manipulations of the transmembrane electrical potential (25).

Transport

Proteins in human plasma capable of binding biotin include α and β-globulin as well as biotinidase. Biotinidase contains two biotin binding sites, one with high biotin binding affinity (K_d = 3 nM) and one with low biotin binding affinity (K_d = 59 nM) (26). The binding characteristics of these two sites predict that a high percentage of the total free biotin pool should be bound. However, new data suggest that less than 10% of the total free pool plus reversibly bound biotin is reversibly bound to plasma macromolecules. Incubation of [^{3}H]biotin with freshly collected human plasma followed by ultrafiltration showed only 8% of the label in the retentate where protein bound biotin is found (27). Recently, Wang et al. showed that the percentage of protein-bound biotin in pig plasma was also <10% (6.9-7.6% depending on the method used) (28).

Uptake of free biotin by tissues has been assessed in 3T3-L1 and HepG2 cells, rat hepatocytes, and basolateral membrane vesicles from human liver and found to be mediated by both diffusion and by a specialized Na^+ gradient, energy and temperature dependent carrier system. Transport is electroneutral (1:1 Na^+:biotin) and specific for a free carboxyl group. However, isolated cultured hepatocytes did not exhibit this carrier mediated transport system (29).

Deficiency

Biotin's role as an essential nutrient in humans has been demonstrated in three situations: prolonged consumption of raw egg-white (30), total parenteral nutrition (TPN) without biotin supplementation (31), and multiple carboxylase deficiency (32). Avidin, a glycoprotein in raw egg-whites, binds biotin (essentially irreversibly, K_d=10^{-15} M) and can cause a severe form of biotin deficiency (frank biotin deficiency) characterized by dermatitis, hair loss, and neuromuscular dysfunction (1). Symptoms of frank biotin

deficiency, whether caused by avidin consumption or non-supplemented TPN, are similar in adults and children. Symptoms gradually appear weeks to months after continuous consumption of avidin or initiation of TPN (30,31). Most subjects present with thinning hair, seborrheic, eczmatous rash distributed around the eyes, nose, and mouth, depression, lethargy, hallucinations, and paresthesias of the extremities. Infants normally present with symptoms earlier than adults possibly due to an increased growth requirement of biotin. Candida is typically cultured from lesions of the rash in infants suggesting a role of biotin in immune function (32).

A secondary biotin deficiency has been seen in individuals not expressing biotinidase (33). This secondary deficiency is thought to occur due to an inborn error of biotin metabolism that causes decreased absorption of biotin concurrent with decreased release of the free vitamin from ingested protein bound forms, and decreased salvage of biotin from the turnover of biotinylated proteins; however, symptoms of a biotinidase deficiency are not limited to those seen in biotin deficiency. Additional symptoms include seizures, hearing loss, and optic atrophy in addition to the other common biotin deficiency symptoms (34). These additional symptoms may indicate other activities of biotinidase that remain unknown.

Deficiencies of the biotin-dependent carboxylases exist and consist of isolated carboxylase deficiencies or multiple carboxylase deficiencies. The most common clinical finding of a carboxylase deficiency is organic acidemia (35-41). Propionyl CoA carboxylase deficiency, although rare, is the most common of the isolated carboxylase deficiencies. During this condition, β-hydroxypropionic acid accumulates. Deficiency of methylcrotonyl CoA carboxylase has also been described. A deficiency in

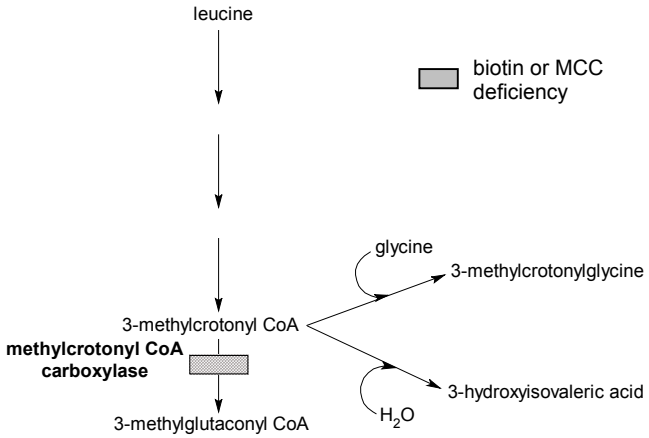

Figure 1-3. Accumulation of 3-Hydroxyisovaleric acid and 3-methylcrotonylglycine due to a biotin deficiency or methylcrotonyl CoA carboxylase (MCC) deficiency.

methylcrotonyl CoA carboxylase causes 3-methylcrotonylglycine (3-MCG), and 3-hydroxyisovaleric acid (3-HIA) to accumulate due to a decrease in activity of methylcrotonyl CoA carboxylase (Figure 1-3). This causes methylcrotonyl CoA to accumulate and then be shunted into an alternate pathway where it is converted to 3-HIA by cronotase and 3-MCG by glycine acylase.

Multiple carboxylase deficiency is described as a characteristically low activity of all biotin-dependent carboxylases. Two forms of multiple carboxylase deficiency exist: a neonatal or infantile form and a late-onset or juvenile form. The neonatal form manifests within the first few days of life and results from a mutation in the gene coding for holocarboxylase synthase (42). The late-onset form presents within 3-6 months of age and is caused by abnormalities in the intestinal absorption of biotin (43). All inborn errors of biotin metabolism are treated with pharmacologic doses of biotin.

There is a prevailing assumption that biotin deficiency is rare unless massive amounts of raw egg-white are consumed, deficiencies in the carboxylases are present, or long-term nonsupplemented TPN is the sole source of nutrition. More recent studies are

now providing evidence that decreased biotin status is seen in other groups of individuals without outward signs of gross deficiency. This condition is being termed marginal biotin deficiency. Accumulating data are providing evidence of marginal biotin deficiency during pregnancy (44) and prolonged use of anticonvulsants (36-38). The most notable finding is that 3-HIA excretion into the urine is increased in both of these states along with a decrease in plasma biotin, accelerated catabolism of biotin, and a decreased absorption of biotin in individuals taking anticonvulsants.

The possibility of marginal biotin deficiency during pregnancy is troublesome considering biotin deficiency during pregnancy has teratogenic effects on the fetuses of chicken, turkey, mouse (45), rat, and hamster (46). Even mild biotin deficiencies that do not produce the typical cutaneous and central nervous system (CNS) symptoms in the dam caused the fetus to have micrognathia, cleft palate, and micromelia. These defects increase with severity of biotin deficiency.

Toxicity

Few studies have measured the toxicity of biotin. When given in excess, biotin demonstrated sterilizing effects in both the Mexican fruit fly and the housefly (47). Pregnant mice receiving 50 mg/kg biotin i.p. through gestation showed no signs of disturbed reproductive function or abnormal embryonic development (48). No toxicity of biotin has been reported with individuals receiving 200 mg orally or 20 mg intravenously for treatment of inborn errors of biotin metabolism (49).

Biotin Status

The excretion of 3-HIA, biotin, and its metabolites into the urine are now identified as early and sensitive markers of biotin status (50). Subjects consuming a biotin-deficient egg-white based diet developed an increased excretion of 3-HIA by day 7

of consumption. By day 14, urinary excretion of biotin and its metabolites had decreased below normal values. Serum biotin levels were still within the normal range 20 days after initiation of the diet, demonstrating that it is not a reliable status indicator.

Role in Immune Function

Studies of immune responses in biotin deficient rodents, and of decreased immunity in patients with multiple carboxylase deficiencies, indicate that biotin is required for normal immune function. Rats fed an egg-white based biotin-deficient diet for 20 weeks had several indicators of decreased immune function. A marked reduction of thymic size and cellularity, depressed immune response to sheep red blood cells, and a lack of allergic encephalomyelitis following immunization with guinea pig myelin basic protein were all seen in the biotin-deficient group when compared to the adequate group (egg-white diet + 2 mg biotin/kg diet) (51).

The role of biotin in human immune function can be demonstrated in patients suffering from biotin-responsive multiple carboxylase deficiencies. In 1979, three siblings presented with CNS dysfunction, candida dermatitis, keratoconjunctivits, absent delayed-type hypersensitivity responses, absent in vitro lymphocyte responses to candida antigen, IgA deficiency, lack of antibody response to pneumococcal polysaccharide immunization, and a subnormal percentage of T lymphocytes. The first two siblings began to show symptoms at 2½ and 3 months of age and died at 8 and 40 months of age, respectively, with progressive CNS deterioration and overwhelming infection. The third sibling began to show symptoms at 3 months of age. Assays of white cell preparations for PC, PCC, and MCC activity were 7, 35, and 28% of controls respectively. After four days of oral biotin (10 mg/d), the values increased to 281, 491, and 120% of controls respectively. Although in vivo and in vitro responses to candida antigen were absent and

T lymphocyte numbers still mildly depressed after four months of treatment, there was no reoccurrence of candida dermatitis or keratoconjunctivitis (52). These data are thought to be consistent with a reduced affinity for biotin either in a transport protein or holocarboxylase synthase (32).

Expression of interleukin-2 (IL-2) and IL-2 receptor γ genes are affected by biotin deficiency. IL-2 is secreted into the extracellular space by helper T-cell lymphocytes responding to stimulation by antigens. The binding of IL-2 to IL-2 receptors triggers intracellular signaling cascades that lead to growth and differentiation of immune cells. Although three forms of IL-2 receptor are known (α, β, and γ), only IL-2 receptor γ plays a role in signal transduction (53). When grown in biotin-deficient medium and exposed to phytohemagglutinin, the human T-cell line Jurkat increases net secretion of IL-2 as compared to cells grown in biotin-adequate and supplemented media (54). This occurs however, without an increase in cell proliferation as expected from high IL-2 levels. It has now been determined that biotin-deficiency appears to increase the net secretion of IL-2 due to a decrease in synthesis of IL-2 receptor γ (55).

Lipopolysaccharide

Structure

Lipopolysaccharide (LPS) is a potent endotoxin found as an integral part of the outer membrane of gram negative bacteria. LPS consists of four domains: lipid A (embedded in the outer membrane of the bacteria unless lysed), the inner core oligosaccharide, the outer core oligosaccharide, and the O-antigenic peptide (extending outward from the bacteria, responsible for complement activation), all of which contribute to the smooth appearance of colonies (56,57). There are approximately 3-4

million molecules of LPS per cell comprising about 3% of total cell dry weight in typical laboratory strains of E. coli and greater than 10% of dry weight in clinical strains (56).

Lipid A is the component attributed with the major biological activities of LPS, and it is the most conserved structure in LPS throughout bacterial species. The backbone of lipid A is composed of a D-gluco-configured hexosamine disaccharide with two phosphate residues attached. The polar head group and acyl chains of lipid A do not share the same homology as the backbone. The polar head group can have a positive or negative charge, or consist of zwitterionic groups. While the polar head group is not known to contribute to the endotoxic properties of lipid A, it may be important in determining the organization and permeability of the membrane as well as antibiotic resistance of the bacteria. Generally, the primary fatty acids of lipid A are β-hydroxylated with very few known exceptions (56).

The outer core of LPS is made of hexose residues and the inner core consists of heptose and two deoxy-manno-octulosonic acid residues that link the lipid A to the core. The O-antigenic peptide consists of repeating oligosaccharide units, and provides the basis of the variability of LPS between bacterial strains (57).

Binding

CD14 and LPS binding protein (LBP) are the two most understood molecules involved in the action of LPS. LBP is a plasma protein made in the liver that is thought to enhance the binding of LPS to CD14 increasing cell sensitivity to LPS (58). LBP accomplishes this by acting as a lipid transfer protein (58), bringing the hydrophobic LPS molecule out of its aggregate to interact with the water-soluble CD14 (56). LBP also

transfers LPS to lipoproteins and may facilitate the transfer of LPS from CD14 into cell membranes by lipid exchange (58).

CD14 exists as either a membrane bound (mCD14) or soluble form (sCD14). Membrane CD14 is expressed mainly on monocytes and macrophages along with polymorphonuclear neutrophils (58). It is a glycerophosphatidylinositol-anchored glycoprotein that lacks a cytoplasmic portion, and so is thought to interact with another signal transducing molecule at the cell membrane. It is now known that the protein responsible for generating the transmembrane signal linked to LPS-induced cell activation is Toll-like receptor 4 (TLR-4) (59). Binding of the LPS-CD14 complex to TLR-4 promotes dimerization of the receptor. The cytoplasmic domain of TLR-4 interacts with the cytoplasmic adapter protein MyD88 which then activates IL-1 receptor associated kinase (IRAK), mitogen-activated protein kinase, and TNF receptor-associated factors (TRAF6), which results in the phosphorylation of IκB and its dissociation from NF-κB (60). NF-κB is then able to migrate to the nucleus and initiate transcription and production of IL-1, IL-6, TNF-α, acute phase proteins, and nitric oxide (61). Cells that do not contain mCD14 are activated when sCD14 forms a bridge between the LPS-LBP molecule and the cell membrane (62), suggesting other receptors on the membrane that are capable of binding LPS or the LPS-CD14 complex (58). Soluble CD14 is produced by macrophages proteolytically cleaving their phosphotidylinositol-linked surface molecules and is thought to be made and secreted from the liver (58).

Activation of cells with LPS through the CD14 receptor is only required at low concentrations of LPS. At higher concentrations, LPS can stimulate cells in a CD14-independent manner. This suggests the involvement of a second molecule that

recognizes LPS. This molecule may be the CD11/CD18 integrins, but the exact pathway is unclear (62). These β2 integrins are leukocyte-restricted adhesion molecules responsible for cell-cell and cell-matrix interactions. These integrins enable LPS responsiveness when transfected into Chinese hamster ovary fibroblasts void of CD14 (58).

Elicited Response

Stress, whether from LPS or any other acute febrile illness, typically stimulates highly predictable metabolic and physiologic responses. These responses lead to both a hypermetabolic and hypercatabolic state that causes loss of somatic cell protein, depletion of body nutrient stores, negative nitrogen balance, and decreased body weight (63). Accelerated proteolysis in the skeletal muscle supplies free amino acids for metabolic energy, the repair of structural damage, the production of new cells, as well as the synthesis of new proteins needed during acute illness for secretion (64,65).

Hyperglycemia is seen during early sepsis due to the accelerated breakdown of glycogen. As sepsis continues, hypoglycemia develops due to the inability to sustain glucose release from glycogen and the continued use of glucose for energy production (66,67). Changes in lipid metabolism are not as drastic as those of protein and carbohydrate; however, fat stores do provide a major source of needed calories (68,69). Mineral and trace element metabolism is also changed. Sepsis stimulates the hepatic uptake of iron and zinc and the hepatic secretion of copper (70). Relatively little is known about the alterations of vitamin metabolism, although the general consensus is that vitamins are utilized in greater amounts than seen in normal individuals (71). Hormonal and endogenous mediators such as adrenocorticotropic hormone,

catecholamines (epinephrine and norepinephrine), aldosterone, and cytokines (IL-1, IL-6, and TNF) provide the driving force for these alterations (62).

These alterations, if severe enough, may lead to multiple organ dysfunction syndrome (MODS). Multiple organ dysfunction syndrome is the progressive dysfunction of two or more organ systems resulting from an uncontrolled inflammatory response. It is thought that multiple inflammatory insults are required to produce MODS. This is now known as the "two-hit hypothesis" and suggests that an initial sensitizing insult primes neutrophils and macrophages through the release of cytokines (primarily IL-1 and TNF). Within a specific period of time, a second insult can then activate these cells to produce an exaggerated response known as secondary MODS. A second hypothesis, known as the gut hypothesis, states that intestinally derived bacteria, or endotoxins released from the bacteria, trigger or exacerbate the septic state that eventually results in organ failure. In this hypothesis, MODS is thought to occur when increased intestinal permeability allows gut bacteria and endotoxins to be released into circulation due to intestinal injury caused by the impairment of oxygen delivery during shock (71).

Rationale and Hypotheses

Dietary Modulation of Biotin Metabolism

Studies characterizing the nutritional status of rats on standardized diets containing deficient or adequate amounts of biotin have used protocols that may not be applicable to the general human population. Previous studies have used extended periods of induced biotin deficiency (>8 weeks) before biotin and its metabolites were measured (72,73). These extended periods of biotin deficiency caused signs and symptoms of a frank biotin deficiency in the rats, a state that is uncommon in today's developed societies.

In studies including a biotin-adequate group, a level of 0.6 mg biotin/kg diet or higher has generally been used (73-75). When adjusted to a per kg body weight basis, this level of consumption is equivalent to a 70 kg human consuming in excess of ten times the estimated safe and adequate daily dietary intake (ESADDI). In this study we hypothesized that a level of biotin supplementation more relative to the human ESADDI would be adequate to prevent biotin deficiency. We also hypothesized that a marginal biotin deficiency, with no outward signs of frank deficiency, would decrease both free and protein-bound metabolic pools of biotin. Finally, we hypothesized that a level of supplementation of 100 mg biotin/kg diet, the highest level of consumption yet tested, would not be toxic.

Modulation of Biotin Metabolism by Endotoxin

The possibility that nutritional support may prevent and treat the profound metabolic alterations seen during stress, whether from wounds, falls, burns, or sepsis, has gained increased attention. Nutrient requirements for energy, protein, fat, and some minerals during stress have been well defined. However, no specific guidelines for the requirements of vitamins, most minerals, or trace elements exist for the metabolically stressed individual even though there is evidence that the requirements of stressed individuals are increased over nonstressed individuals (71).

Vitamin and mineral metabolism and uptake are affected after endotoxin exposure. Enhanced flux of iron and zinc from the plasma to storage compartments such as the liver are thought to protect the host and combat infection by limiting nutrients needed for bacterial growth (76). In contrast to plasma iron and zinc, serum copper concentrations gradually increase, reflecting an increased synthesis and/or release of ceruloplasmin (77). Endotoxin has been shown to significantly decrease ascorbic acid

transport in mouse fibroblasts (78) and rat adrenocortical cells (79) in a dose dependent manner. Endotoxin also decreases the level of ascorbic acid in the heart (80) and aqueous humor of the eye, which has one of the highest concentrations of ascorbic acid (81).

As discussed previously, decreases in immune function are seen in rats and mice on biotin deficient diets as well as individuals with inborn errors of biotin metabolism. Outside of deficiency, biotin and its role in immune function have never been tested. Nothing is known as to the behavior of free and protein bound pools of biotin in healthy animals during immune challenge. However, studies on the effects of endotoxin on gluconeogenesis and de novo fatty acid biosynthesis provide clues that biotin metabolism may change during endotoxin exposure. An endotoxin-induced mediator from cells stimulated with endotoxin regulated key anabolic activities in adipose cells (82). To examine the effect of the mediator substance on the activities of ACC and fatty acid synthetase (FAS), 3T3 L1 cells were exposed to conditioned medium from mouse peritoneal exudate cells cultured in the presence of endotoxin. ACC and FAS activities were determined in digitonin released cytosolic fractions of the cells along with protein synthesis using an [^{35}S] methionine pulse following 3, 6, or 20-hr of incubation with the mediator. The activities of both enzymes decreased over the 20-hr period to approximately 25% of initial values. [^{35}S] methionine incorporation into FAS and ACC were down 80 and 95% respectively. These results are consistent with the concept that the mediator depresses the activity of ACC and FAS by interfering with the synthesis of the enzymes. However, these results do not show if degradation of the enzymes occurred with the possible release of biotin from ACC into the free pool.

The flux of pyruvate through PC is inhibited in hepatocytes prepared from endotoxin injected rats (83). Sprague Dawley rats were injected with 4 mg/kg LPS 16-hr prior to hepatocyte preparation. Flux of 1 mM pyruvate through PC significantly diminished by 50% with all subsequent steps showing a similar percentage decrease in flux. This suggests that the site of action of endotoxin resides predominately at the level of PC. It was also shown that the decreased flux through PC was not due to an increased flux of pyruvate through pyruvate dehydrogenase. The cytosolic concentration of oxaloacetate is known to rise during treatment with endotoxin. This is consistent with a decreased rate of utilization by phosphoenolpyruvate carboxykinase (PEPCK). It was suggested that an inhibition of PEPCK may explain the decreased flux through PC as a result of feedback inhibition, possibly through the increased mitochondrial and cytosolic concentrations of oxaloacetate. Again, no characterization of biotinylation of the enzymes was performed.

We hypothesize that administration of endotoxin to rats will induce changes in biotin metabolism. These changes will provide possible mechanisms for the known alterations in carbohydrate, protein, and fatty acid metabolism that follow endotoxin exposure. We further hypothesize that a reduced biotin status will modify the described normal effects of endotoxin on biotin metabolism. Overall immune function will be monitored by the flux of Zn and glucose into and out of the serum. We hypothesize that biotin deficiency during endotoxin challenge will delay the recovery of serum Zn and glucose. Although less sensitive to LPS than humans, the rat is a good model of human biotin metabolism. The egg-white fed rat is the most commonly used model of human

biotin deficiency. This model has been shown to mimic the human profiles of excretion of biotin and its metabolites into the urine (84), and lack of excretion into the bile (85).

CHAPTER 2
GENERAL MATERIALS AND METHODS

Preparation of Radiolabeled and Nonradiolabeled Bisnorbiotin and Biotin Sulfoxide Standards

Radiolabeled and nonradiolabeled BNB and BSO metabolites serve as both chromatography retention time standards (radiolabeled) and as independent quantitation standards (nonradiolabeled) for unlabeled metabolites when used with the competitive assay. Labeled and unlabeled metabolites were prepared by the same method, except [^{14}C] biotin or [^{3}H] biotin was used for radiolabeled metabolites. Adapted from Mock 1997 (86).

Bisnorbiotin

The fungal strain, Rhodotorula rubra, converts biotin to BNB through β-oxidation of the valeric acid side chain. Two 3 ml subcultures (one each for radiolabeled conversion and unlabeled conversion) of R. rubra (American Type Culture Collection, Manassas, VA) were grown in YPD broth (10 g yeast extract, 20 g peptone, 20 g dextrose per liter) plus 9 g KH_2PO_4, 0.3 g $CaCl_2$, 0.5 mg $MgSO_4 \cdot 7\ H_2O$, and 6 g $(NH_4)_2SO_4$ (per L). Cells were collected, washed, and resuspended in 1 ml phosphate buffered saline (PBS, 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 per liter, (pH 7.4)) containing 87.5 mCi of [^{14}C] biotin (radiolabeled, Amersham Pharmacia, Piscataway, NJ, 54 mCi/mmol) or 3 μM biotin (unlabeled) and the culture incubated at 24-25^{o}C for 48-hr while shaking at 190-200 rpm. Cells were then centrifuged and the supernatant

containing [^{14}C] or unlabeled BNB collected. Unlabeled BNB was purified by high performance liquid chromatography (HPLC) as described below.

Biotin Sulfoxide

Biotin sulfoxide was synthesized through oxidation of the sulfur atom of the tetrahydrothiophene ring with hydrogen peroxide in an acidic environment. Equal volumes of acid peroxide (0.1 M HCl, 1% v/v H_2O_2) and 100 μM [^{3}H] (32 Ci/mmol) or unlabeled biotin were mixed and incubated for 24-hr at room temperature. The reaction was stopped by the addition of 1.5 volumes 0.1 M NaOH. The standards were dried under N_2 at 37^{o}C and resuspended in 10 mM KP_i, pH 7.0.

Biotin, Bisnorbiotin, Biotin Sulfoxide, and Biocytin Separation by High Performance Liquid Chromatography

This procedure was adapted from Mock 1997 (86). Prior to HPLC separation, all tissue cytosols and sera were ultrafiltered using a centrifugal filtration device with a nominal molecular weight cut off (NMWCO) of 5 kDa (Millipore, Billerica, Massachusetts). This removes all protein bound biotin so that only free biotin is separated and subsequently measured. Separation of biotin and its metabolites from a sample was carried out by HPLC (Waters, Milford, MA) using a reversed phase C18 SphereClone 5m column (250 x 4.6 mm, Phenomenex, Torrance, CA) initially equilibrated in 0.05% (v/v) trifluoroacetic acid (TFA) adjusted to pH 2.5 with ammonium acetate (solution A). A constant flow rate of 1 ml/min was used throughout the separation. The sample was first acidified with 1/10 volume 6 N HCl and then injected into the HPLC. A linear gradient was then started with the subsequent mixing of solution A with acetonitrile-0.05% TFA (1:1, v/v) (solution B). This gradient begins at 0% (v/v) solution B and reaches 40% (v/v) by 35 min. Nonpolar constituents of the sample were

eluted from the column after the analytical gradient by increasing to 100% solution B over 5 min and holding at 100% solution B for 5 min. The column was then re-equilibrated to initial conditions by returning to 100% A for 10 min. The system was then ready for injection of the next sample. Total run time was 60 min/sample.

The fractions in which biotin, BNB, and BSO were resolved were determined by retention times of the radiolabled standards prepared above, injected prior to separation of samples. There is no radiolabeled standard available for biocytin, so its retention time was determined by measurement of each fraction by the competitive assay described below. Since each metabolite has a discrete retention time, the fraction(s) corresponding to that time were collected. If a radioactive sample was run, the fractions were collected and counted in a liquid scintillation counter (Beckman). If the sample was not radioactive, the fractions were dried under N_2 to remove the chromatography solvents and resuspended in HEPES buffer (0.1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0, 1M NaCl) before measuring by competitive assay (see Competitive Assay of Biotin).

Preparation of Biotinylated Bovine Serum Albumin

Biotinylated bovine serum albumin (BBSA) was used in the competitive assay for biotin in which it competes against biotin in the sample for avidin binding as described in Mock 1997 (86). Biotin N-hydroxysuccinimide ester (60 mg, NHS-biotin) was dissolved in 5 ml of dimethyl sulfoxide (Sigma, St. Louis, MO) and added to 500 mg of fatty acid free bovine serum albumin (fafBSA, Sigma) dissolved in 50 ml of ice cold 0.1 M $NaHCO_3$, pH 7.5. This was incubated overnight at 4^{o}C while stirring and then placed in dialysis tubing (30,000 NMWCO, Fisher Scientific, Pittsburgh, PA) and dialyzed against

PBS for 48-hr at 4°C with gentle stirring. The PBS was changed every 12-hr during the 48-hr incubation.

Competitive Assay of Biotin

The measurement of biotin and its metabolites BNB, BSO, and biocytin in all samples was performed after HPLC separation (86). Each well of a 96-well microplate was coated with 200 µl of a 1:50 dilution of BBSA to coating buffer (50 mM bicarbonate, pH 9.0, 2.5 mg/ml BSA). The plate was covered and incubated at 4°C for 1-hr to 4 days. After coating, the plate was washed twice with HEPES buffer (0.1 M HEPES, pH 7.0, 1 M NaCl). To a separate plate, 100 µl of biotin or metabolite standard (1500, 750, 375, 186.5, 93.25, 46.63, 23.32, 11.67 pM), unknown from HPLC separation, or HEPES buffer (blank) was added. Avidin buffer (50 µl; NeutrAvidin-HRP (Pierce, Birmingham, AL) diluted 1:25,000 in 0.1 M HEPES, pH 7.0, 1 M NaCl, 0.1% (w/v) BSA) was added to each well and mixed thoroughly. The plate was incubated for one hr at room temperature. The contents of these wells (100 µl) were transferred into the corresponding wells of a BBSA coated plate that had been washed three times with 0.05% Tween 20 (Fisher Scientific Pittsburgh, PA) and incubated at room temperature for one hr. The plate was washed three times with 0.05% Tween 20. Substrate solution (200 µl, 0.1 M citric acid, 0.2 M sodium phosphate, final pH 5.0, 4.5×10^{-3} M o-phenylenediamine (Fisher Scientific), 0.012% (v/v) H_2O_2) was added and the plate incubated for 45 min. At exactly the end of 45 min 100 µl of 2 M H_2SO_4 was added and the absorbance in the wells measured at 490 nm – 650 nm in a spectrophotometer (Molecular Devices, Sunnyvale, CA).

Preparation of Avidin AlexaFluor 430

NeutrAvidin (Pierce), an isoelectrically neutral and deglycosylated form of avidin, was conjugated to the succinimidyl ester form of AlexaFluor 430 (Molecular Probes, Eugene, OR). NeutrAvidin, 10 g/L in 50 mM sodium bicarbonate (pH 8.3), was mixed 5:1 (v/v) with a solution of AlexaFluor 430 (10 g/L) in dimethyl sulfoxide (DMSO) while vortexing. The solution was incubated while slowly vortexing for 1-hr at room temperature. Unconjugated dye was removed by size exclusion chromatography over a DG-10 column (Bio-Rad, Hercules, CA) equilibrated with PBS (20 mM sodium phosphate, pH 7.2, 150 mM NaCl). Using PBS, the conjugate NeutrAvidin-AlexaFluor 430 was eluted from the column and the fractions with the highest absorbance at 280 nm combined. Sodium azide (0.02% w/v) was added for preservation, and the conjugate stored at 4^{o}C protected from light.

Markwell Assay for Protein Concentration

Protein concentrations were measured using the modified Lowry procedure known as the Markwell Assay (87). Tissue samples were first homogenized in 10 volumes of HEM (300 mM mannitol, 10 mM HEPES, 1 mM EDTA). Standards (0, 0.025, 0.05, 0.075, 0.1 mg BSA) or sample were added to test tubes in duplicate and brought to a final volume of 100 µl with Milli Q water. Solution C (1 ml, 100:1 solution A (2% Na2CO3, 0.4% NaOH, 0.16% Na+, K+ Tartrate, 1.0% Sodium Dodecyl Sulfate (SDS) (w/v) to solution B (4% CuSO4)) was then added to each test tube and incubated at room temperature for 10 min. Folin phenol (100 µl, 1 N) was then added and incubated for 45 min. The absorbance at 650 nm was then measured in a spectrophotometer (Molecular Devices).

Carboxylase Separation by SDS-PAGE

Acetyl CoA Carboxylase

Acetyl CoA carboxylase isoforms 1 and 2 were separated using a 3% stacking gel (0.125 M Tris HCl, pH 6.8, 0.1% SDS, 3% acrylamide, 0.05% APS, 30 µl tetramethylethylenediamine (TEMED, Fisher Scientific)) and a 5% separating gel (0.375 M Tris HCl, pH 8.8, 0.1% SDS, 5% acrylamide, 0.05% ammonium persulfate (APS), 30 µl TEMED). Sample homogenate (75 µg total protein) was diluted into 20 µl of sample dilution buffer (0.375 M Tris HCl, pH 6.8, 0.23% SDS, 35% (v/v) glycerol, 0.035 mg/ml bromophenol blue, 1.43 mM β-mercaptoethanol). The sample was incubated at 37^{o}C for 5 min and then loaded onto the gel in an electrophoresis apparatus (Gibco BRL, Gaithersburg, MD) filled with running buffer (0.025 M Tris base, 0.2 M glycine, 0.1% SDS). The gel was electrophoressed at 37 volts overnight or until the dye front was approximately 1 cm from the bottom.

Pyruvate Carboxylase

Pyruvate carboxylase was separated as ACC1 and 2 except that 10% acrylamide was utilized in the separating gel.

Methylcrotonyl-CoA Carboxylase, and Propionyl-CoA Carboxylase

Methylcrotonyl-CoA carboxylase and PCC were separated as ACC1 and 2 except that 8% acrylamide and a pH of 8.0 was utilized in the separating gel. This allows the separation of MCC and PCC despite their close molecular weight.

Detection of Carboxylases After SDS-PAGE

Each gel was equilibrated in cold transfer buffer (0.03M Tris base, 0.2 M glycine, 60% (v/v) methanol) for 5-15 min. The gel was then transferred onto polyvinylidene fluoride (PVDF, Millipore, Billerica, MA) using an electroblotter (Idea Scientific,

Corvallis, OR) at 12 volts for 2-hr. The PVDF was then stained with amido black stain (50% (v/v) methanol, 10% (v/v) glacial acetic acid, 0.02% (w/v) naphthol blue black) and then destained with amido destain (50% (v/v) methanol, 10% (v/v) glacial acetic acid). The blot was rinsed in methanol and allowed to dry.

The blot was then blocked for 10 min in 0.5% nonfat dry milk (NFDM)- TBST (0.5% NFDM, 20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4). NeutrAvidin – alkaline phosphatase (Pierce, ACC1 and 2 and P61; 1:1000 dilution) or NeutrAvidin-AlexaFluor 430 (PC, MCC, and PCC; 1:500 dilution) was then added and incubated for 45 min. The blot was washed 3 x 5 min in TBST no NFDM, and then developed using ECF (Amersham Pharmacia, ACC1 and 2 and P61 only; PC, MCC, and PCC are scanned directly with no substrate addition). The blot was then scanned on a fluorescent imager (STORM 840, Molecular Devices).

Activity Assays of Pyruvate Carboxylase and Methylcrotonyl CoA Carboxylase

Adapted from Suormala et al, 1985 (88).

Pyruvate Carboxylase

Pyruvate carboxylase activity was determined through the measurement of [^{14}C] bicarbonate incorporated into the end product of the reaction catalyzed by pyruvate carboxylase, oxaloacetate. Approximately 100 μg total protein (25 μl) from rat liver homogenized in 50 volumes HEM, was mixed with 100 μl PC activity master mix containing a final concentration of 100 mM Tris HCl (pH 8.0), 3.8 mM $MgCl_2$, 0.5% triton X-100 (v/v), 0.32 mM acetyl CoA, 3.14 mM ATP, 0.5 mM [^{14}C] bicarbonate, 3.5 mM bicarbonate, and 15 mM pyruvate. Pyruvate was left out of the master mix for blanks. The samples were incubated for 15 min at 37^{o}C in a water bath. The reaction

was stopped through protein precipitation by the addition of 50 µl of 20% trichloroacetic acid (TCA). The samples were centrifuged at 10,000 x g (Beckman) for 5 min to pellet the precipitated protein. The supernatant was removed and placed into scintillation vials. TCA (100 µl of a 10% solution) was added to the protein pellet to remove any remaining radioactivity. The wash was combined with the supernatant and then evaporated at 65^{o}C under a constant stream of N_2. The evaporated samples were resuspended in 500 µl of water and 3 ml of scintillation cocktail was added. The amount of [^{14}C] labeled oxaloacetate was then determined in a liquid scintillation counter.

Methylcrotonyl CoA Carboxylase

Methylcrotonyl CoA carboxylase activity was determined through the measurement of [^{14}C] bicarbonate incorporated into the end product of the reaction catalyzed by methylcrotonyl CoA carboxylase, methylglutaconyl CoA. The assay was identical to the PC assay except for the master mix. The MCC master mix contained 100 mM Tris HCl (pH 8.0), 100 mM KCl, 0.75 mM dithiothreitol, 6 mM $MgCl_2$, 0.1% triton X-100 (v/v), 0.5 mM EDTA, 31.4 mM ATP, 0.5 mM [^{14}C] sodium bicarbonate, 1.5 mM sodium bicarbonate, and 2.8 mM methylcrotonyl CoA.

Isolation of P61 by Monomeric Avidin Column

Immobilized monomeric avidin (Pierce) was first poured into a plastic column (15 x 85 mm) and allowed to settle. The column was flushed with 10 ml PBS (0.1 M NaP_i (pH 7.2), 0.15 M NaCl). The column was washed 3 x 5 ml with biotin blocking and elution buffer (PBS + 2 mM biotin) to block any irreversible biotin binding sites. The column was then washed with 3 x 10 ml of regeneration buffer (0.1M glycine (pH 2.8)) to remove reversibly bound biotin. Finally, the column was equilibrated with 3 x 10 ml

of PBS. P61 containing supernatant (5 ml) was then placed onto the column and incubated at room temperature for 1-hr. The column was then washed with 8 x 5 ml PBS until all unbound protein was removed (no absorbance detected at 280 nm). Bound biotin containing proteins and peptides were then eluted with 8 x 5 ml biotin blocking and elution buffer until no absorbance was detected at 280 nm. The column was then regenerated with 4 x 5 ml regeneration buffer. The biotin containing fractions (80 µl of sample) were then separated on a 10% SDS-PAGE (pH 8.8), transferred to PVDF, and stained with amido black (50% (v/v) methanol, 10% (v/v) glacial acetic acid, 0.02% (w/v) naphthol blue black) and then destained with amido destain (50% (v/v) methanol, 10% (v/v) glacial acetic acid). Stained fractions containing P61 were concentrated 4 fold and 240 µl of concentrate separated on a 10% SDS-PAGE (pH 8.8). The gel was stained for 3-hr in coomassie stain (50% methanol, 6% glacial acetic acid, 0.05% coomassie blue G250 (w/v)) and destained overnight (10% methanol, 15% glacial acetic acid).

CHAPTER 3
DIETARY BIOTIN INTAKE MODULATES THE POOL OF FREE AND PROTEIN-BOUND BIOTIN IN RAT LIVER

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Introduction

Biotin is present in organisms in two distinct forms, i.e. unassociated (free), and protein bound. In the protein-bound form, biotin is covalently bound to the ε-amino group of lysine residues occurring in a specific amino acid motif (89). In contrast, free biotin exists as a distinct pool in several compartments, including urine, plasma, and tissue. Free biotin undergoes substantial metabolism in lower as well as higher organisms (90-94). In urine, biotin and its associated metabolites are all free due to normal glomerular filtration of protein. The majority (~90%) of serum biotin is also free, with the remainder either reversibly or covalently bound (27,95). Indirect evidence for an intracellular pool of free biotin has been demonstrated in several ex vivo systems, including cultured hepatocytes and peripheral blood mononuclear cells (29,96). The free biotin pool in the tissues of intact animals has not been previously characterized.

Biotin deficiency markedly reduces urinary biotin excretion, with onset beginning ~2-3 weeks after consumption of a biotin-free diet (72). In a similar manner, biotin deficiency effectively reduces serum and plasma biotin levels (72-74). The depleting effect of biotin deficiency on tissue biotin levels, including brain, liver and pancreas, has also been analyzed in several studies (97-99). As might be expected, consumption of a biotin-supplemented diet or administration of biotin markedly elevates urinary biotin

excretion as well as serum or plasma levels in both humans and rodents (50,100). Biotin supplementation also raises the concentration of the biotin metabolites, including biotin sulfoxide and bisnorbiotin (101). To our knowledge, the effect of dietary biotin intake on the free pool of biotin in tissues has not been investigated.

The relationship between dietary biotin intake and protein bound biotin has also not been analyzed. Biotinylation is required for carboxylase function, and therefore conditions that reduce protein biotinylation reduce enzymatic activity (72,97,98). It has been proposed that during biotin deficiency, a pool of apocarboxylases is present because the administration of biotin to biotin-deficient rats results in a rapid restoration of the abundance of biotinylated polypeptide (75).

We sought to determine whether alterations in dietary biotin intake regulate tissue biotin pools in a manner similar to plasma and urine. In this study, the free pool of biotin in the liver and its relationship to protein biotinylation were analyzed separately in biotin-deficient, biotin-adequate, biotin-supplemented, and pharmacologically supplemented rats. The results have implications for understanding the relationship between dietary biotin intake and the maintenance of biotin-dependent cellular function in higher organisms.

Previous studies of biotin metabolism have used a purified diet with spray-dried egg-white as the sole protein source and a biotin level of ~2 mg biotin/kg diet. Avidin which is present in the egg-whites, binds ~ 1.44 mg of biotin/kg diet. This leaves ~0.6 mg biotin/kg diet available for absorption by the rat. This level equals a consumption of 900 ng biotin/day for a 0.15 kg rat, which is equivalent to a 70 kg human consuming 4.2 mg biotin/day. The biotin requirement for both the rat and human is unknown; however,

this equivalent level of consumption in humans is 45 times higher than the estimated safe and adequate daily dietary intake (ESADDI) of 30-100 μg/day for humans. The purpose of this study was to determine if a relative physiologic dose of biotin, more equivalent to the human ESADDI, was adequate to prevent biotin deficiency in the rat.

Table 3-1. Modified AIN 76A diet

INGREDIENT	g/1000 g
Egg-White	200
Corn Starch	150
Sucrose	503
Cellulose	50
Corn Oil	50
AIN 76A Mineral Mix	35
AIN 76A Vitamin Mix (without biotin and choline)	10
Choline Bitartrate	2
Biotin (deficient)	0
Biotin (adequate)	1.5
Biotin (supplemented)	2.04
Biotin (pharmacologic)	101.44

Methods

Animals and Dietary Treatments

Purified biotin-free rodent diet based on a modified AIN 76A formulation was obtained from Research Diets (New Brunswick, NJ). Although similar to diets used in other studies on biotin nutriture, this diet differs significantly from those used in other studies in terms of carbohydrate source and amount, fatty acid composition and some

vitamins and minerals (72-75,102,103). This diet has been modified to include spray-dried egg-white as its sole protein source (Table 3-1). The protein avidin found in egg-whites binds ~1.44 mg biotin/kg diet, inhibiting absorption (104). The levels of dietary biotin designated in this study represent the amount of biotin in the diet in excess of the binding capacity of avidin in the diets. Male Sprague-Dawley rats (50-75 g initial weight, n=20), obtained from Harlan (Indianapolis, IN), were housed individually in hanging wire-bottom cages in an environmentally controlled room with constant temperature (22^{o}C) and a 12-hr light:dark cycle. After a four-day acclimation period during which all rats consumed the 0.6 mg biotin/kg diet, rats were randomly assigned to one of five groups (n=5/group) consuming an AIN 76A-based egg-white powdered diet containing 0 mg biotin/kg diet (deficient), 0.06 mg biotin/kg diet (adequate), 0.6 mg biotin/kg diet (supplemented), or 100 mg biotin/kg diet (pharmacologic). The diets were consumed for three weeks. Body weight and food intake were measured three times weekly. Twenty-four hr before the end of the study, the rats were placed into metabolic chambers to allow the discrete collection of urine for biotin analysis. Rats were anesthetized under halothane vapor and killed by exsanguination. The University of Florida Institutional Animal Care and Use Committee approved all procedures.

Sample Preparation and Analysis

Blood was withdrawn using an EDTA-coated syringe to inhibit clotting and centrifuged at 10,000 x g for 10 min to collect plasma. For plasma biotin, samples were ultrafiltered using a 5000 dalton nominal molecular weight cut-off (NMWCO) filter (Millipore, Bedford, MA) centrifuged at 12,000 x g for 1-hr at 4^{o}C. This removes all protein bound biotin allowing for a true free biotin measurement. Liver (~500 mg) was removed and homogenized in 10 volumes ice-cold homogenization buffer (HEM, 300

mM mannitol, 10 mM HEPES (pH 7.2), 1 mM EDTA, and 1:100 (v/v) protease inhibitor cocktail (Sigma, St. Louis, MO)) and centrifuged at 200,000 x g for 30 min at 4^{o}C. The supernatant was also ultrafiltered as above using a 5000 dalton NMWCO filter before the competitive binding assay. The pellet was resuspended in homogenization buffer to a concentration of ~40 g/L. All samples were immediately frozen in a mixture of dry ice and isopropanol and stored at –80^{o}C until analyzed.

Measurement of Free Biotin

The measurement of free biotin in urine, plasma, and liver was performed using the coupled HPLC/competitive binding assay as described previously in Chapter 2.

Measurement of Protein-Bound Biotin

Using the avidin blotting techniques described in Chapter 2, we specifically detected five distinct proteins in the liver using a fluorescent imager, corresponding to the five carboxylase enzymes ACC1, ACC2, PC, MCC, and PCC. We have validated the limits of detection of the carboxylases using a fluorescent imager. To determine sensitivity of our three detection methods (NeutrAvidin-AlexaFluor 430, NeutrAvidin-HRP/ECL-Plus, NeutrAvidin-AP/ECF), we resolved varying amounts of BBSA (0.0001-1 ng; produced in Chapter 2) by SDS-PAGE, transferred the gel to PVDF, and visualized the BBSA using the appropriate reagent system. The NeutrAvidin-AlexaFluor 430 proved to be the least sensitive of the three methods, being able to detect to 0.245 ng BBSA (Figure 3-1A). The NeutrAvidin-HRP/ECL-Plus system exhibited much higher sensitivity, and was able to detect to 0.004 ng BBSA (Figure 3-1B). The NeutrAvidin-AP/ECF system demonstrated the most sensitivity, as evidenced by the ability to detect 0.0009 ng BBSA.

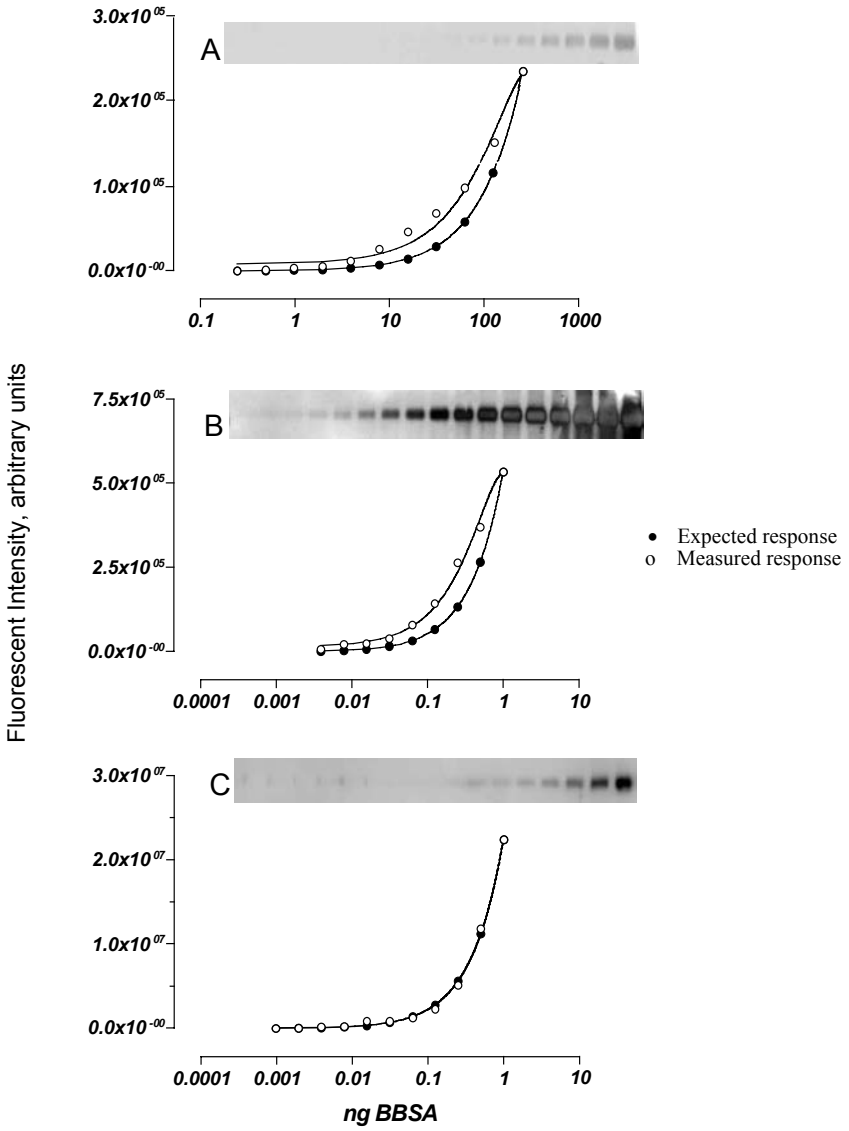


Figure 3-1. Detection and quantification of biotinylated bovine serum albumin by three fluorescent methods. A. Avidin-AlexaFluor 430, B. Avidin-HRP/ECL-Plus, C. Avidin-AP/ECF.

In order to assess the detection and quantification of biotinylated proteins in complex protein mixtures, the linearity and sensitivity of the three detection systems was analyzed in rat liver extracts. Rat liver endogenously expresses the biotinylated enzymes ACC1 and 2, PC, MCC, and PCC. Although the exact abundance of these proteins is not known in relation to the total amount of protein resolved by the gel, the molar ratio of protein biotinylation is known to be 1:1, since only one biotinylation site exists in each of

these enzymes (89). Various amounts of rat liver homogenate extracts (100 - 0.78 μg by serial dilution for NeutrAvidin – AlexaFluor 430 and the ECL-Plus system, 25-0.091 μg by serial dilution for the ECF system) were resolved and transferred to PVDF prior to being probed by one of the detection systems. Blots were then scanned and quantified using a fluorescent imager.

The NeutrAvidin-AlexaFluor 430 conjugate was able to detect pyruvate carboxylase, methylcrotonyl CoA carboxylase, and propionyl CoA carboxylase down to a mass of 1.56 μg total protein, but failed to detect either isoform of ACC. For the carboxylases that were detected, the observed response was linear for pyruvate carboxylase and fit a second order polynomial curve for the methylcrotonyl CoA and propionyl CoA carboxylase bands (Figure 3-2A). As expected, the ECL-Plus system exhibited a much higher yield of fluorescence, but sensitivity was not markedly increased for any of the carboxylases (1.56 μg total protein; Figure 3-2B). Again, ECF exhibited the highest yield of fluorescence and sensitivity (0.19 μg total protein) compared to NeutrAvidin-AlexaFluor 430 and the ECL-Plus system (Figure 3-2C). These results suggested the use of NeutrAvidin-AlexaFluor 430 to detect carboxylases present in high abundance, i.e. PC, MCC, and PCC. For the lower abundant isoforms of ACC, these results suggest the use of NeutrAvidin-AP/ECF due to its ability to detect very low levels of biotinylated proteins.

In this study, protein-bound biotin was measured as described in Chapter 2. Pyruvate Carboxylase, MCC, and PCC were all measured using NeutrAvidin-AlexaFluor 430. Acetyl CoA carboxylase isoforms 1 and 2 were measured using NeutrAvidin-AP/ECF.

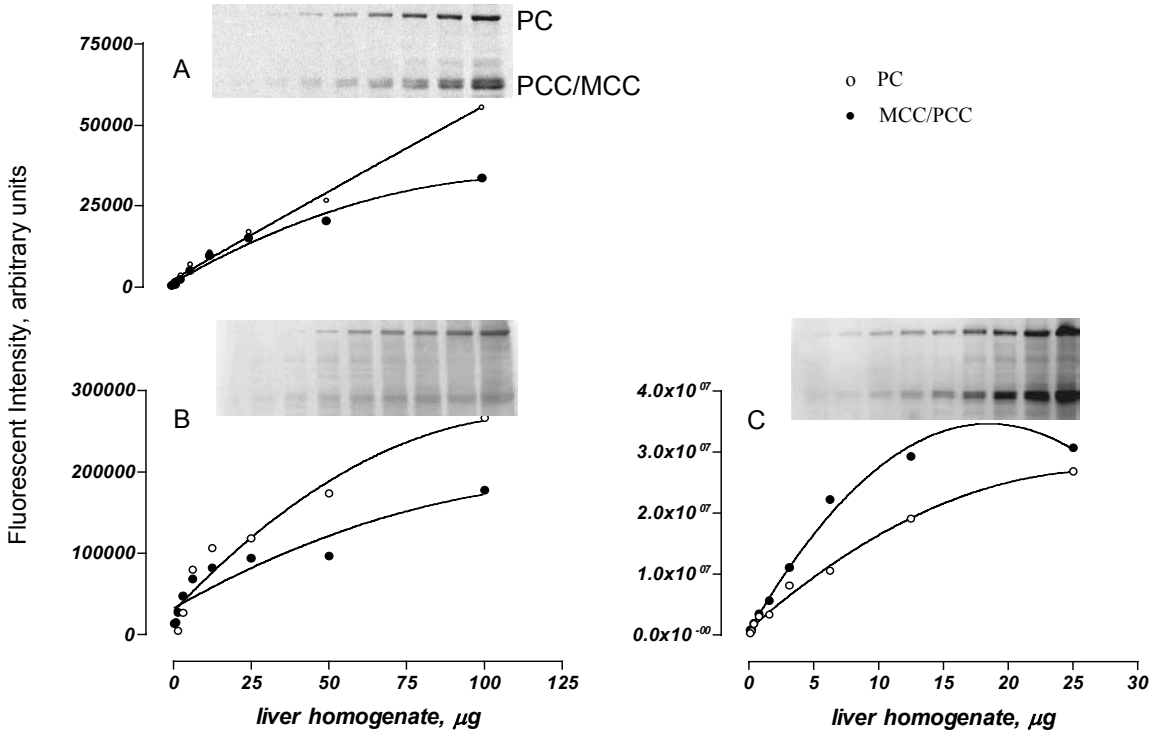


Figure 3-2. Detection and Quantification of biotinylated polypeptides in rat liver homogenate. A. Avidin-AlexaFluor 430, B. Avidin-HRP/ECL-Plus, C. Avidin-AP/ECF .

Statistical Analysis

Results are expressed as means ± SEM. The significance of differences ($P<0.05$) was tested by one-way ANOVA with Newman-Keuls post test.

Results

Effect of Dietary Biotin Intake on Rat Growth and Food Intake

At the observed rate of food intake, rats consumed 0 (deficient), 0.9 (adequate), 9 (supplemented), or 1502 (pharmacologic) µg biotin/day. The dietary biotin level in the AIN 76A had no significant effect on either growth or food intake (Figure 3-3). Additionally, no outward signs of biotin deficiency were found in rats consuming the biotin-free diet. Toxicity was not evident in pharmacologically supplemented rats.

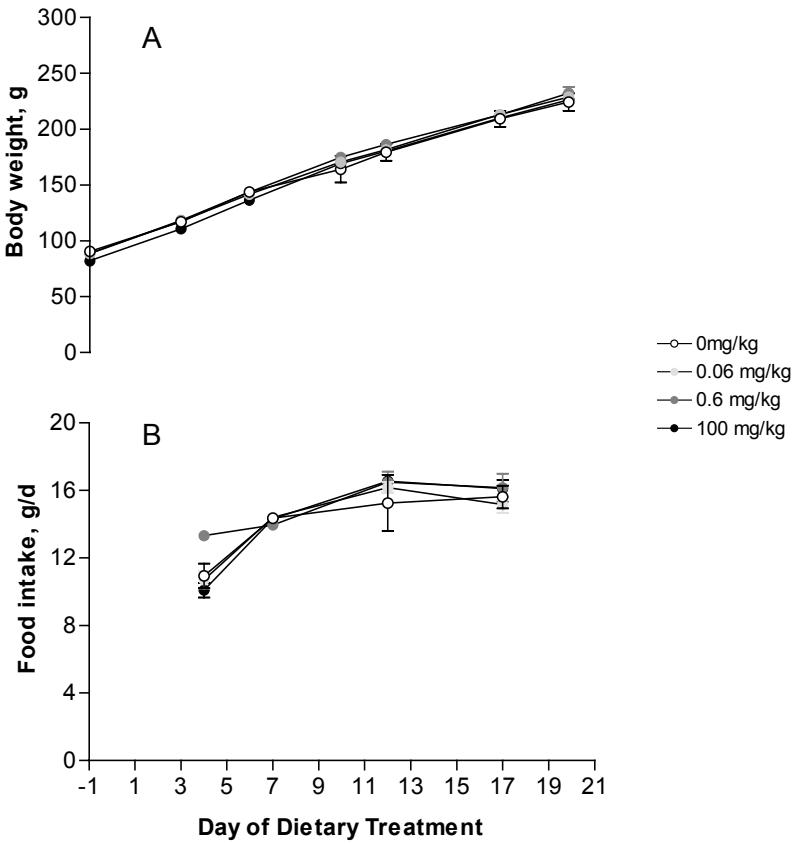


Figure 3-3. Effect of dietary biotin intake on rat growth (A) and food intake (B).

Effect of Dietary Biotin Intake on Biotin Status

Plasma biotin concentration increased with dietary biotin intake (Figure 3-4A). Biotin deficient rats exhibited ~10% the biotin concentration found in biotin-adequate rats ($P<0.05$, 2.0 ± 0.9 and 38.2 ± 11 nmol/L, respectively). Biotin-supplemented rats exhibited a 3.6 fold increase in plasma biotin concentration over biotin-adequate rats ($P<0.05$, 38.2 ± 11 and 176.7 ± 16.3, respectively). Rats consuming the pharmacologic level of biotin exhibited a 137 fold elevation in plasma biotin ($P<0.001$, $5{,}239 \pm 270$ nmol/L) compared to biotin-adequate rats.

The concentration of hepatic free biotin, normalized to wet organ weight, was 32 ± 7 pmol/g tissue in biotin-adequate rats (Figure 3-4B). Biotin-deficient rats exhibited a

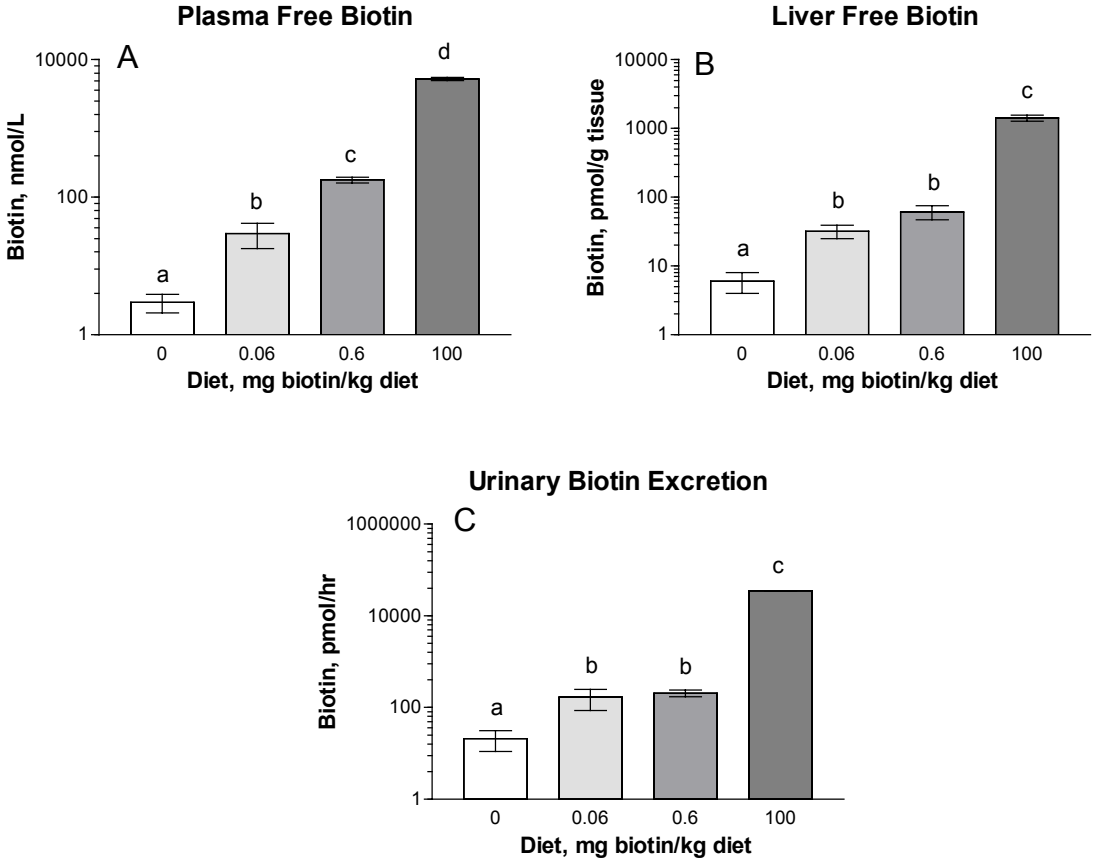


Figure 3-4. Plasma (A), Liver (B), and Urinary (C) biotin levels following consumption of varying levels of dietary biotin for 3 weeks. Bars with different letters are significantly different ($P<0.05$).

78% reduction in free biotin ($P<0.05$, 6 ± 2 pmol/g tissue). Unlike plasma free biotin, hepatic free biotin did not differ between biotin-adequate and biotin-supplemented rats (32 ± 7 and 61 ± 14 pmol/g tissue). Pharmacologically supplemented rats had a 44 fold elevation in free liver biotin concentration compared to biotin-adequate rats ($P<0.01$, 1414 ± 138 pmol/g tissue).

Rats consuming the biotin-free diet for 21 days excreted significantly less biotin in the urine compared with biotin-adequate rats ($P<0.05$, 21 ± 10 and 168.4 ± 82 pmol/hr, respectively) (Figure 3-4C). Rats consuming the diet containing a pharmacologic level of biotin had a greatly increased urinary excretion rate ($P<0.001$, 35,090 ± 59 pmol/hr). As

with liver free biotin, there was no significant difference in the urinary biotin excretion rate between biotin-adequate and biotin-supplemented rats (168.4 ± 82.6 and 206.2 ± 36 pmol/hr, respectively).

Effect of Dietary Biotin Intake on the Relative Abundance of Biotinylated Carboxylases

The effect of dietary biotin intake on the relative abundance of biotinylated polypeptides was assessed using avidin blotting. Under these SDS-PAGE conditions, MCC and PCC could not be separated and so results for these carboxylases are presented as the combined fluorescence of MCC and PCC labeled MCC/PCC. Rats consuming

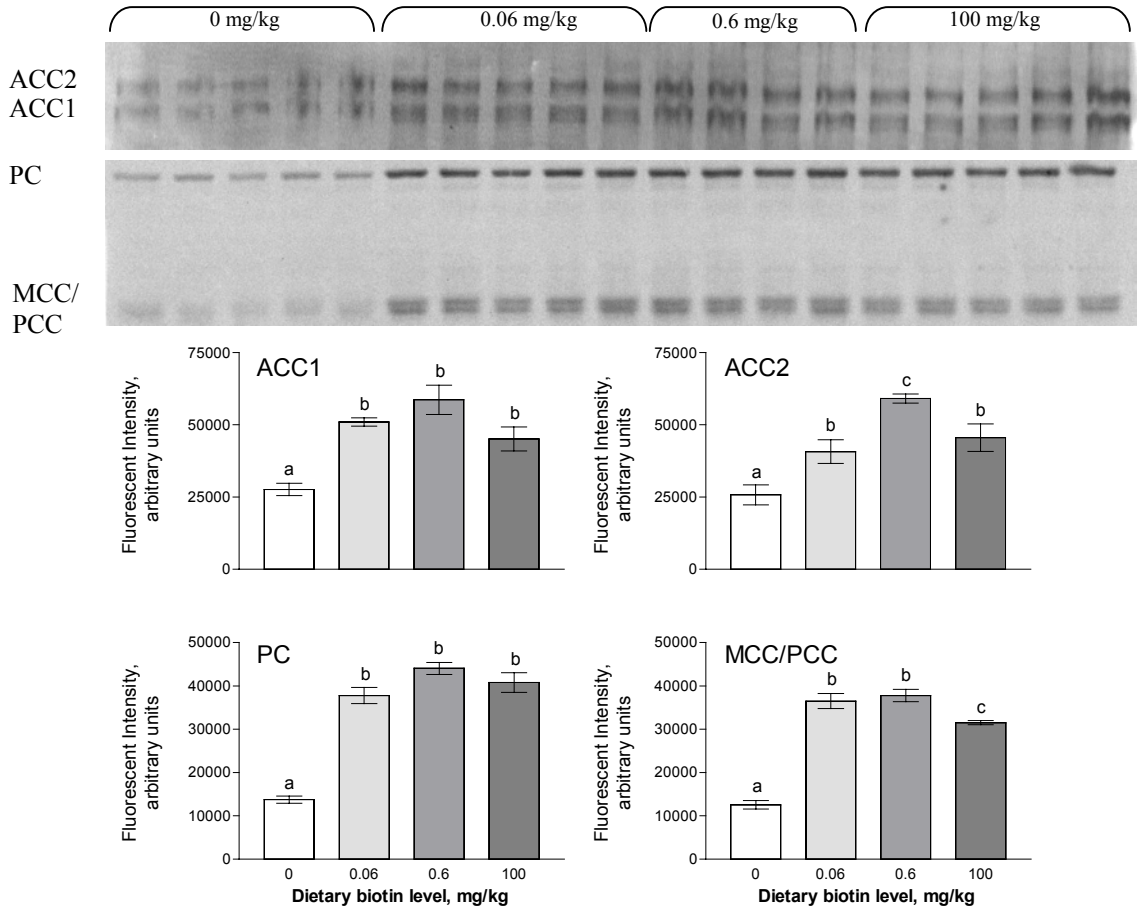


Figure 3-5. Relative abundance of biotinylated ACC1, ACC2, PC, and MCC/PCC following consumption of varying levels of dietary biotin for 3 weeks. Bars with different letters are significantly different ($P<0.05$).

biotin-free diets had less of the biotinylated forms of all carboxylases after 21 days ($P<0.05$). The magnitude of the reduction ranged from 50% for ACC isoforms 1 and 2, to 60% for PC, and MCC/PCC (Figure 3-5). The abundance of ACC isoform 2 was significantly elevated by 40% in biotin-supplemented rats compared to biotin-adequate rats ($P<0.05$). There was no difference in the abundance of biotinylated PC, MCC/PCC, and ACC1 between biotin-adequate and biotin supplemented rats. Pharmacologic biotin supplementation significantly reduced the abundance of biotinylated ACC2, and MCC/PCC ($P<0.05$) compared to biotin-supplemented rats.

Discussion

Despite the observation that frank signs of biotin deficiency are rare, there is growing appreciation of genetic, physiologic, and pharmacologic conditions that impair biotin status (36,38,44,105-108). This suggests that the lack of physiologic manifestations of biotin deficiency may not be a reliable measure by which to gauge biotin status in the general population. The effects of marginal biotin status, in the absence of physiologic signs of deficiency, have not been analyzed as well as those of more severe deficiency. In this study, the relationship between dietary intake and intracellular pools of free and protein-bound biotin was analyzed in a rat model of biotin nutriture. Our studies extend previous work in this area in three important ways: the use of physiologically relevant intakes, the use of marginal biotin deficiency, and the measurement of the free-biotin pool in the liver.

The experimental diets chosen in this study model several relevant states of biotin nutriture. Dietary biotin intake had no effect on growth rate or food intake, suggesting that severe deficiency was absent. This is in contrast to earlier studies in which loss of growth was observed relatively early in the dietary manipulation, and may represent an

improvement in the use of the AIN 76A diet design (72,73,109). In these experiments, rats fed the 0.06 mg biotin/kg diet exhibited an intake of ~0.9 μg biotin/d. This is equivalent to an intake of ~400 μg biotin/d for a 70 kg human. Although still four to ten times higher than the ESADDI, this is the lowest amount of biotin used in a biotin status study. A biotin supplemented condition was obtained in rats consuming the 0.6 mg/kg diet, or ~100 times the ESADDI. Previous to this study, this level of biotin was the lowest level used and considered an adequate intake. This level represents a supplemented dietary intake easily obtained in self-selected biotin supplements or in the treatment of biotin-responsive disorders, such as biotinidase deficiency or multiple carboxylase deficiency (110-112). To determine whether pharmacologic doses of biotin might lead to adverse effects on physiology, some rats were given a diet containing 100 mg biotin/kg, a dietary level that is the highest intake yet tested, equivalent to an intake of 667 mg for a 70 kg person.

Despite the lack of outward signs of deficiency, biochemical measurements clearly indicated a change in biotin status. We confirmed previous studies that demonstrated a direct relationship between dietary biotin intake and urinary biotin excretion (72,91). Urinary biotin excretion was significantly depressed in marginally biotin-deficient rats, suggesting the induction of a homeostatic mechanism attempting to conserve biotin pools. There was no difference in the urinary excretion of biotin in rats consuming either the adequate or supplemented diets, suggesting that biotin pools were not yet sufficiently large to spill over into the urine. Alternatively, biotin reabsorption capacity in the kidney might not be saturated, preventing an increase in urinary excretion. This was an unexpected finding because the adequate diet in our studies corresponded to

a relatively high human intake, and the supplemented diet represented a 10-fold increase over that level. It is possible that, consistent with earlier studies in humans, excess biotin was catabolized to BNB or BSO (100). Biotin catabolites were not measured in these studies. We observed the expected large increases in urinary biotin excretion and plasma biotin concentrations in rats consuming the pharmacologic doses of biotin, in agreement with human studies that demonstrate a rapid and large excretion of biotin in excess of required levels (100,101,113).

Plasma biotin pools were similarly influenced by dietary biotin intake. Marginal biotin deficiency was remarkably effective in reducing plasma biotin levels, suggesting that in this limited case, plasma biotin was a useful index of biotin status. In fact, plasma biotin might be argued to be a better marker of biotin status in this study because a significant elevation of plasma biotin was observed in the biotin-supplemented rats, in contrast to urinary biotin excretion for which no such difference was found. Whether such a conclusion can be extended to biotin-supplemented humans is unclear because, during biotin deficiency, urinary biotin excretion appears to be a more reliable indicator of biotin status (114). Plasma biotin was significantly elevated in pharmacologically supplemented rats compared with all other dietary groups, demonstrating that a very high circulating biotin concentration did not elicit any outward effects on physiology.

The soluble fraction of liver, which in our experiments reasonably represents the cytosolic cellular fraction, also demonstrated a free biotin pool responsive to dietary biotin intake. The intracellular biotin pool in biotin-deficient rats was 20% that in biotin-adequate rats. Like plasma biotin, it was possible to greatly expand the hepatic free biotin pool possibly available for protein biotinylation or other proposed functions of

biotin (115) using pharmacologic supplementation. Although high plasma biotin concentrations may have contaminated the liver samples and falsely elevated the biotin level, the ratio of liver to plasma free biotin (data not shown) was very small in all dietary groups, suggesting that contamination was not contributing substantially to the liver biotin measurement.

After establishing the biotin status of each dietary group, the effect of biotin intake on the abundance of biotinylated polypeptides was analyzed. We believe that changes in the abundance of biotinylated polypeptides are due to the deficiency per se, rather than changes in the apoprotein form of the polypeptide. Although we cannot rule out the latter possibility because Western blot analysis of each carboxylase was not performed, our assumption is supported by the following findings: 1) biotin deficiency does not alter the abundance of carboxylase mRNA, and 2) in biotin-deficient rats, large pools of apocarboxylases are present that are immediately available for biotinylation upon return of biotin to the system (20). Post-transcriptional regulation of MCC and PCC has been proposed, but the relevant studies did not directly analyze the amount of MCC or PCC polypeptide (75).

Biotin-deficient rats, even those not exhibiting outward signs of deficiency, have a significantly reduced abundance of the biotinylated form of all carboxylases. This demonstrates that loss of carboxylase activity is occurring before the overt signs of biotin deficiency emerge. Biotin-adequate rats, consuming close to the suggested human ESADDI, appear to demonstrate full biotinylation of PC, MCC, PCC, and ACC1 because biotin-supplemented rats did not have significantly more biotinylated carboxylases. Interestingly, we found a significant difference in the abundance of biotinylated ACC2

between biotin-adequate and biotin-supplemented rats, consistent with an earlier report of higher sensitivity of ACC biotinylation to dietary biotin supply (103). We find this observation to be of interest because it suggests that in rats consuming near the human ESADDI for biotin, the function of ACC2 may not be maximal. The physiologic or functional implications for incomplete biotinylation of ACC2 under presumably adequate conditions cannot be inferred from this study. Several other studies have clearly demonstrated alterations in fatty acid metabolism in biotin-deficient rats, but have usually focused on fatty acid biosynthesis rather than β-oxidation (116-127).

Another unexpected finding of this study was the effect of a pharmacologic intake of dietary biotin on protein biotinylation. We observed that in pharmacologically supplemented rats, the abundance of some biotinylated carboxylases was significantly reduced. Whether this represents a physiologically relevant detrimental effect of pharmacologic biotin intake has yet to be determined.

Changes in biotin status have been demonstrated to affect a range of metabolic processes, from changes in carboxylase activity to changes in the expression of nonbiotin-dependent enzymes such as glucokinase, ornithine transcarbamylase, and phosphoenolpyruvate carboxykinase (12,74,128-130). The sensitivity of this regulation has not been addressed, but the possibility that marginal biotin status at the cellular level regulates gene expression cannot be ruled out and is, in fact, supported by this work because marginal biotin deficiency and supplementation resulted in altered tissue free biotin pools. Finally, the use of rats as a model for biotin metabolism in humans has been validated (85,131); thus, our data suggest that our current knowledge and

recommendations concerning biotin intake and the maintenance of biotin function may not be in agreement and require further investigation.

CHAPTER 4
LIPOPOLYSACCHARIDE INDUCED INFLAMMATORY RESPONSE ALTERS CIRCULATING BIOTIN AND THE RELATIVE ABUNDANCE OF THE BIOTIN-DEPENDENT CARBOXYLASES

Introduction

Several lines of investigation provide evidence that biotin is required for normal function of the immune system, including antibody production, immunological reactivity, T and B cell differentiation, and cytotoxic T-cell response (49). Biotin's role in human immune function can be demonstrated in patients suffering from biotin-responsive multiple carboxylase deficiencies. Cowan et al., assessed the biotin responsiveness of three siblings who presented in early childhood with central nervous system dysfunction, candida dermatitis, intermittent ataxia, and alopecia (52). Analysis of urine samples from these individuals demonstrated elevated levels of β-hydroxypropionate, β-methylcrotonylglycine, and β-hydroxyisovalerate, strongly suggesting an insufficiency in biotin-dependent enzyme function. Two of the siblings expired ultimately to overwhelming infection, but the third improved after the administration of high doses of biotin. Concomitant with the improvement in pathology, the urinary organic acids drastically decreased.

A strong link between the importance of biotin in immune function has also been obtained from the case study of a 12 month old boy who exhibited multiple carboxylase deficiency likely due to biotinidase deficiency (132). This individual had decreased levels of prostaglandin E_2 (PGE_2), which was associated with an absence of monocyte suppressor activity. Prostaglandin synthesis is one facet of the downstream signal

transduction cascades in the inflammatory response. Oral biotin supplementation restored biotin dependent enzyme activity as well as PGE_2 levels, strongly suggesting an important role for the biotin dependent enzyme acetyl CoA carboxylase in these cells. Candidiasis is a common finding in biotinidase and multiple carboxylase deficiency patients, suggesting a generalized lack of immune competence (32,133-137).

Further evidence of the importance of biotin in the immune system comes from a recent study by Manthey and colleagues (54). This study evaluated the effects of biotin supply on cell proliferation, cell death, and IL-2 secretion in the Jurkat human T-cell line. While cell proliferation and death did not change between cells incubated in deficient, adequate, or pharmacologic levels of biotin, the concentration of IL-2 was inversely related to the concentration of biotin in the media. IL-2 produced by T-cells functions in an autocrine manner to stimulate T-cell proliferation as well as to potentiate the apoptotic cell death of antigen activated T-cells. IL-2 also stimulates the proliferation of natural killer cells and B-cells (138). It has now been determined that the higher levels of IL-2 seen in cells grown in deficient medium was due to a decrease in IL-2 receptor γ (55). This in part explains the lack of ability of high IL-2 levels to induce cell proliferation. In vivo, the differing levels of IL-2 receptor γ caused by deficient, physiologic, or pharmacologic amounts of biotin may have affected the proliferation rates of natural killer cells, B-cells, or proliferation rates and apoptosis of activated T-cells.

In a study of the effect of biotin status on immune function in rodents, experimentally induced biotin deficiency significantly reduced spleen size as well as the absolute number of spleen cells (139). Additionally, the percentage of immune cells presenting T and B-cell surface markers were altered. The percentage of cells expressing

surface immunoglobulin decreased significantly (B-cells), while the percentage of cells expressing CD3, CD4, and CD8 were increased significantly (T-cells). This suggests that biotin deficiency may affect B and T-cells differently. The decrease in cells expressing surface immunoglobulin could correlate with the decreased antibody production seen in biotin deficiency. Spleen cell proliferation stimulated by the mitogen concanavalin A was also decreased in biotin-deficient rodents.

While immune dysfunction during biotin deficiency is well known, investigations into the effect of an immune response on biotin metabolism are just now being researched. Recently, the uptake and metabolism of biotin in human lymphocytes has been investigated. Mitogen stimulation results in a substantial increase in biotin uptake by proliferating lymphocytes through an increase in the number of biotin transporters per cell; there is good evidence that the purpose of this increased intake is to support biotinylation of an increased abundance of biotin-dependent enzymes (96,115,140). While these in vitro data show biotin metabolism is changed during stimulation of immune cells, no research into the effect of an in vivo immune response on whole body biotin metabolism has been performed. The purpose of the present study was to determine if the metabolism and function of biotin are altered during an inflammatory response elicited by the administration of the bacterial endotoxin lipopolysaccharide, and how a marginal biotin deficiency modulates this response.

Methods

Materials

Male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Purified biotin-free rodent diet based on a modified AIN 76A formulation (described previously in Chapter 3) was obtained from Research Diets (New Brunswick, NJ). d-

Biotin, protease inhibitor cocktail, LPS (catalogue # L3129 lot # 20K4047), and o-phenylenediamine dihydrochloride were purchased from Sigma (St. Louis, MO); avidin-horseradish peroxidase (Avidin-HRP) and avidin-alkaline phosphatase (Avidin-AP) were purchased from Pierce Chemical Company (Birmingham, AL); 96-well microtiter plates (Nunc Maxisorb) and bovine serum albumin (BSA) were purchased from Fisher Scientific (Pittsburgh, PA). Enhanced chemifluorescence (ECF) reagent was purchased from Amersham-Pharmacia (Piscataway, NJ). Biotinylated BSA, and Avidin-AlexaFluor 430 conjugate were synthesized as previously described in Chapter 2.

Animals and Dietary Treatments

Male Sprague Dawley rats, 50-74 g initial weight, were housed individually in hanging wire-bottom cages in an environmentally controlled room with constant temperature (22^{o}C) and a 12-h light:dark cycle. The University of Florida Institutional Animal Care and Use Committee approved all procedures.

Dose-response experiments

Rats (n=20) were fed a modified AIN 76A diet containing 0.06 mg biotin/kg diet for one week prior to the study to standardize their biotin status as previously described (Chapter 3). This diet has been modified to include spray-dried egg-white as its sole protein source. The protein avidin, contained within the egg-white, binds ~1.44 mg biotin/kg purified diet. The amount of biotin expressed in the diet is the amount above that bound by avidin as determined by the HPLC – avidin binding assay. Rats were randomly assigned to receive i.p. 0, 0.1, 1, or 10 mg LPS/kg body weight dissolved in endotoxin free 0.9% saline. Rats were immediately placed in metabolic cages for discreet collection of urine for biotin and metabolite analysis. Rats were then anesthetized under halothane vapor and killed by exsanguination 6-hr post LPS injection.

Time-dependent and dietary status experiments

Rats (n=36) were acclimated on a modified AIN 76A diet described in Chapter 3 containing 0.06 mg biotin/kg diet for five days and then randomly divided into two groups: biotin deficient and biotin adequate. The biotin deficient diet was the modified AIN 76A diet used above without biotin. The biotin adequate diet was the same diet used during acclimation containing 0.06 mg biotin/kg diet. Rats consumed the diets for three weeks before being further divided into rats receiving 5 mg/kg LPS dissolved in 0.9% saline or 0.9% saline i.p. only. Three rats from each group (LPS deficient, saline deficient, LPS adequate, saline adequate) were then anesthetized under halothane vapor and killed by exsanguination 6, 12, or 24-hr after injection with LPS.

Sample Preparation

Dose-response experiments

Blood was withdrawn, allowed to coagulate for 30 min, and centrifuged at 10,000 x g for 5 min to collect serum. Liver (~500 mg) was removed and homogenized in 10 volumes of ice-cold HEM (300 mmol/L mannitol, 10 mmol/L HEPES (pH 7.2), 1 mmol/L EDTA, and 1:100 (v/v) protease inhibitor cocktail). A portion of the homogenate was set aside for protein biotinylation analysis and the remainder centrifuged at 200,000 x g for 30 min at 4^{o}C to collect the soluble fraction. All samples were immediately frozen in a mixture of dry ice and isopropanol and stored at –80^{o}C until needed.

Time-dependent and dietary status experiments

Blood was withdrawn, allowed to coagulate for 30 min, and centrifuged at 10,000 x g for 5 min to collect serum. The serum was immediately frozen and stored at –80^{o}C until needed. Liver was removed, divided into 6 equal pieces, immediately frozen on dry

ice, and stored at –80°C until needed. When needed, individual liver pieces were thawed and homogenized in 10 volumes of ice-cold HEM.

Competitive Binding Assay of Biotin

The measurement of biotin and its metabolites in urine, serum, and liver was performed with a coupled HPLC/competitive binding assay as previously described in Chapter 2.

Detection and Quantification of Biotinylated Proteins

The five biotin-dependent carboxylases, acetyl CoA carboxylase isoforms 1 and 2 (ACC1, ACC2), pyruvate carboxylase (PC), propionyl CoA carboxylase (PCC), and methylcrotonyl CoA carboxylase (MCC) were detected using the avidin blotting technique described previously in Chapter 2 with slight modifications. Because of their similar molecular weights, PCC and MCC are not separated under normal SDS-PAGE conditions (10% separating gel pH 8.8). Using an 8% separating gel at pH 8.0, we have separated these proteins from one another so that individual quantification of each band could occur.

Serum Zn and Serum Glucose

Serum Zn was measured using an AAAnalyzer (Perkin-Elmer, Shelton, CT) after a 1:4 dilution with highly purified water. Serum glucose was measured using the Infinity Glucose Reagent kit (Sigma).

PC and MCC Activity

The measurement of PC and MCC activity was performed using PC and MCC activity assays described in Chapter 2.

Statistical Analysis

Dose-response experiments

Results are expressed as means ± SEM. The significance of differences ($P<0.05$) was tested by one-way ANOVA with Newman-Keuls post test.

Time-dependent and dietary status experiments

Results are expressed as mean ± SEM. The significant differences ($P<0.05$) was tested by three-way ANOVA or t-test (weight gain, diet consumption, and in vitro biotinylation).

Results

Dose Dependent Effects of an LPS Induced Inflammatory Response

Serum zinc and glucose

In rats consuming a purified diet containing 0.06 mg free biotin/kg diet and injected with saline, the serum Zn concentration was 1.71 ± 0.08 ppm. The administration of lipopolysaccharide significantly reduced serum Zn concentration approximately 75% at all doses tested ($p<0.05$) (Table 4-1). The serum Zn of one animal in the 1 mg/kg LPS group did not decrease below control levels (1.56 ppm). This animal also showed no outward signs of illness and was therefore excluded from all subsequent analyses. Serum glucose, while lower in LPS treated animals, was not significantly reduced (Table 4-1).

Table 4-1. Changes in serum Zn and glucose during exposure to varying doses of endotoxin

	LPS Dose, mg/kg			
	0	0.1	1	10
Zn, ppm	1.71 ± 0.08	0.44 ± 0.03[a]	0.42 ± 0.03[a]	0.36 ± 0.02[a]
Glucose, mg/dl	198 ±19	175 ± 8	181 ± 4	170 ± 10

Urine, serum, and tissue free biotin

Urinary free biotin excretion could not be reliably measured over this short time frame due to large alterations in urine volume and creatinine excretion. The administration of lipopolysaccharide significantly elevated serum free biotin concentration over controls at the 0.1 and 10 mg LPS/kg BW doses by 270% (Figure 4-1A). Serum BNB was significantly increased over control values only at the highest LPS dose (10 mg/kg, Figure 4-1B). No differences were detected in serum BSO or biocytin (Figure 4-1C, D). Liver free biotin was significantly increased by 47 % in rats injected with 1 mg LPS/kg BW and 53% in rats injected with 10 mg LPS/kg BW (Figure 4-2A). The liver biotin metabolites were not significantly different (Figure 4-2 B, C, D).

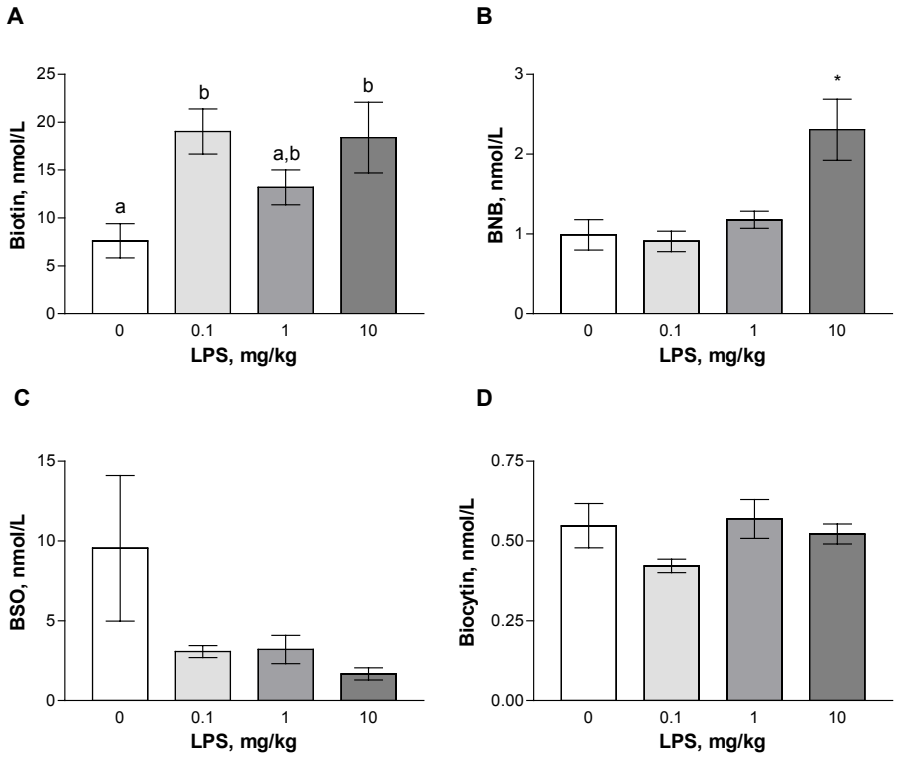


Figure 4-1. Changes in serum free biotin and biotin metabolites during exposure to varying doses of endotoxin. A. Biotin, B. BNB, C. BSO, D. Biocytin. Bars with different letters are significantly different ($P<0.05$).

Relative abundance of hepatic biotinylated carboxylases

Administration of LPS significantly reduced the relative abundance of biotinylated ACC isoform 1 at the 1 and 10 mg/kg body weight doses (Figure 4-3). Biotinylated ACC isoform 2 was significantly reduced in abundance at all doses of LPS analyzed (Figure 4-3). The abundance of hepatic biotinylated PC was significantly decreased at all doses of LPS by ~50% following LPS administration (P<0.0001; Figure

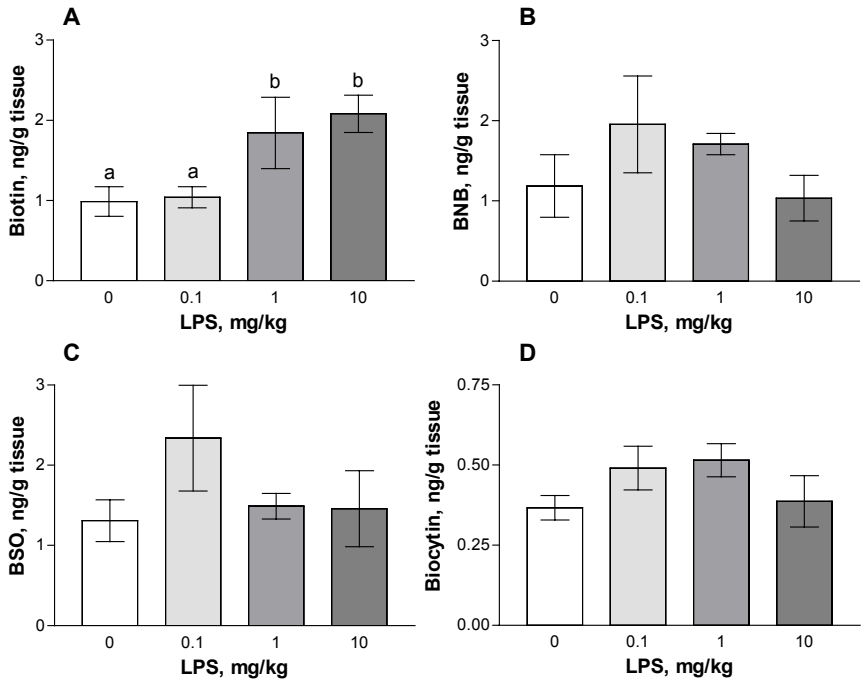


Figure 4-2. Changes in liver free biotin and biotin metabolites during exposure to varying doses of endotoxin. A. Biotin, B. BNB, C. BSO, D. Biocytin. Bars with different letters are significantly different (P<0.05).

4-4). In contrast, the abundance of hepatic biotinylated MCC was significantly increased ~100% after LPS administration (P=0.0001; Figure 4-5A). No significant difference was detected in the abundance of hepatic biotinylated liver PCC (P>0.05; Figure 4-5B).

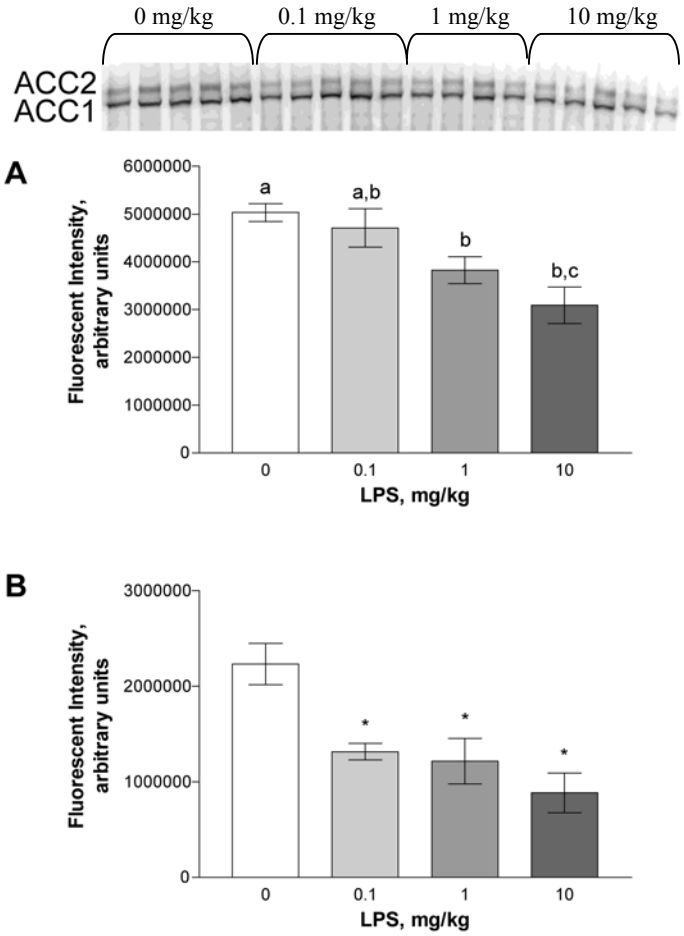


Figure 4-3. Changes in the relative abundance of biotinylated ACC1 and ACC2 during exposure to varying doses of endotoxin. A. ACC1, B. ACC2.

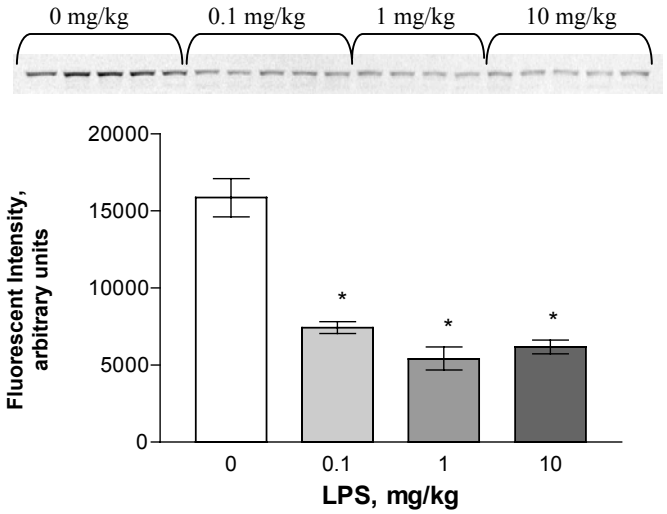


Figure 4-4. Changes in biotinylated PC during exposure to varying doses of endotoxin. * Indicates significant difference (P<0.05).

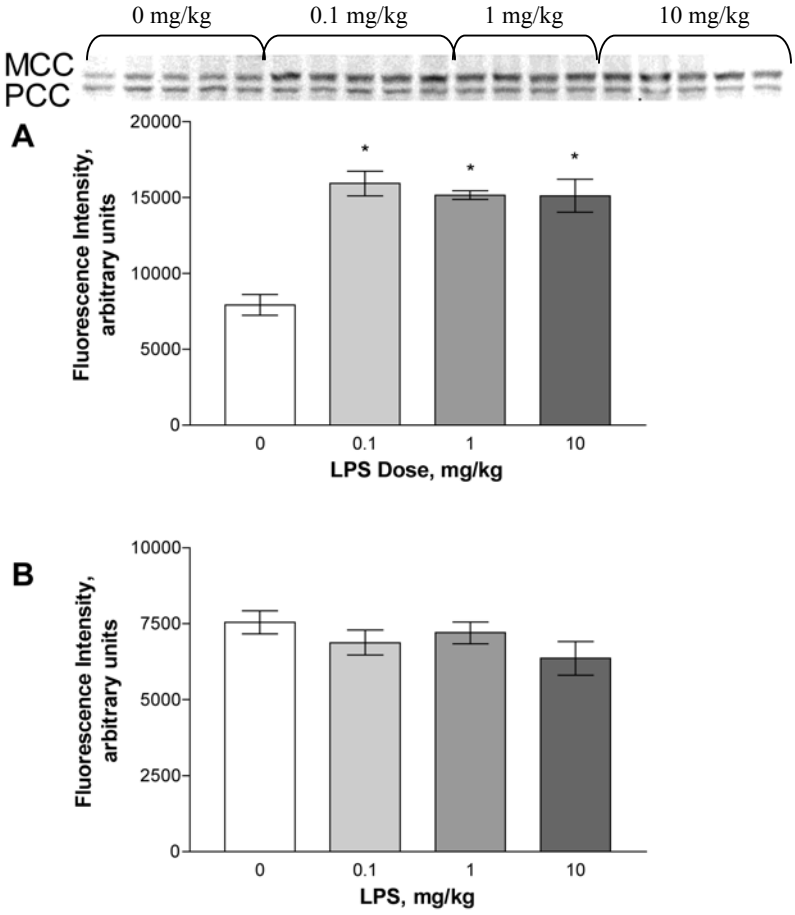


Figure 4-5. Changes in the relative abundance of biotinylated MCC and PCC during exposure to varying doses of endotoxin. A. MCC, B. PCC. * Indicates significant difference (P<0.05).

Time and Diet Dependent Effects of an LPS Induced Inflammatory Response

Animal weight and diet consumption

Beginning body weight did not differ significantly in biotin-adequate versus biotin-deficient animals. There continued to be no difference in body weight throughout the study. There was no difference in diet consumption between groups through day 16 ($P>0.05$). Biotin-deficient animals consumed significantly less diet than biotin-adequate animals on day 16 ($P<0.001$). Consumption returned to biotin-adequate levels by the next measurement on day 20 (Data not shown).

Serum zinc and glucose

Regardless of diet, serum Zn was significantly decreased after LPS administration at all time points ($P<0.001$). At 24-hr post LPS administration, serum Zn was

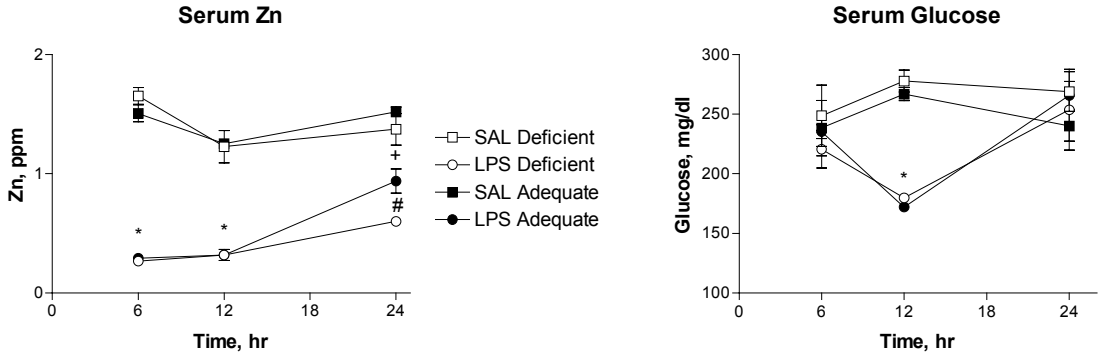
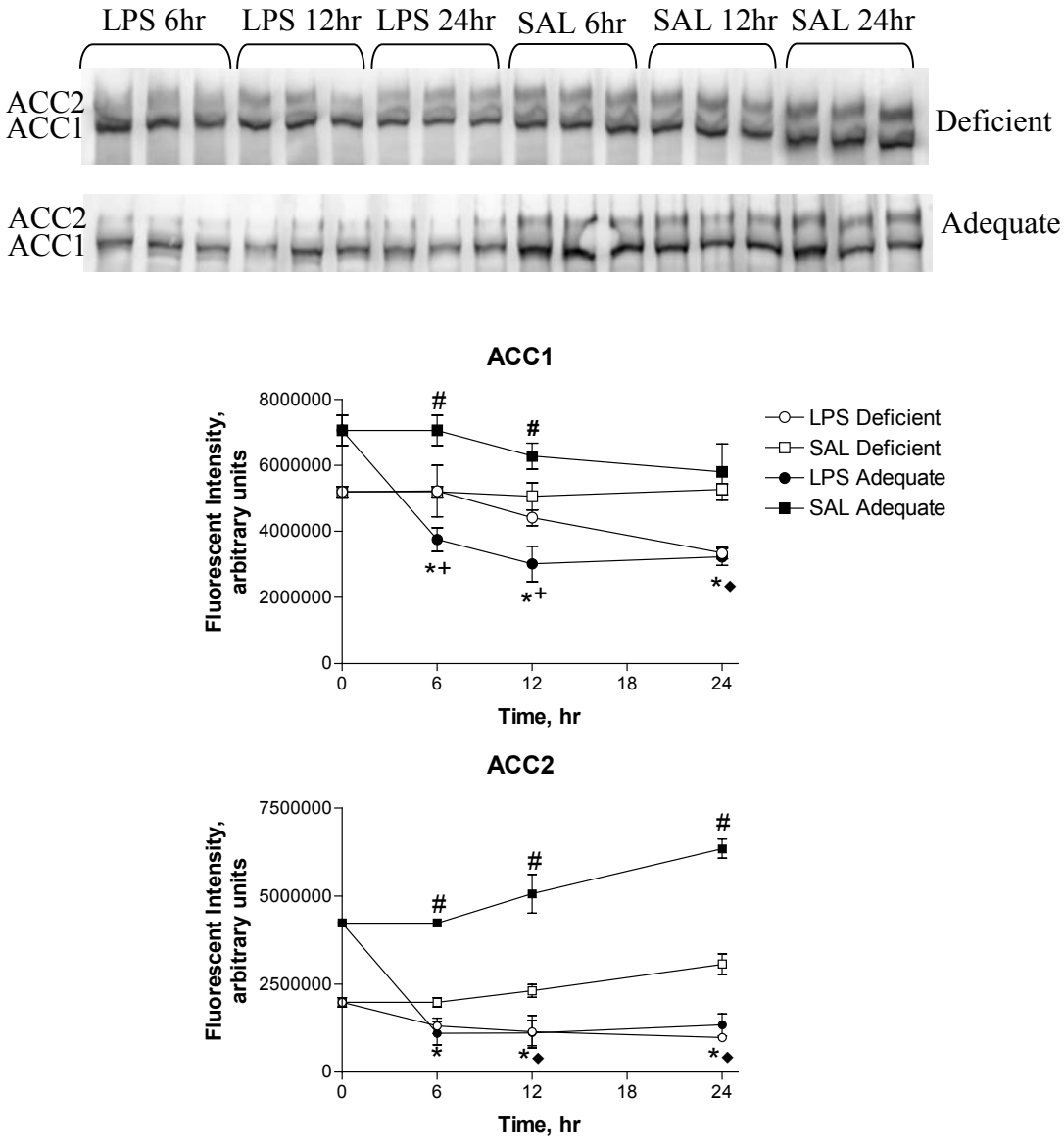


Figure 4-6. Changes in serum Zn and glucose in biotin-deficient and adequate rats exposed to endotoxin. Different symbols indicate significant difference ($P<0.05$).

significantly higher in biotin-adequate animals compared to biotin-deficient animals ($P=0.006$; Figure 4-6). Serum glucose was not significantly different between any treatment group at 6-hr. Serum glucose significantly decreased by 35% at 12-hr in all animals exposed to LPS regardless of diet ($P<0.001$). Serum glucose returned to saline values by 24-hr and was not significantly different between groups (Figure 4-6).

Relative abundance of hepatic biotinylated carboxylases

Acetyl CoA carboxylase isoform 1 was significantly decreased in biotin-deficient saline treated animals at the 6 and 12-hr time points compared to biotin-adequate saline



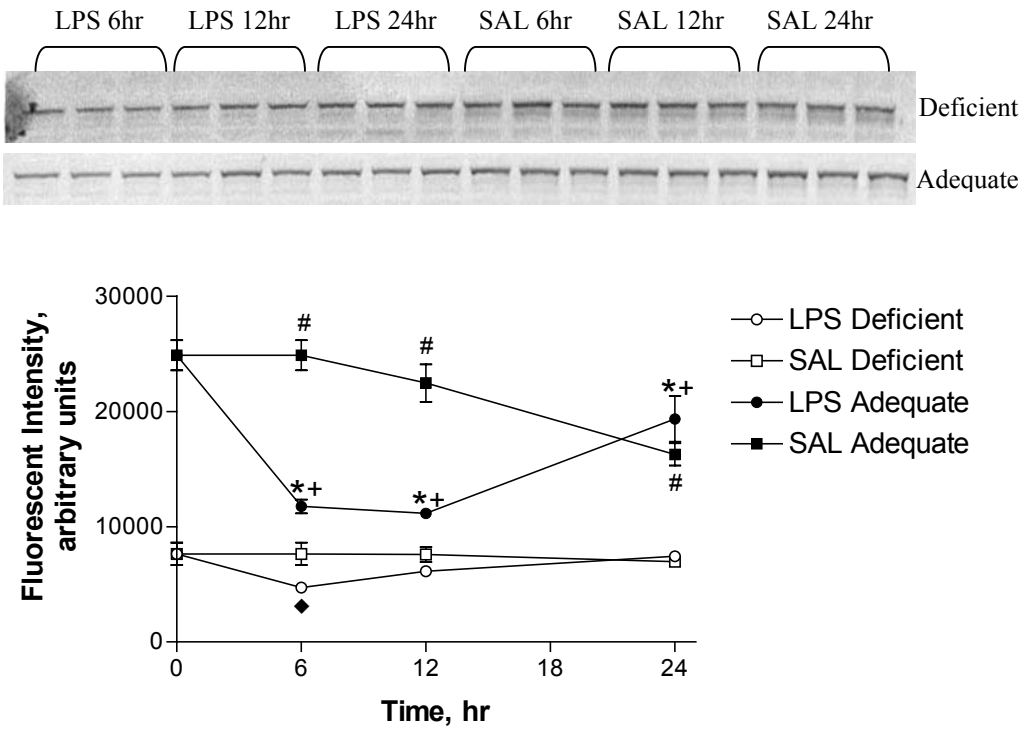
Statistical Key: * LPS Adequate significantly different than SAL Adequate, ◆ LPS Deficient significantly different than SAL Deficient, + LPS Adequate significantly different than LPS Deficient, # SAL Adequate significantly different than SAL Deficient

Figure 4-7. Changes in the relative abundance of biotinylated ACC1 and 2 in biotin-deficient and adequate rats after exposure to endotoxin.

treated animals (P<0.001). LPS administration to biotin-adequate animals significantly decreased the relative abundance of biotinylated ACC1 at all time points by 50% (P<0.005). The relative abundance of biotinylated ACC1 began to decrease at the 12-hr time point in biotin deficient LPS treated animals compared to biotin-deficient saline treated animals; however, this trend was not significantly different until the 24-hr time point (P=0.005). The relative abundance of biotinylated ACC1 was significantly decreased at the 6 and 12-hr time points in biotin-adequate LPS treated animals compared to biotin-deficient LPS treated animals (Figure 4-7; P<0.001). By 24-hr, the relative abundance of biotinylated ACC1 in biotin-deficient LPS treated animals had decreased to biotin-adequate LPS treated animals.

Acetyl CoA carboxylase 2 was significantly decreased by 55% in biotin-deficient saline treated animals at all time points compared to biotin-adequate saline treated animals (P<0.001). LPS administration to biotin-adequate animals significantly decreased the relative abundance of biotinylated ACC2 by 80% at all time points compared to biotin-adequate saline treated animals (P<0.001). LPS administration to biotin-deficient animals significantly decreased the relative abundance of biotinylated ACC2 at the 12 and 24-hr time points compared to biotin-deficient saline treated animals (50%, P<0.01, 12-hr; 68%, P<0.001, 24-hr). There was no significant difference in the relative abundance of biotinylated ACC2 between biotin-adequate LPS treated and biotin-deficient LPS treated groups (Figure 4-7).

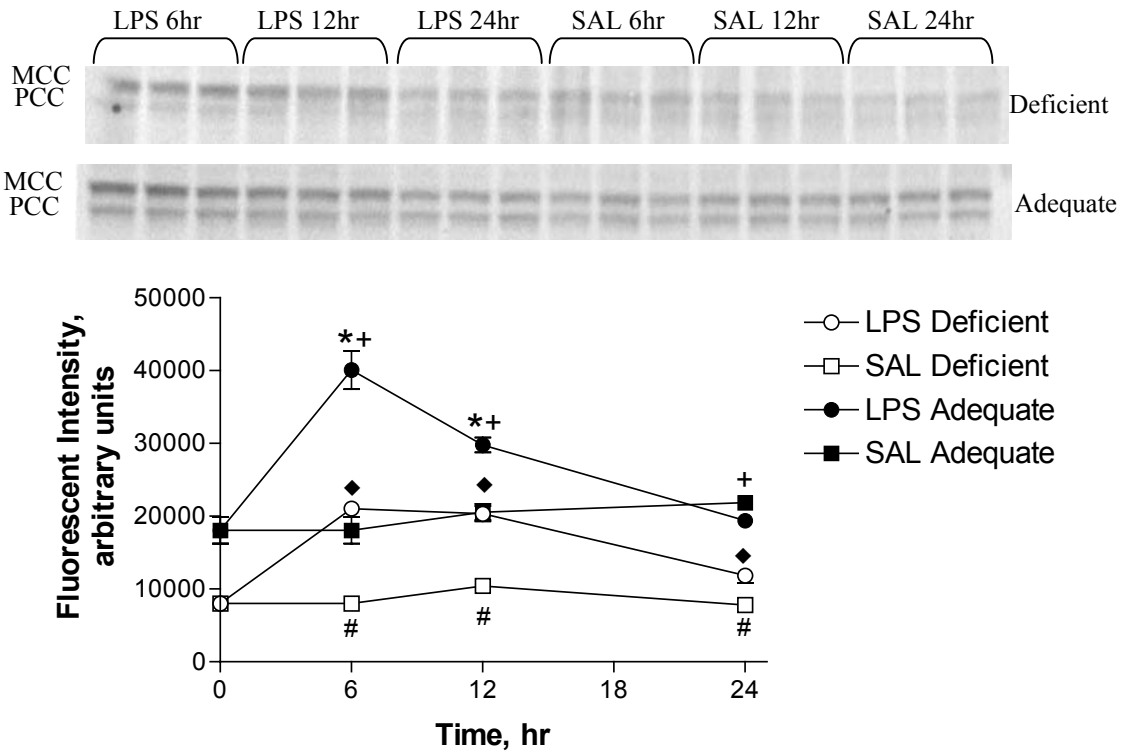
Pyruvate carboxylase was significantly reduced in biotin-deficient compared to biotin-adequate animals by 65% regardless of treatment or time after treatment (P<0.001). The relative abundance of biotinylated PC was significantly decreased at the



Statistical Key: * LPS Adequate significantly different than SAL Adequate, ◆ LPS Deficient significantly different than SAL Deficient,+ LPS Adequate significantly different than LPS Deficient, # SAL Adequate significantly different than SAL Deficient

Figure 4-8. Changes in the relative abundance of biotinylated PC in biotin-deficient and adequate rats after exposure to endotoxin.

6-hr time point in LPS treated animals compared to saline treated animals regardless of diet (53% $P<0.001$, biotin-adequate; 38% $P<0.05$, biotin-deficient). At 12-hr, biotinylated PC remained significantly decreased in biotin-adequate LPS treated animals by 50% compared to biotin-adequate saline treated animals ($P<0.001$), while the relative abundance of biotinylated PC in biotin-deficient LPS treated animals returned to biotin-deficient saline animal levels at the 12 and 24-hr time points. At the 24-hr time point, the relative abundance of biotinylated PC in biotin-adequate LPS treated animals was significantly increased over biotin-adequate saline treated animals by 20% ($P<0.001$, Figure 4-8).



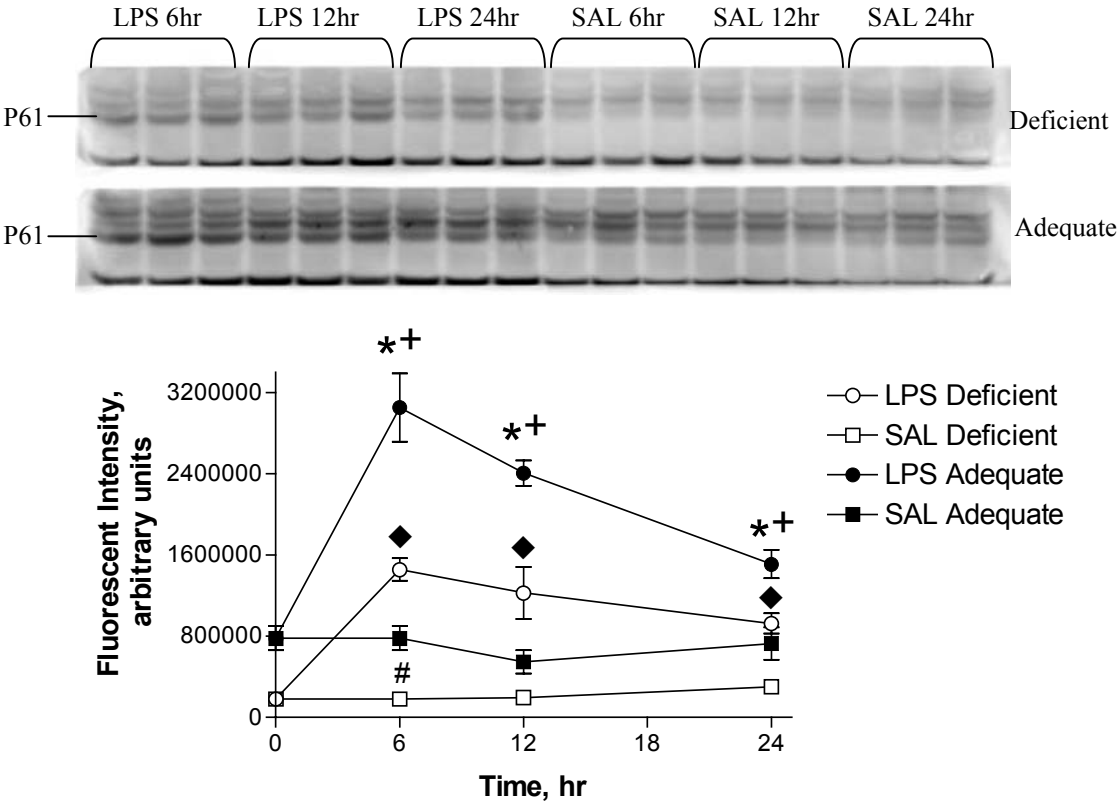
Statistical Key: * LPS Adequate significantly different than SAL Adequate, ◆ LPS Deficient significantly different than SAL Deficient,+ LPS Adequate significantly different than LPS Deficient, # SAL Adequate significantly different than SAL Deficient

Figure 4-9. Changes in the relative abundance of biotinylated MCC in biotin-deficient and adequate rats after endotoxin exposure.

MCC was significantly decreased in biotin-deficient saline treated animals by 50% at all time points compared to biotin-adequate saline treated animals ($P<0.001$). LPS administration significantly increased the relative abundance of biotinylated MCC at the 6 and 12-hr time points regardless of diet compared to saline treated animals (127% 6-hr adequate, 45% 12-hr adequate, 163% 6-hr deficient, 95% 12-hr deficient; $P<0.001$). Interestingly, LPS treatment increased MCC levels in the biotin-deficient animals to the same levels seen in biotin-adequate saline controls. By 24-hr, MCC levels in LPS treated biotin-adequate animals had returned to the level of biotin-adequate saline treated animals; however, biotin-deficient LPS treated animals were still significantly increased by 51% at 24-hr versus biotin-deficient saline treated animals ($P=0.02$, Figure 4-9).

Relative abundance of biotinylated P61

Administration of LPS to rats increased the relative abundance of an unknown biotinylated protein with an apparent molecular weight of 61 kDa. The relative abundance of biotinylated P61 was significantly lower in saline treated biotin-deficient compared to biotin-adequate rats at the 6-hr time point only (P=0.01). At 6, 12, and 24-



Statistical Key: * LPS Adequate significantly different than SAL Adequate, ◆ LPS Deficient significantly different than SAL Deficient,+ LPS Adequate significantly different than LPS Deficient, # SAL Adequate significantly different than SAL Deficient

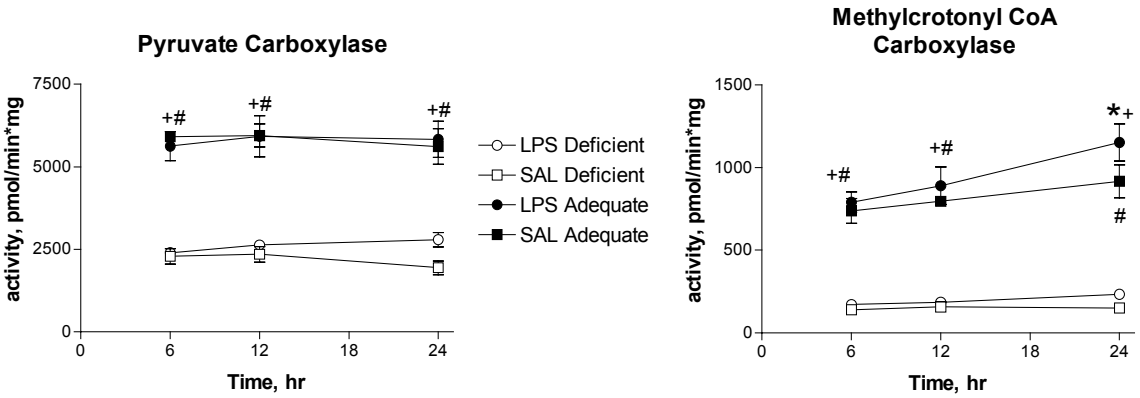
Figure 4-10. Changes in the relative abundance of biotinylated P61 in biotin-deficient and adequate rats after endotoxin exposure.

hr, the relative abundance of biotinylated P61 was significantly increased in LPS treated animals versus saline treated animals regardless of diet (300%, 340%, 100% adequate 6, 12, and 24-hr; 700%, 530%, 200% deficient 6, 12, and 24-hr; P<0.001). P61 was significantly increased in biotin-adequate LPS treated animals versus biotin-deficient

LPS treated animals at all time points (110% 6-hr, 96% 12-hr, 63% 24-hr; P<0.001) (Figure 4-10).

PC and MCC Activity

PC activity, expressed as pmol oxaloacetate formed/min*mg protein, was significantly reduced at all time points in biotin-deficient compared to biotin-adequate animals by 60% regardless of treatment (P<0.001). There was no change in PC activity



Statistical Key: * LPS Adequate significantly different than SAL Adequate, ◆ LPS Deficient significantly different than SAL Deficient,+ LPS Adequate significantly different than LPS Deficient, # SAL Adequate significantly different than SAL Deficient

Figure 4-11. Changes in the activity of pyruvate carboxylase and methylcrotonyl CoA carboxylase in biotin-deficient and adequate rats after endotoxin exposure.

in biotin-deficient or biotin-adequate animals with LPS administration at any time point (P>0.05, Figure 4-11).

MCC activity, expressed as pmol methylglutaconyl CoA formed/min*mg protein, was significantly reduced at all time points in biotin-deficient compared to biotin-adequate animals by 80% regardless of treatment (P<0.001; Figure 4-11). In biotin-adequate animals, MCC activity was significantly increased by 26% at only the 24-hr time point in LPS treated animals compared to saline treated animals (P=0.01). There

was no difference in MCC activity with LPS treatment in biotin-deficient animals at any time point ($P>0.05$, Figure 4-11).

Discussion

It is well established that biotin deficiency affects immunity (31,52,139,141,142). However, very little is known on how biotin metabolism is changed during immune stimulation. Recently, studies have shown that isolated PBMC's stimulated with mitogens increase their biotin uptake possibly to supply biotin for increased formation of the holocarboxylases (140). This indicates that changes in biotin metabolism may occur, but this has not been evaluated in vivo. In the present study, endotoxin was administered to Sprague-Dawley rats in order to determine the in vivo effects of an acute inflammatory response on the metabolism and function of biotin.

The animals in these studies were first acclimatized on purified AIN 76A based diet with spray-dried egg-white as the sole protein source and 0.06 mg biotin/kg diet. This level of biotin was chosen because it closely approximates the physiologic daily intake of biotin for humans when adjusted to the amount per kg body weight, and has been shown to be an adequate amount of biotin for rats over this time period (143). Commercial rodent diet contains much higher amounts of biotin (~ 0.22 mg biotin/kg diet). When adjusted to the amount per kg body weight intake, this is a supplemented dose of biotin for humans, which may in itself affect biotin metabolism. After acclimation, endotoxin was administered immediately in the dose-response experiment.

In the time and diet dependent study, the rats were first acclimatized on the purified AIN 76A diet containing egg-white as the sole protein source and 0.06 mg biotin/kg for 5 days. After acclimation, the rats consumed this same diet containing 0.06 mg biotin/kg diet or the same diet with no biotin for 3 weeks before endotoxin

administration. Serum Zn and glucose were measured to validate the efficacy of the administration of LPS. It is well established that these parameters are decreased during endotoxin exposure (83,144). At all doses of LPS, serum Zn was significantly reduced. In biotin-adequate LPS injected animals, serum Zn was significantly decreased at all time points. At 24-hr, serum Zn had recovered to 62% of saline levels. Serum Zn in biotin-deficient rats treated with LPS was also significantly lower than biotin-deficient saline treated rats; however, by 24-hr had only recovered to 44% of saline animals. Serum glucose was significantly decreased in LPS treated rats at 12-hr only regardless of diet. This explains why we saw no change in serum glucose in the dose response study at 6-hr. The decreased recovery of serum Zn indicates that biotin-deficient rats may recover more slowly than biotin-adequate animals; however, the same trend might be expected in serum glucose, which does not occur.

Administration of varying doses of endotoxin decreased the relative abundance of the biotinylated forms of ACC1 and ACC2 (Figures 4-3). ACC1 is a cytosolic enzyme responsible for catalyzing the conversion of acetyl CoA to malonyl CoA during de novo fatty acid synthesis (49). ACC2 is a mitochondrial membrane bound enzyme that also catalyzes the conversion of acetyl CoA to malonyl CoA an inhibitor of the carnitine palmitoyl CoA shuttle that transports long chain fatty acids into the mitochondria for β-oxidation (9). The decreased relative abundance of the biotinylated form of ACC1 and ACC2 may indicate that the liver is decreasing fatty acid synthesis and increasing fatty acid β-oxidation. This detection method measures only the amount of ACC1 and 2 to which biotin is covalently linked. It provides no information on the amount of total

ACC1 and 2 in the liver; however, an earlier study indicated that the biotin-dependent carboxylases may be affected by endotoxin exposure.

In 1983, Pekala et al. used endotoxin to study how infection induced cachexia, shock, and death. It was determined that an endotoxin induced product (now known to be cytokines and other inflammatory mediators) secreted into culture supernatant by stimulated macrophages could modify fatty acid metabolism in 3T3-L1 adipocytes. When incubated with this endotoxin induced macrophage product, 3T3-L1 cells suppressed ACC activity by 75% and ACC synthesis by 95% (82). These data indicate that the normal response of biotin-adequate rats to endotoxin is to decrease fatty acid synthesis and increase fatty acid β-oxidation. This normal adaptation may be perturbed in biotin-deficient animals. In adequate animals, the reduction of biotinylated ACC1 and 2 with endotoxin exposure was detected as early as 6-hr after administration and remained reduced through 24-hr. Biotin deficiency delayed this decrease until 24-hr in ACC1 and 12-hr in ACC2 (Figure 4-7). This could reduce the ability of the deficient rat to decrease fatty acid synthesis and increase β-oxidation possibly depriving the deficient rats a valuable energy source during the inflammatory response.

The relative abundance of the biotinylated form of pyruvate carboxylase was decreased with endotoxin exposure (Figures 4-4 and 4-8). These data in part, suggest a possible mechanism for the decreased gluconeogenesis seen during endotoxin exposure (83). Endotoxin is known to decrease gluconeogenesis from lactate, pyruvate, and alanine in the livers of endotoxin-treated rats. Several enzymes in the gluconeogenic pathway have been suggested as contributing to the inhibition of gluconeogenesis from these substrates, in particular pyruvate carboxylase. Pyruvate carboxylase catalyzes the

conversion of pyruvate to oxaloacetate in gluconeogenesis as well as to replenish the TCA cycle. Jones and Titheradge (83) provided evidence that pyruvate carboxylase is inhibited during endotoxin-induced inflammation. In hepatocytes isolated from rats injected with endotoxin, the flux of pyruvate through PC decreased 50%. They concluded that the reduced flux may be due to feedback inhibition of PC by high levels of oxaloacetate produced by the inhibition of PEPCK. However, our data indicate that the reduced flux of PC may be due to a reduction in biotinylated PC, the only form capable of carboxylating pyruvate to oxaloacetate.

In biotin-adequate animals, PC was reduced 50% at 6 and 12-hr after endotoxin administration and had recovered to slightly above saline levels by 24-hr. PC was also reduced in biotin-deficient animals at 6-hr but to a lesser degree (38%) and had recovered to saline values by 12-hr. The relative abundance of PC in saline injected biotin-deficient rats was reduced by 65% compared to saline injected biotin-adequate animals. This follows the same trend seen in previous studies of deficient rats having 50% less biotinylated PC than adequate rats when fed a biotin-deficient diet for 3 weeks (143). This indicates that the anapleurotic reaction catalyzed by PC is already reduced in biotin-deficient rats to levels lower than the reduction caused by endotoxin administration in adequate rats. This may explain the ability of the LPS treated biotin-deficient rats to quickly return to saline treated biotin-deficient rat levels. The reduction in biotinylated PC in biotin-deficient LPS treated animals at 6-hr may be an initial reaction to LPS that does not need to be sustained due to already reduced PC levels.

To determine if the decrease in the relative abundance of biotinylated PC during an inflammatory response corresponded to a decrease in the ability to catalyze the

carboxylation of pyruvate to oxaloacetate, the activity of PC was measured. The activity of PC in biotin-deficient rat liver was reduced 60% compared to biotin-adequate rats. This value is comparable to the 65% reduction in biotinylated PC abundance seen in biotin-deficient rats compared to biotin-adequate rats. While the abundance of biotinylated PC decreased due to endotoxin administration, PC activity did not change. This indicates that other factors influencing PC activity may be involved. This could include the allosteric activation of PC from increasing levels of acetyl CoA from the decreased activities of ACC1 and 2 seen during endotoxin exposure, or dilution effects of PC (145,146).

The relative abundance of the biotinylated form of MCC significantly increased with varying doses of endotoxin (Figures 4-5). MCC is involved in the degradation of leucine by converting methylcrotonyl CoA to methylglutaconyl CoA (49). Our observed increase in the relative abundance of biotinylated MCC could indicate that the liver may also be increasing its degradation of leucine as seen in the muscle. Protein catabolism occurs during endotoxin-induced inflammation to release branched-chain amino acids in the muscle, which can be immediately used by muscle cells as a source of energy or to generate amino nitrogen groups that are combined with pyruvate or other carbon sources for the synthesis of alanine and glutamine (147). As a result, the amino acid composition of muscle is altered. It has recently been demonstrated that leucine levels double in liver perfusate of rats treated with endotoxin; however, it is still unknown if leucine degradation is increased (148).

Consumption of a biotin deficient diet for 3 weeks decreased the abundance of biotinylated MCC by 50% compared to biotin-adequate rats (Figure 4-9). This

corresponds with results presented in Chapter 3 showing decreased levels of biotinylated MCC after consumption of a biotin deficient diet for 3 weeks (143). In biotin-adequate rats, the relative abundance of biotinylated MCC was significantly increased by 127% in endotoxin treated rats compared to saline treated rats at 6-hr. MCC levels began to return to saline levels in endotoxin treated rats by 12-hr but were still elevated by 45%. By 24-hr, MCC levels had returned to saline levels. Biotin deficiency did not prevent the elevation of MCC with endotoxin exposure; however, this increase was only to biotin-adequate saline treated levels. At 24-hr, MCC had not returned to saline levels in biotin-deficient rats. These data may indicate that because of the reduced ability to increase active levels of MCC, deficient rats require MCC levels to remain increased for longer periods of time to cope with increased leucine loads.

To determine if the decrease in the relative abundance of biotinylated MCC during an inflammatory response corresponded to a decrease in the ability to catalyze the carboxylation of methylcrotonyl CoA to methylglutaconyl CoA, the activity of MCC was measured. The activity of MCC in biotin-deficient rat liver was reduced 80% compared to biotin-adequate rats. This value is consistent with a 50% reduction in biotinylated MCC seen in biotin-deficient rats compared to biotin-adequate rats. Although biotinylated MCC levels were increased at the 6 and 12-hr time points in LPS injected biotin-adequate rats and had returned to saline levels by 24-hr, MCC activity was only increased at the 24-hr time point. This indicates that there may be a delay between the increase in active MCC and the concurrent increase in MCC activity. In contrast, MCC activity did not increase at any time point measured in biotin-deficient rats. It can not be ruled out that MCC activity may be increased at later time points in biotin-deficient rats.

In contrast to the other carboxylases, no change in the relative abundance of biotinylated PCC was detected (Figure 4-5). Propionyl CoA carboxylase catalyzes the conversion of propionyl CoA to methylmalonyl CoA during the degradation of odd-chain fatty acids and the side chain of cholesterol, and the catabolism of isoleucine, methionine, threonine, and valine (49). The fact that the relative abundance of biotinylated PCC did not change during endotoxin exposure indicates that the changes in the relative abundance of the biotinylated form of the other carboxylases is specific, not a general change of all carboxylases due to endotoxin exposure.

Serum biotin increased with increasing doses of endotoxin (Figure 4-1A). This effect is opposite that seen in serum Zn. This increase may be a result of the changes in the biotinylated forms of the carboxylases seen in the liver of endotoxin-injected rats. The decreases in the relative abundance of biotinylated ACC1 and 2, and PC could result in an increase of liver free biotin that could then be released into the serum. However, other possible explanations exist. Several tissues in the body are known to contain pools of free biotin. LPS stimulation could cause a redistribution of these free biotin pools from one or more organs to the serum. In this study, only the free pool of biotin in the liver was measured. Liver free biotin increased significantly with LPS administration and could provide a source of biotin for the serum (Figure 4-1B). Although the free pool did change it could not be evaluated whether the flux of free biotin into or out of the liver changed. Free biotin released from ACC1, ACC2, and PC, which showed decreases in the relative abundance of their biotinylated forms, could explain the increase in free biotin in the liver. The free biotin released from these proteins could have been used to increase the relative abundance of MCC and P61. The liver free biotin could have been

released into the serum supplying free biotin for proliferating immune cells. Other explanations include a reduction in urinary excretion of biotin or a reduction in the conversion of serum biotin to its metabolites. Urinary excretion of biotin could not be accurately measured in this study due to the severe alterations in the urine volume excreted by the LPS treated animals. Normally this could be accounted for by adjusting the measured value to the normally constant excretion of creatinine into the urine. However, it is well known that LPS administration effects urinary creatinine excretion (149) which was confirmed in our study. Finally, although highly variable between animals, no changes were detected in the profile of biotin metabolites in the serum of LPS treated animals, except BNB arguing against a reduction in biotin's conversion to its metabolites. Serum BNB increased significantly at the 10mg/kg dose possibly due to an increased state of β-oxidation.

Endotoxin administration also increased the relative abundance of a previously undescribed biotinylated protein with a molecular weight of 61 kDa (Figure 4-10). This protein was increased in both biotin-deficient and adequate animals exposed to LPS; however, biotin-adequate LPS injected animals had a significantly increased level of biotinylated P61 compared to biotin-deficient LPS injected animals. The characterization of this protein is described in the next chapter.

This study reports several novel observations of biotin metabolism during endotoxin-induced inflammation. It is clear that changes in both free and protein-bound pools of biotin are normal in the response to LPS-induced inflammation. What these changes signify remains unknown. Are they a normal beneficial response of the body to clear the inflammatory stimulus, or one of the many detrimental responses brought on by

the inflammatory stimulus that leads to the many derangements seen during sepsis? Many times in sepsis, these two possibilities are one and the same with cytokines and other inflammatory mediators released by the body to fight the infection eventually overwhelming the body and causing damage and death. These studies also show that biotin deficiency delays or decreases the changes in carboxylase abundance and activity induced by endotoxin. It is well known that biotin deficiency impairs immune function. However, the only indication of a decreased inflammatory response to endotoxin was a slower recovery of serum Zn in biotin-deficient LPS rats. The biotin-deficient rats showed no outward signs of increased sickness due to endotoxin exposure and their serum glucose closely followed endotoxin exposed biotin-adequate animals. Further research evaluating other immune parameters will be required to determine if biotin-deficiency impairs recovery from endotoxin exposure.

CHAPTER 5
ISOLATION AND CHARACTERIZATION OF P61

Introduction

In Chapter 4, a previously undescribed protein with an apparent molecular weight of ~55-61 kDa was visualized by avidin blot in liver homogenate of rats treated with endotoxin. This protein is also present in liver homogenate from saline treated animals; however, at a very light intensity previously thought to be background from nonspecific binding of avidin. With endotoxin exposure, the intensity of this band increases 6-10 fold.

In animals, biotin is thought to be covalently bound only to the five biotin-dependent carboxylases: ACC isoforms 1and 2, PC, MCC, and PCC. In bacteria, several other proteins covalently attached to biotin have been described including transcarboxylase, geranyl-CoA carboxylase, urea carboxylase, methylmalonyl-CoA decarboxylase, and oxaloacetate decarboxylase (26). It is possible P61 is the mammalian form of one of these bacterial proteins. In fact, when molecular weights of these bacterial proteins are compared to P61, the α-chain of oxaloacetate decarboxylase stands out with a molecular weight of 63.5 kDa.

Oxaloacetate decarboxylase from several bacterial species has been well characterized (150-154). Oxaloacetate decarboxylase catalyzes the reaction of oxaloacetate to pyruvate, which is coupled to the translocation of Na^+. It contains an α, β, and γ subunit. The α-subunit is a peripheral membrane protein containing both carboxyltransferase activity and the biotin carrier function. The β-subunit is an integral

membrane protein that catalyzes the decarboxylation of carboxybiotin, which is coupled to Na^+ pumping. Finally, the Zn containing γ-subunit is thought to keep the complex together and accelerate the carboxytransfer reaction.

In this chapter, we describe the characterization and isolation of P61. We first determined if P61 was a membrane bound protein using differential centrifugation and detergent extraction. We then isolated P61 using a monomeric avidin column capable of reversibly binding biotin containing compounds. The isolated P61 was sent to Emory University School of Medicine, Microchemical Facility (Atlanta, GA) for sequencing.

Materials and Methods

Localization of P61 by Differential Centrifugation

Animal treatment

Sprague Dawley rats (n=6) were acclimated on standard rodent diet for 5 days. The rats were then injected with 5 mg LPS/kg BW in 0.9% saline (n=4) or 0.9% saline only (n=2). After 6-hr, the animals were euthanized by cardiac puncture and 0.5 g liver removed.

Differential centrifugation

The liver pieces were homogenized in 10 volumes ice-cold HEM (300 mmol/L mannitol, 10 mmol/L HEPES (pH 7.2), 1 mmol/L EDTA, and 1:100 (v/v) protease inhibitor cocktail) and processed by differential centrifugation as described below. An aliquot of homogenate was saved for each animal at this point. The remainder was centrifuged at 500 x g for 10 min to isolate a crude nuclear fraction. This fraction contains primarily cellular debris, plasma membrane, and nuclei. The pellet was resuspended in 2 ml HEM and the supernatant centrifuged at 3,000 x g for 10 min to isolate a heavy mitochondrial fraction. The pellet was resuspended in 1 ml HEM and the

supernatant centrifuged at 17,000 x g for 15 min to isolate a light mitochondrial fraction. The pellet was resuspended in 1 ml HEM and the supernatant centrifuged at 200,000 x g to isolate both microsomal and cytosolic fractions. The cytosolic fraction was saved and the microsomal pellet resuspended in 1 ml HEM. All samples were stored at –80^{o}C until analyzed.

Separation and detection of P61

Samples from each group were pooled and 100 μg of protein from each fraction were separated on a 10% SDS-PAGE (pH 8.8) overnight. The gel was then transferred to PVDF, probed with avidin-AP, and P61 detected with ECF on a fluorescent imager as described in Chapter 2.

Inhibition of Detection of P61 after Competition with Free Biotin

Homogenate samples from each group in the differential centrifugation experiment were pooled and 75 μg of protein were separated on a 10% SDS-PAGE (pH 8.8) overnight. The gel was then transferred to PVDF. Avidin-AP was then incubated in 0.5% NFDM-TBST, 1 μM biotin for 1-hr. The free biotin binds all free avidin-binding sites so that only nonspecific interactions remain. The PVDF blot was then incubated with the avidin-AP, NFDM-TBST, biotin mixture for 45 min and detected with ECF on a fluorescent imager.

Solubilization of P61

Liver samples from LPS and saline treated animals were homogenized in 10 volumes of HEM. The homogenates were centrifuged at 200,000 x g for 30 min at 4^{o}C. The cytosolic fractions were saved and the pellets homogenized in HEM containing 1% Triton X-100 or 2% SDS. The resuspended samples were incubated on ice for 20 min

and then centrifuged at 200,000 x g for 30 min at 4°C. The supernatant from each sample was saved and the pellets resuspended in HEM without detergents. Each sample (80 μg protein) was separated on a 10% SDS-PAGE, transferred to PVDF, probed with avidin-AP, and detected with ECF on a fluorescent imager as described in Chapter 2.

Solubilization of Peripherally Bound Constituents of P61

A liver sample from a LPS treated animal was homogenized in 10 volumes HEM. The homogenate was centrifuged at 17,000 x g for 15 min at 4°C. The pellets were resuspended in HEM with and without 50 mM sodium carbonate (unadjusted pH). Each sample (75 μg protein) was separated on a 10% SDS-PAGE, transferred to PVDF, probed with avidin-AP, and detected with ECF on a fluorescent imager as described in Chapter 2.

P61 Isolation

Three rat livers removed from biotin-adequate animals euthanized 6-hr after LPS administration in the LPS: Time-dependent and Dietary Status Experiments in Chapter 4 were homogenized together in 10 volumes (of combined weight) of phosphate buffer (20 mM KP_i (pH 7.5), 0.5 M NaCl, 1 mM EDTA, 1:100 protease inhibitors). The sample was centrifuged at 17,000 x g for 15 min at 4°C and the supernatant removed. The pellet was resuspended in phosphate buffer containing 2% Triton X-100 and incubated on ice for 30 min. The resuspended pellet was then centrifuged at 200,000 x g for 30 min at 4°C. The supernatant was collected and the pellet resuspended in phosphate buffer. Streptomycin sulfate (3% wt/volume) was added to the supernatant to precipitate nucleic acids and the sample centrifuged at 28,000 x g for 20 min at 4°C. The supernatant was saved and the pellet resuspended in phosphate buffer. Ammonium sulfate was added to

the supernatant to 30% saturation and the sample centrifuged again at 28,000 x g for 20 min at 4^{o}C. The precipitate was removed and resuspended in phosphate buffer and the supernatant removed and biotin containing proteins isolated on a monomeric avidin column as described in Chapter 2. Fractions eluted from the avidin column were separated on a 10% SDS-PAGE and stained with coomassie blue for detection of P61. The detected band was then sliced from the gel and sent to Emory University School of Medicine, Microchemical Facility for sequencing. The Microchemical Facility first attempted to perform N-terminal sequencing by Edman degradation; however, the protein did not yield any sequence, as the amount of the sample was below the detection limit of the instrument (~200 fmol). Mass spectrometry analysis (MALDI TOF) of the protein was then attempted. The sample was concentrated four fold in an Amicon stirred cell (Millipore, regenerated cellulose membrane, 10 kDa NMWCO) and separated by SDS-PAGE as described in Chapter 2. The P61 band was excised from the gel and washed 3 x 5 min with 50% acetonitrile in 10 mM ammonium bicarbonate. The washed gel was then dried for 30 min in a Speed-Vac, rehydrated in 10 mM ammonium bicarbonate containing 0.25 μg trypsin, and digested overnight at 37^{o}C. The reaction was stopped by the addition of 1.5 μl 10% TFA. Peptides were extracted from the gel by vortexing the gel in 60% acetonitrile/10 mM ammonium bicarbonate, and the supernatant concentrated in the Speed-Vac to 10 μl. The sample was then drawn into a C18 Zip tip. The Zip tip was washed three times with 0.1% TFA and the sample eluted with 70:30 acetonitrile:0.1% TFA. The eluent was mixed with α-cyano-4-hydroxycinnamic acid matrix and spotted onto a sample plate for MALDI TOF analysis. The sample was then analyzed by MALDI TOF in positive reflector mode.

Results

Localization of P61 by Differential Centrifugation

P61 was subjected to differential centrifugation in order to characterize its distribution within the liver cell. P61 was detected in the nuclear, heavy mitochondrial, and light mitochondrial fractions. This indicates that P61 is either an integral or peripherally bound membrane protein (Figure 5-1).

Competitive Inhibition of the Detection of P61

The possibility exists that P61 is not a biotinylated protein but a protein detected through nonspecific interactions with avidin. To determine if P61 is a biotinylated protein, P61 was detected using avidin as well as avidin that had been preincubated with

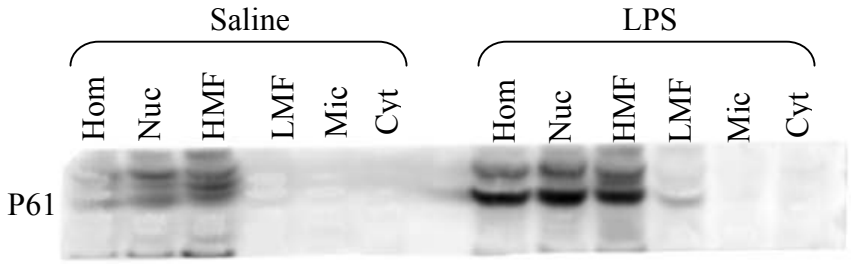


Figure 5-1. Differential centrifugation and detection of P61 in saline and LPS treated rats. Hom: Homogenate, Nuc: Nuclear fraction, HMF: Heavy mitochondrial fraction, LMF: Light mitochondrial fraction, Mic: Microsomes, Cyt: Cytosol.

1 μM biotin. P61 was detected only when avidin alone was used. Avidin preincubated with biotin eliminated the detection of P61 indicating the specific nature of avidin binding to P61 (Figure 5-2).

Solubilization of P61

Many times membrane bound proteins can be solubilized using detergents. P61 could be solubilized using 1% Triton X-100 or 2% SDS. While P61 was solubilized by SDS, Triton X-100 seemed to be the better choice (Figure 5-3).

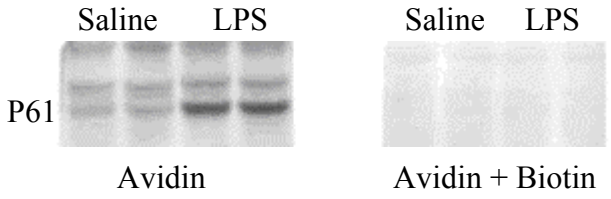


Figure 5-2. Detection of P61 and its competition by the incubation of biotin with avidin.

Solubilization of Peripherally Bound Constituents of P61

In oxaloacetate decarboxylase, the α-chain of the complex contains biotin covalently attached to the biotinylation motif. The α-chain is considered a peripherally bound membrane protein attached to the integral membrane β and γ-chains through ionic

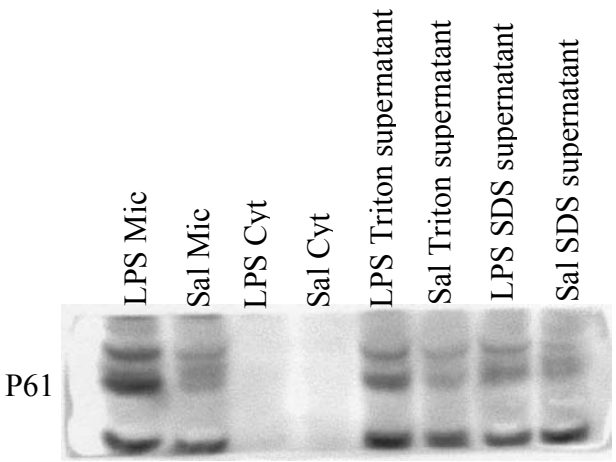


Figure 5-3. Solubilization of P61 using 1% Triton X-100 or 2% SDS.

interactions. If P61 is a peripherally attached membrane protein, incubation of the membrane pellet with high salt concentrations should disrupt these ionic interactions releasing P61 into the supernatant. Figure 5-4 shows that P61 is only detectable in liver homogenate from rats treated with LPS. When this homogenate is centrifuged at 17,000 x g, P61 is found in the membrane pellet and not the supernatant. If the membrane pellet is resuspended in HEM and centrifuged at 200,000 x g, P61 is still found only in the membrane pellet. If resuspended in 50 mM Na_2CO_3 and centrifuged at 200,000 x g, P61 was released from the membrane pellet to the supernatant. This indicates that P61 is a peripherally attached membrane protein.

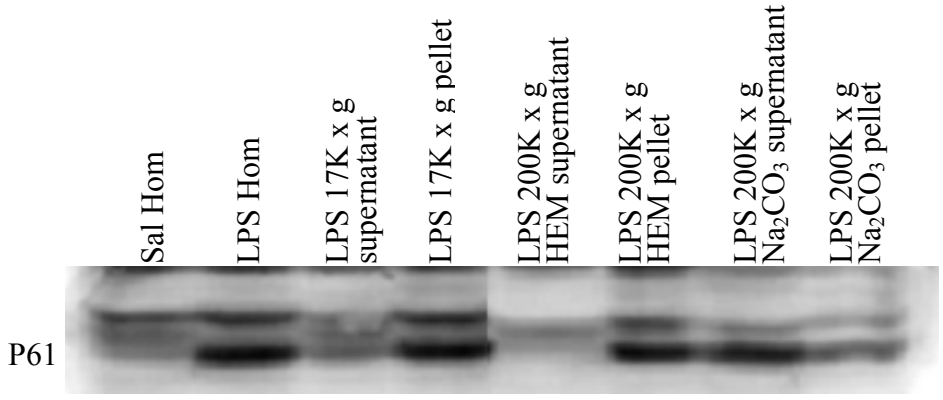


Figure 5-4. Solubilization of P61 using 50 mM sodium carbonate.

Isolation of P61

The characterization of P61 as a biotin containing protein allows the use of the binding characteristics of a monomeric avidin column for isolation. In contrast to tetrameric avidin, monomeric avidin has a reduced affinity for biotin allowing for the release of bound biotinylated proteins using gentle nondenaturing conditions. Solubilized P61 resolved on a monomeric avidin column eluted in fractions B2 and R2 as shown in Figure 5-5.

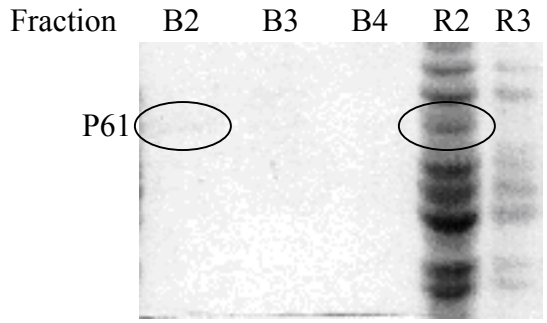


Figure 5-5. Purification of P61 on a monomeric avidin column.

Sequencing of P61

Isolated P61 was sent to the Microchemical Facility of Emory University for N-terminal sequencing by Edman degradation. The protein did not yield any sequence, as the amount of the sample was below the detection limit of the instrument (~200 fmol). The sample was concentrated 4 fold and peptide sequencing was attempted using MALDI-TOF mass spectrometry analysis following trypsin digestion. Twenty-seven monoisotopic masses were detected. The predicted amino acid sequences from these masses were searched for matching proteins in the NCBInr database, using PROWL. Two primary protein candidates emerged: 1. myosin-binding protein C, 2. propionyl CoA carboxylase (β-chain) (Table 5-1).

Discussion

P61 was first detected in the liver of rats treated with endotoxin. Although present in saline treated rats, the very light detection level was mistaken for background fluorescence from the nonspecific binding of avidin. Once treated with endotoxin, rat liver P61 increased 5-10 fold in intensity. This lead us to investigate the localization of P61 in the liver cell as well as its binding specificity for avidin.

Differential centrifugation of liver homogenate allows the separation and collection of individual sub-cellular fractions. Although not studied here, marker enzymes exist whose activity levels can be followed to verify the purity of the subcellular fractionation. After differential centrifugation, P61 was localized to the nuclear, heavy mitochondrial, and light mitochondrial fractions. This indicates that P61 is a membrane bound protein.

If the detection of P61 with avidin were a specific interaction and not nonspecific binding, then prior incubation of avidin with biotin would block the binding of avidin to P61. To determine if the detection of P61 by avidin was a specific interaction, competition of the binding of P61 with avidin was performed using 1 μM free biotin. Incubation of avidin with biotin prior to the detection of P61 completely inhibited the fluorescent signal produced by ECF when avidin-AP binds P61.

As a membrane-bound protein, P61 must be solubilized before any chromatographic isolation techniques can be used. Membrane-bound proteins require detergent treatment in order to solubilize the protein and many detergents are available with various useful qualities. The two detergents chosen for the solubilization of P61 were SDS and Triton X-100. P61 was best solubilized by Triton X-100.

When the molecular weight and localization of P61 were compared to known sequenced proteins, the α-chain of bacterial oxaloacetate decarboxylase was very similar. Oxaloacetate decarboxylase consists of a biotin containing α-chain peripherally attached to the bacterial membrane by interactions with the integral membrane subunits β and γ.

Table 5-1. P61 Monoisotopic Masses Determined by MALDI-TOF

Rat Myosin-Binding Protein C

Measured Mass	Computed Mass	Peptide Sequence
888.502	888.51	LGKEVCLK
1088.542	1088.575	AIMEGSGRIR
1110.492	1110.603	EEIVPGPKSR
1513.722	1513.871	ITTPLTDQTVKLGK
1703.782	1703.787	SMEWFAVIEHYHR
1779.852	1779.863	DDGNAAITGYTIQKADK
1850.912	1850.889	AESYPDSSTLVIDVAER

Unmatched Masses

1157.580, 1197.550, 1357.640, 1383.690, 1465.790, 1536.720, 1541.660, 1548.660
1561.720, 1568.820, 1601.860, 1673.790, 1735.820, 1772.930, 1830.960, 1880.970
1965.030, 2005.000, 2115.990, 2155.090

Mouse Propionyl CoA Carboxylase (beta chain)

Measured Mass	Computed Mass	Peptide Sequence
1088.542	1088.576	FANPFPAAVR
1464.782	1464.666	AFDNDVDALCNLR
1567.812	1567.813	DTSYLFITGPEVVK
1672.782	1672.768	GHQDVEAAQAEYVEK
1771.922	1771.892	AYNMLDIIHAVIDER
1879.962	1879.967	IQEGVESLAGYADIFLR
2154.082	2154.109	IMDQAITVGAPVIGLNDSGGAR

Unmatched Masses

889.510, 1111.500, 1157.580, 1197.550, 1357.640, 1383.690, 1514.730, 1536.720
1541.660, 1548.660, 1561.720, 1601.860, 1704.790, 1735.820, 1780.860, 1830.960
1851.920, 1965.030, 2005.000, 2115.990

Ionic interactions between subunits are the primary forces involved with keeping peripherally attached membrane proteins in contact with the membrane and other protein subunits. In most cases, these interactions can be disrupted by high salt concentrations or alterations of pH. To determine if P61 was a peripherally attached membrane protein, solubilization of P61 with 50 mM Na_2CO_3 was used. This technique uses the high salt concentration and high pH to disrupt any ionic interactions that may be holding P61 to the membrane. Resuspension of the 17,000 x g pellet of liver homogenate in 50 mM Na_2CO_3 followed by centrifugation at 200,000 x g successfully interrupted ionic interactions connecting P61 to the membrane fraction allowing its solubilization into the

200,000 x g supernatant. This provided further evidence that P61 may be the mammalian form of bacterial oxaloacetate decarboxylase α-chain.

Definitive evidence that P61 is the mammalian form of the α-chain of oxaloacetate decarboxylase would come from comparison of sequence of the two proteins. Several bacterial species of oxaloacetate decarboxylase have been sequenced; however, P61 must be isolated from the liver before sequence can be obtained. All biotin containing proteins solubilized by Na_2CO_3 were first isolated by affinity chromatography using immobilized monomeric avidin. The reduced binding affinity of monomeric avidin compared to tetrameric avidin allows for the reversible binding of biotinylated proteins and polypeptides. Once biotin-containing proteins were isolated using the monomeric avidin column, the fractions containing the eluted biotinylated proteins were further separated by SDS-PAGE. The molecular weight of P61 is less than the other biotin containing proteins allowing the discrete isolation of P61 for sequencing.

Peptide sequencing of isolated P61 produced 27 monoisotopic masses. When these masses were searched in the NCBInr database using PROWL, no confidant matches could be made. Only 8 of the 27 masses matched any previously sequenced protein. The top three matches were: 1. myosin-binding protein C, 2. propionyl CoA carboxylase (β-chain), and 3. phosphatidylinositol phosphate kinase. Of these, myosin-binding protein and phosphatidylinositol phosphate kinase can be dismissed due to discrepancies in molecular weight of these proteins compared to P61 (68 and 47 kDa respectively).

Propionyl CoA carboxylase (β-chain) has a similar molecular weight (58 kDa) to P61; however, the complete sequence of both α and β-chains of PCC have been determined revealing that the α-chain is the only portion of PCC that contains the

biotinylation motif recognized by holocarboxylase synthase. As shown by avidin blot, P61 is a biotin containing protein and so is not likely to be PCC (β-chain). Also, only a small portion of the peptide sequences were matched during the analysis of P61. The peptide profile of rat PCC β-chain is contained within the NCBInr database, and if PCC β-chain was truly the identity of P61, matching of all monoisotopic masses would be expected.

At this time, the identity of P61 remains unknown. Possible explanations include: lack of database information on all inherent rat proteins, lack of homology between rat and bacterial oxaloacetate, or that P61 is in fact a previously undescribed protein. While an identity of P61 is lacking, preliminary evidence described in this research indicates that P61 is possibly the mammalian form of bacterial oxaloacetate decarboxylase. Future amino acid sequence and function determination will be required to determine the identity of P61.

CHAPTER 6
SUMMARY

As a cofactor for the biotin-dependent carboxylases, the importance of biotin in intermediary metabolism is indisputable; however, it is debatable whether concern of biotin deficiency in the general public is warranted. In advanced societies, a frank biotin deficiency is unheard of unless caused by the consumption of a large quantity of egg-whites or the placement of a patient with short bowel syndrome on unsupplemented total parenteral nutrition. Even disorders of biotin metabolism such as biotinidase deficiency and single or multiple carboxylase deficiencies are rare. However, with the advancement of analytical techniques of biotin measurement, new evidence indicates even a marginal biotin deficiency with no outward signs of deficiency may have metabolic consequences.

In animals, a marginal biotin deficiency can have devastating effects on the fetus (45,46). Marginally biotin deficient hens produce chicks with higher mortality rates, reduced hatchability, parrot beak deformity, perosis, micromelia, and syndactyly. Fetal malformations in marginally biotin deficient hamsters and rats are similar including cleft palate, micrognathia, and micromelia. Recently, evidence has emerged indicating an increased biotin requirement and a marginal biotin deficiency during human pregnancy as shown by increased urinary excretion of 3-HIA and decreased excretion of urinary biotin and bisnorbiotin (44).

Outside of gestation, few deficiency studies have looked at a marginal deficiency. Measurements of biotin metabolism have normally occurred after several weeks of avidin induced biotin deficiency and outward signs of frank biotin deficiency have emerged. In

Chapter 3, we sought to add to the current body of literature by inducing a marginal biotin deficiency in male Sprague Dawley rats and comparing levels of free and protein-bound biotin to those of biotin-adequate, supplemented, and pharmacologically supplemented rats. Plasma, urine, and liver free biotin as well as liver protein-bound biotin were significantly reduced in rats fed an egg-white based AIN 76A biotin deficient diet compared to rats fed the same diet but with adequate, supplemented, or pharmacologically supplemented levels of biotin. This suggests that the lack of physiologic manifestations of frank biotin deficiency may not be a reliable measure by which to gauge biotin status.

Previous studies of biotin metabolism have used a purified diet with spray-dried egg-white as the sole protein source and a free biotin level of 0.6 mg biotin/kg diet (72,73,109). At this level of supplementation, the rat consumes 900 ng biotin/day, which is equivalent to a 70 kg human consuming 4.2 mg/day. This level of consumption is highly supplemented when compared to the ESADDI of 30-100 μg/day. In this study we compared a more relevant dietary intake of 0.06 mg biotin/kg diet to the previously used intake of 0.6 mg biotin/kg diet as well as to a pharmacologically supplemented level of 100 mg biotin/kg diet. Only plasma free biotin and the relative abundance of liver ACC2 were significantly lower in rats consuming 0.06 mg biotin/kg diet than in supplemented rats consuming 0.6 mg biotin/kg diet. Since urinary excretion was not reduced and ACC1, PC, MCC, and PCC were fully biotinylated, it was concluded that 0.06 mg biotin/kg diet was an adequate amount of biotin for Sprague-Dawley rats over the three week time period. We also saw no indication of toxicity in rats consuming 100 mg biotin/kg body weight over this time period.

The importance of biotin in normal immune function is well documented. Biotin deficiency leads to reduced antibody production, immunological reactivity, T and B-cell differentiation, and cytotoxic T-cell response (30,52,132,133). Recently, the uptake and metabolism of biotin during mitogen stimulation was investigated to determine the effect of an immune response on biotin metabolism in vitro. Mitogen stimulation resulted in a substantial increase in biotin uptake into proliferating lymphocytes through an increase in biotin transporters per cell with evidence suggesting that this increase is to support biotinylation of biotin-dependent carboxylases (96,115). Chapter 4 sought to extend the literature by measuring biotin metabolism in vivo during an immune response triggered by the administration of endotoxin to rats.

In biotin-adequate animals (Figure 6-1), endotoxin administration significantly reduced the relative abundance of biotinylated liver ACC1, ACC2, and PC while significantly increasing biotinylated MCC and P61. A reduction in these carboxylases provides possible mechanisms by which the body controls fatty acid synthesis, fatty acid β-oxidation, and gluconeogenesis. Acetyl CoA carboxylase 1 and 2 in the liver would indicate a mechanism by which the body is able to reduce fatty acid synthesis and increase fatty acid β-oxidation, a known occurrence during endotoxin exposure. The decrease in biotinylated PC would further corroborate evidence for decreased gluconeogenesis during endotoxin exposure. Leucine levels are known to increase in the liver during endotoxin exposure and the increase in MCC, the biotin-dependent carboxylase involved in leucine degradation, may occur to help remove the extra leucine. The significance of the increase in P61, a previously undescribed biotinylated protein,

remains unknown at this time but reinforces the fact that other functions of biotin in the body remain undiscovered.

We were also able to compare the normal response of biotin metabolism to that of biotin-deficient animals exposed to endotoxin (Figure 6-2). For the majority of the carboxylases, the response to endotoxin was blunted or delayed compared to biotin-adequate animals. What this means to recovery of the animal from endotoxin exposure remains unknown; however, data from serum Zn levels from biotin-deficient rats indicate an impaired immune response. Other indicators of immune response should be measured to determine how biotin-deficiency impairs the body's response to endotoxin.

Of particular interest was the appearance of a novel biotinylated protein (P61) in liver from rats exposed to endotoxin. In Chapter 5, we sought to characterize this novel protein through sub-cellular localization, specificity to avidin detection, definition of membrane interactions, and finally sequencing of the protein. Through these experiments, P61 was localized to the nuclear and mitochondrial membrane fractions. It was determined to specifically bind avidin as shown by competition of the detection of P61 when avidin was preincubated with free biotin. P61 was also determined to be a peripherally attached membrane protein due to the ability of high ionic strength and pH to solubilize this protein. Although isolated, no confident matches of P61 could be made to existing proteins in the NCBInr database. Amino acid sequence will be required to obtain a true identity of P61.

These data reinforce the importance of biotin in intermediary metabolism and immune function. They also agree with old and new findings showing how biotin may have functions other than as the cofactor for the biotin-dependent carboxylases. This

includes gene regulation, DNA packaging, and cell proliferation (11,15). These data begin to provide new understanding of the metabolic changes associated with biotin intake as well as the changes in biotin metabolism during endotoxin exposure on carbohydrate, protein, and fatty acid metabolism. Further study will lead to a better understanding of biotin's role in these processes and may begin to elucidate even more undiscovered functions.

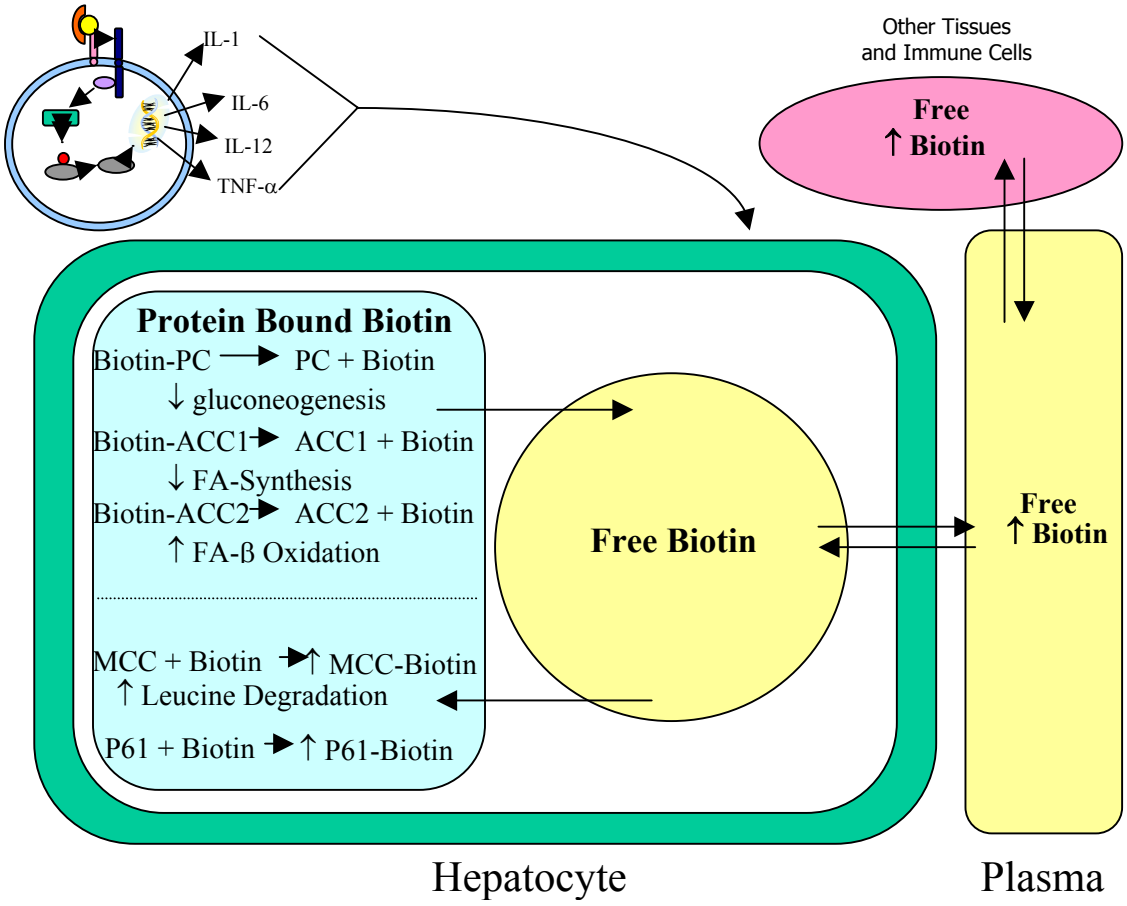


Figure 6-1. The effect of endotoxin on biotin metabolism in a biotin-adequate rat. After endotoxin exposure the hepatocyte is affected by cytokines secreted by activated immune cells. The hepatocyte reduces the levels of biotinylated PC, ACC1, and ACC2 releasing biotin to the cytosolic free pool of biotin in the hepatocyte. This raises the level of free biotin within the hepatocyte and provides a source of biotin to increase plasma levels of free biotin as well as provides a supply of biotin to increase other biotinylated proteins within the hepatocyte.

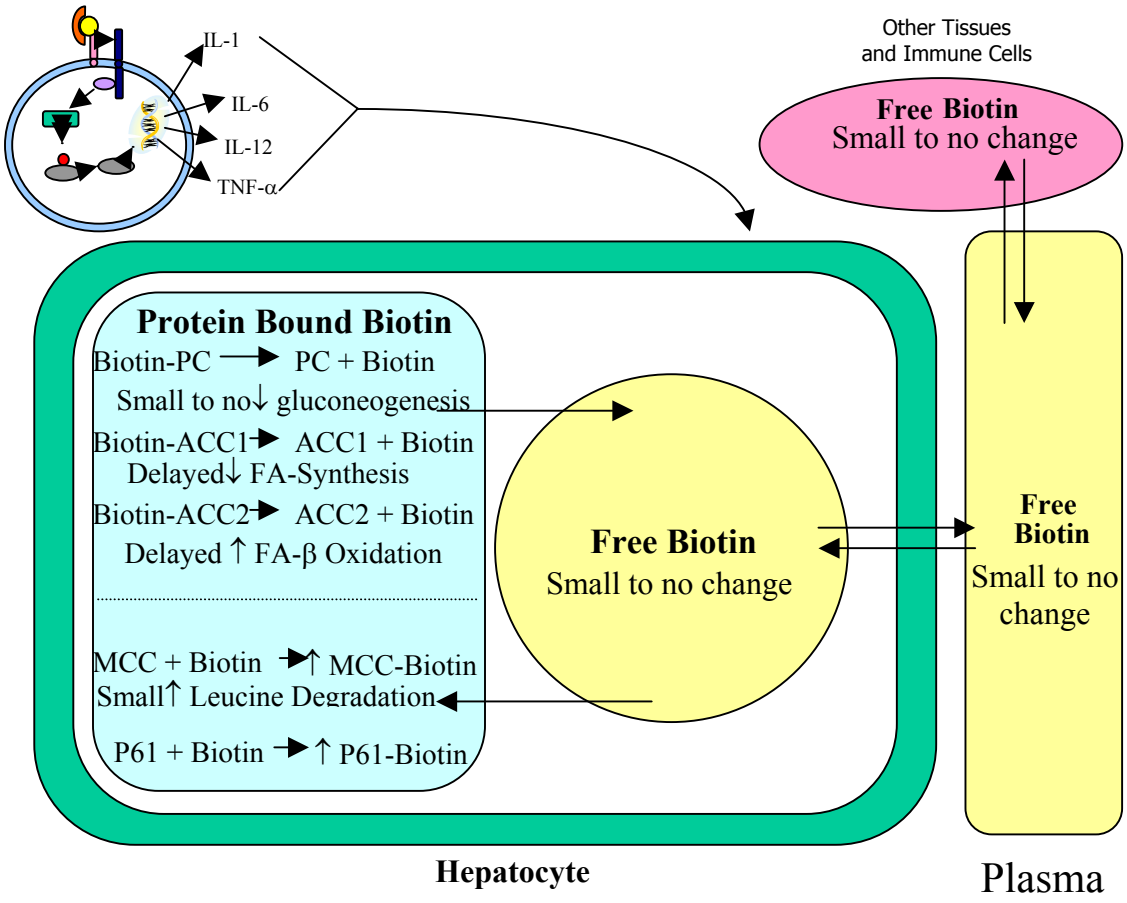


Figure 6-2. The effect of endotoxin on biotin metabolism in a biotin-deficient rat. After endotoxin exposure the hepatocyte is affected by cytokines secreted by activated immune cells; however, in the biotin-deficient hepatocyte, the changes in the levels of biotinylated proteins are smaller in magnitude and delayed compared to the biotin adequate hepatocyte.

APPENDIX
LIST OF ABBREVIATIONS

ACC1	Acetyl CoA carboxylase 1
ACC2	Acetyl CoA carboxylase 2
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BBSA	Biotinylated bovine serum albumin
BNB	Bisnorbiotin
BSO	Biotin sulfoxide
BW	Body weight
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECF	Enhanced chemifluorescence
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ESADDI	Estimated safe and adequate daily dietary intake
FafBSA	Fatty acid free bovine serum albumin
FAS	Fatty acid synthase
HEM	HEPES, EDTA, Mannitol
HEPES	4-(2-hydroxyethyl)-1-piperarzineethanesulfonic acid
3-HIA	3-hydroxyisovaleric acid
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IL	Interleukin
IRAK	IL-1 receptor associated kinase
K_d	Dissociation constant
kDa	Kilodalton
kg	Kilogram
LBP	LPS binding protein
LPS	Lipopolysaccharide
MALDI TOF	Matrix-assisted laser-desorption ionization time of flight
MCC	Methylcrotonyl CoA carboxylase
3-MCG	3-methylcrotonyl glycine
mg	Milligram
MODS	Multiple organ dysfunction syndrome
mRNA	Messenger ribonucleic acid

NFDM	Nonfat dry milk
NHS-biotin	N-hydroxysuccinimide ester
NMWCO	Nominal molecular weight cut off
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC	Pyruvate carboxylase
PCC	Propionyl CoA carboxylase
PEPCK	Phosphoenolpyruvate carboxykinase
PGE_2	Prostaglandin E_2
PVDF	Polyvinylidene fluoride
SAL	Saline
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
TBST	Tris buffered saline, tween 20
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF6	TNF receptor associated factor 6

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BIOGRAPHICAL SKETCH

Brandon Lewis was born in Pensacola, FL, where he graduated from B. T. Washington High School in 1993. He attended the University of Florida and was awarded a Bachelor's of Science degree in food science and human nutrition in 1997. He then began the combined MS/DI program from which he progressed to the Doctor of Philosophy in nutritional sciences program at the University of Florida.