HEART- AND LIVER-TYPE FATTY ACID BINDING PROTEINS IN LIPID AND GLUCOSE METABOLISM

A Dissertation

by

ERDAL EROL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2004

Major Subject: Veterinary Microbiology

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Approved as to style and content by:

Bert Binas (Chair of Committee)	Jeremy Wasser (Member)
Gale Wagner (Member)	Stephen Safe (Member)
Ann B. Kier (Head of Department)	

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ABSTRACT

Heart- and Liver-Type Fatty Acid Binding Proteins in Lipid and Glucose Metabolism.

(August 2004)

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Chair of Advisory Committee: Dr. Bert Binas

Heart-type Fatty Acid-Binding Protein (H-FABP) is required for high rates of skeletal muscle long chain fatty acid (LCFA) oxidation and esterification. Here we assessed whether H-FABP affects soleus muscle glucose uptake when measured in vitro in the absence of LCFA. Wild type and H-FABP null mice were fed a standard chow or high fat diet before muscle isolation. With the chow, the mutation increased insulin-dependent deoxyglucose uptake by 141% ($P<0.01$) at 0.02 mU/ml of insulin, but did not cause a significant effect at 2 mU/ml insulin; skeletal muscle triglyceride and long chain acyl-CoA (LCACoA) levels remained normal. With the fat diet, the mutation increased insulin-dependent deoxyglucose uptake by 190% ($P<0.01$) at 2 mU/ml insulin, thus partially preventing insulin resistance, and completely prevented the threefold ($P<0.001$) diet-induced increase of muscle triglyceride levels; however, muscle LCACoA levels showed little or no reduction. With both diets, the mutation reduced the basal (insulin-independent) soleus muscle deoxyglucose uptake by 28% ($P<0.05$). These results establish a close relationship of FABP-dependent lipid pools with insulin sensitivity, and indicate the existence of a non-acute, antagonistic, and H-FABP-dependent fatty acid regulation of basal and insulin-dependent muscle glucose uptake.

Liver fatty acid binding protein (L-FABP) has been proposed to limit the availability of chain LCFA for oxidation and for peroxisome proliferator-activated receptor (PPAR-alpha), a fatty acid binding transcription factor that determines the capacity of hepatic fatty acid oxidation. Here, we used L-FABP null mice to test this hypothesis. Under fasting conditions, this mutation reduced β-hydroxybutyrate (BHB) plasma levels as well as BHB release and palmitic acid oxidation by isolated hepatocytes. However, the capacity for ketogenesis was not reduced: BHB plasma levels were restored by octanoate injection; BHB production and palmitic acid oxidation were normal in liver homogenates; and hepatic expression of key PPAR-alpha target (MCAD, mitochondrial HMG CoA synthase, ACO, CYP4A3) and other (CPT1, LCAD) genes of mitochondrial and extramitochondrial LCFA oxidation and ketogenesis remained at wild-type levels. These results suggest that under fasting conditions, hepatic L-FABP contributes to hepatic LCFA oxidation and ketogenesis by a nontranscriptional mechanism.

DEDICATION

I dedicate this dissertation to my parents and family, who encouraged me and who never stopped believing in my pursuit of this work. And to my father, Ibrahim Erol, whose untimely death during this time did not allow him to share in the celebration of this accomplishment.

ACKNOWLEDGEMENTS

I give special thanks to my graduate advisor and mentor, Dr. Bert Binas, for his continuous support, creative insights, contributions and patience during my study. I also thank my graduate committee members, Drs. Gale Wagner, Stephen Safe and Jeremy Wasser for their support throughout the doctoral program. I give special thanks to Dr. Gale Wagner for his great help and advice during my Ph.D. program.

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CHAPTER I

INTRODUCTION

LCFAs and their metabolism

Lipids are a chemically diverse group of compounds. Their common feature is insolubility in water. They are classified into different categories and have diverse biological functions; fatty acids (FAs), which are carboxylic acids, are the principle stored form of energy in many organisms whereas phospholipids and sterols are partitioned into biological membranes. Other lipids are found in relatively small amounts in cells and play crucial roles as enzyme cofactors (vitamin K), detergents (bile acids) pigments (retinal), hydrophobic anchors (covalently attached fatty acids and phosphatidylinositol), hormones (vitamin D derivatives) and intracellular messengers (eicosanoids).

FAs are highly reduced compounds with hydrocarbon chains of 4 to 36 carbons with an energy of complete oxidation (~38kJ/g) more than twice that for the same weight of carbohydrate or protein. In some FAs, this chain is fully saturated (contains no double bonds) and unbranched; others contain one or more double bonds. Because of their hydrophobicity and extreme insolubility in water, they segregate into lipid droplets in the form of triacylglycerols (TG), which do not raise the osmolarity of the cytosol. TGs contain three FA molecules esterified to the three-hydroxyl groups of glycerol. The relative chemical inertness of TGs allow their intracellular storage in large quantities,

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This dissertation follows the style and format of Research in Veterinary Science.

but not extreme quantities, without the risk of undesired chemical reactions with other cellular components.

Cells that derive energy from the oxidation of FAs may obtain FAs from three sources, namely, fats in diet, fat stores in cells and fats in newly synthesized in cells (de novo synthesis). Because of their insolubility in water, TGs taken by food must be emulsified before they can be digested by water-soluble enzymes in intestines. Bile salts synthesized from cholesterol in liver help convert dietary fats into mixed miscelles of bile salts and TGs. Lipid molecules are more accesible to the action of water-soluble lipases in intestines. Here, TGs are converted into monoglycerides, DGs, free FAs and glycerol. These compounds are small enough to diffuse into the epithelial cells lining the intestinal mucosa, where they are reconverted into TGs and packaged with dietary cholesterol and lipid-binding proteins, called apoliproteins, agregating into chylomicrons. These chylomicrons go through from the lymphatic system, from which they enter the blood and are carried to muscle, adipose tissues and liver. The extracellular enzyme lipoprotein lipase is activated by apoprotein C-II, enabling hydrolysis of TGs into mainly long chain FAs (LCFAs) and glycerol. FAs are ultimately oxidized for energy or reesterified for storage as TGs, depending on the physiological condition of the individual. When the diets contain more FAs than are needed immediately for fuel or precursors, they are converted into TGs in the liver, and TGs are packaged with specific apoliproteins into very-low density lipoproteins (VLDLs). VLDLs are transported in blood from the liver to adipose tissues, where the TGs are removed and stored in lipid droplets within adipocytes.

Although most cells are capable of de novo FA synthesis, cells with large requirements for FA beta-oxidation, such as cardiac and skeletal myocytes, rely on import of exogenous LCFAs to provide this critical metabolic fuel. Cells that store potential energy in the form of TGs, such as adipocytes, have efficient mechanisms for both LCFA import and export. Since most tissues contain only small amounts of storage lipids, energy production depends on a continuous supply of FAs, mostly from adipose tissues. The capacity of mammalians to store these LCFAs as triglycerides (TGs) in cytosolic lipid droplets is an important adaptation for survival during periods of nutritional deprivation. In adipose tissue, FAs are produced by lipolysis, transported bound to albumin in blood and taken up by tissues through passive diffusion or in a process mediated by transport proteins in the plasma membrane, as explained above. Once within the cells, free FAs are reportedly bound to fatty acid binding proteins (FABPs) which are abundant in the cytosol (Ockner and Manning, 1982), depending on the tissue and its metabolic demand. FAs are either converted to TGs, diglycerides (DGs), membrane phospholipids or oxidized in mitochondria for energy production.

For a long time it was believed that the transport of FAs into the muscle cell was purely a passive process. This was based on early observations that FA uptake increased linearly with FA concentration. Recently, specific carrier proteins have been identified in various tissues, including skeletal muscle. In the sarcolemma, two proteins have thus far been identified that are involved in the transport of FAs across the membrane but their functional significances are still unclear. These proteins are a specific plasma membrane FA binding protein (FABPpm) (Sorrentino et al., 1988) and a FA translocase protein (FAT/CD36) (Abumrad et al., 1993). A third protein has been identified (FA transport

protein, FATP) (Schaffer and Lodish, 1994), but its transport role is still under investigation. It is worth noting, however, that not only LCFAs but also far more hydophobic compounds, such as phospholipids and cholesterol, are fully capable of inter-membrane transfer in the absence of any binding protein whatsoever (Brown, 1992).

Cytosolic transport of LCFAs to various intracellular organelles including mitochondria may be mediated by FABPs (Figure I.1). In considering a transport function for the FABPs in the cytoplasm, the intracellular FABPs have been proposed as transporters of LCFAs in the soluble cytoplasm (Storch and Thumser, 2000). The notion that these proteins function as intracellular transporters originally derived from the assumption that hydrophobic LCFAs would require a transport protein for transit in an aqueous milieu such as cytoplasm. LCFAs are released from the LCFAs-FABP complex when they reach their final destinanation in the cytoplasma and either esterified in the cytoplasma or oxidized in mitochondria.

LCFAs in the cytoplasma may be activated by the enzyme acyl-CoA synthethase to form an acyl-CoA complex. For oxidation, this acyl-CoA complex can be bound to carnitine under the influence of the enzyme carnitine palmitoyl transferase I (CPT I), which is located at the outside of the outer mitochondrial membrane (Schulz, 1985). The binding of carnitine with the activated LCFA is the first step in the transport of the LCFA into the mitochondria. As carnitine binds to the acyl-CoA moiety, free CoA is released. The acyl-carnitine complex is transported with a translocase and reconverted into acyl-CoA at the matrix side of the inner mitochondrial membrane by the enzyme carnitine palmitoyl transferase II (CPT II). The carnitine that is released diffuses back across the

into acyl- CoA at the matrix side of the inner mitochondrial membrane by the enzyme carnitine palmitoyl transferase II (CPT II). The carnitine that is released diffuses back across the mitochondrial membrane into the cytoplasm and thus becomes available again for the transport of other LCFAs.

The oxidation of LCFAs to Acetyl-CoA in mitochondria is a central energy-yielding pathway in mammals. The electrons removed during FA oxidation pass through the mitochondrial respiratory chain, beta-oxidation, driving ATP synthesis. The acetyl CoA produced from the FAs may be completely oxidized to carbon dioxide via the citric acid cycle, resulting in further energy production. Acetyl CoA can also be converted in liver into ketone bodies-water soluble fuels exported to the extrahepatic tissues such as brain and muscles when glucose is not available.

Regulation of LCFA metabolism

It has been proposed that the regulation of FAs take place at several levels such as the cell membrane, the mitochondrial membrane and through transcriptional regulation. FA transporters are likely to be responsible for some of the transport of FA across the sarcolemma, and these transporters can be regulated both acutely and chronically. Recently, Bonen et al. (2000) demonstrated that FAT/CD36 can translocate from intracellular vesicles to the cell membrane in a similar manner as the GLUT-4 protein, indicating that FA transport can be regulated. Along with a higher density of FAT/CD36 at the cell membrane, an increased LCFA transport into the cell has been observed. At present, however, it is not known if there are any physiological situations in which this transport becomes limiting. It is also not known what the triggers are inside the cell for the up- or down-regulation of transport proteins such as cytosolic FABPs.

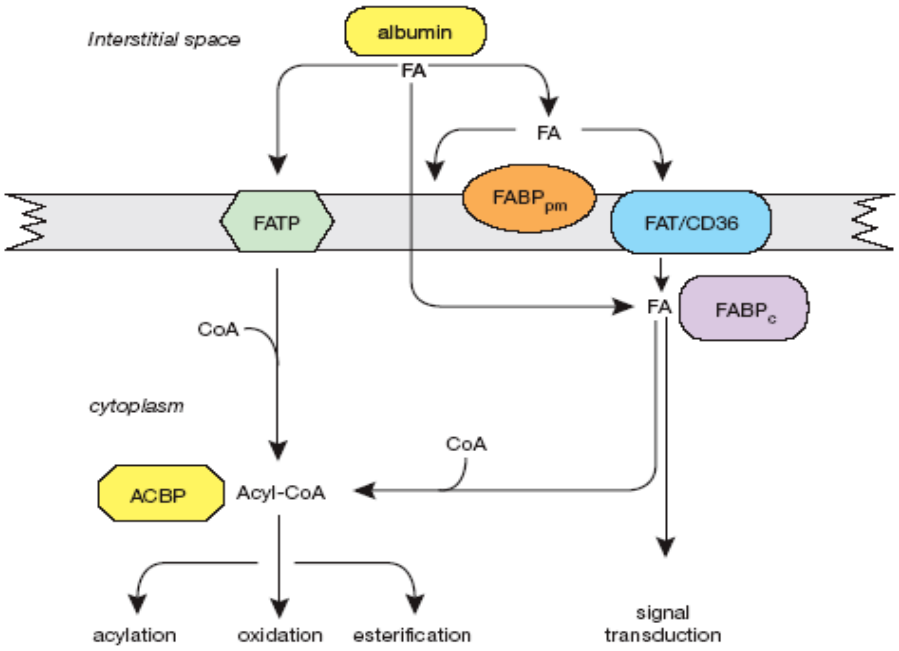

Figure I.1: Fatty acid metabolism and FABPs (from Glatz et al., 2003).

It is generally believed that CPT I is the rate-limiting enzyme in the transport of LCFAs across the mitochondria and may be even rate-limiting for FA oxidation (Ruderman and Dean, 1998). There is substantial evidence that CPT I activity is influenced by numerous regulators. The most important regulator of CPT I activity is malonyl-CoA concentration.

A large number of in vitro studies have now established a role for malonyl-CoA in regulating the entry of LCFAs into the mitochondria in a variety of tissues, including skeletal muscle (Rewieved by Saha and Ruderman, 2003). Malonyl-CoA is a potent inhibitor of CPT I and is thus a potential candidate for the regulation of fat metabolism **(**McGarry and Brown, 1997). Malonyl-CoA is formed from acetyl-CoA, a reaction catalyzed by the enzyme acetyl-CoA carboxylase (ACC). Malonyl-CoA levels decrease in rodent skeletal muscle from rest to moderate intensity exercise, when energy production from fat increases. It is believed that the resting concentrations of malonyl-CoA are sufficiently high to inhibit CPT I, and a decrease in the malonyl-CoA concentration would therefore result in a relief of the inhibition of CPT I and increased LCFA transport into the mitochondria. It is also well known that acetyl-CoA concentration in the muscle increases rapidly at the onset of high-intensity exercise, which will stimulate the activity of ACC as it is the primary substrate for this enzyme. The resulting increased concentration of malonyl-CoA could possibly explain a reduced FA uptake into the mitochondria. It should also be mentioned that activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK)-activated protein kinase increases fatty acid oxidation by lowering the concentration of Malonyl-CoA (Ruderman et al., 2003).

There are now many evidences that peroxisome proliferator-activated receptors (PPARs), members of the ligand-activated nuclear receptor superfamily, exert their major influences on lipid and glucose metabolism (reviewed by Ferre, 2004). PPARs selectively modulate cellular and tissue capacities for FA acid oxidation by acting as lipid sensors. They bind to specific recognition sites known as peroxisome proliferator-activated receptor response elements- PPREs, located in regulatory regions of certain target genes. Most PPREs have been identified in the promoters of genes encoding proteins related to FA oxidation and transport of FA synthesis. At least three different PPARs have now been identified, i.e. PPAR- alpha, PPAR-gamma, and PPAR-delta), which exert their regulatory function directly at the level of gene expression (Kliewer et al., 1997). It has been suggested that PPAR- alpha activation stimulates FA catabolism by inducing L-FABP expression (Kaikaus et al., 1993; Isseman et al., 1992). PPAR-delta has been suggested to mediate the effects of LCFAs in pre-adipocytes, thereby inducing adipogenesis. However the effects of FAs may be mediated exclusively through PPAR-gamma (Hu et al., 1995), or perhaps a combination of PPAR-delta and PPAR-gamma (Ailhaud, 1997).

Regarding regulation of FA synthesis, the reaction catalyzed by acetyl CoA carboxylase (ACC) is the rate-limiting step in the synthesis of fatty acids and this enzyme has important regulatory function. When there is an increase in the concentration of mitochondrial acetyl-CoA and of ATP, citrate is transported out of the mitochondria and becomes both the precursor of cytosolic Acetyl-CoA and an allosteric signal for the activation of ACC.

ACC is also regulated by covalent alteration. Hormones like glucagon and epinephrine trigger phosphorylation, which slows down FA synthesis. Other enzymes in the FA synthesis pathway are also regulated. The pyruvate dehydrogenase complex and citrate lyase, both of which supply acetyl-CoA, are activated by insulin through a cascade of protein phosphorylation. The rate of TG biosynthesis is profoundly altered by the action of several hormones. Insulin promotes the conversion of carbohydrates into TGs. People with severe diabetes, due to the failure of insulin secretion or action, not only are unable to use glucose properly but also fail to synthesize fatty acids from carbohydrates or amino acids. TG metabolism is also influenced by glucagon, growth hormone and adrenal corticol hormones.

Recent data strongly suggest that sterol regulatory element binding protein-1c (SREBP-1c) is a key transcription factor that activates transcription of genes involved with fatty acid synthesis (reviewed by Horton, 2002). SREBPs directly activate the expression of more than 30 genes related to the synthesis and uptake of FAs, cholesterol, TGs and phospholipids (PLs). It has been suggested that SREBP-1c mediates insulin's lipogenic action in liver. Insulin decreases the transcription of genes encoding gluconeogenic enzymes and increases lipogenic enzymes such as fatty acid synthase and ACC. Recent studies suggest that many of these transcriptional changes may be mediated by an increase in the levels of transcripton factor SREBP-1c, whose mRNA levels are increased by insulin (Kim et al., 1998; Foretz et al., 1999; Shimomura et al., 1999). The liver is the responsible organ for convertion of excess carbohydrates to FAs to be stored or burned in muscle. In carbohydrate excess, insulin stimulates the FA synthesis in liver and insulin's stimulatory effect on lipid synthesis is mediated by an

increase in SREBP-1c. Dominant negative forms of SREBP1 can block expression of these gluconeogenic and lipogenic enzymes (Foretz et al., 1999), whereas overexpression can increase their expression (Shimomura et al., 1999).

FABPs

FABPs in mammalian cells were discovered more than 30 years ago as a small (13-15 kDa), non-enzymatic cytoplasmic proteins which bind long-chain fatty acids in vitro (Ockner et al., 1972; Mishkin et al., 1972). So far, they have been now classified in at least eight distinct types (Table I.1). FABPs from intestine (I-FABP) (Ockner et al., 1972), liver (L-FABP) (Mishkin et al., 1972) and heart (H-FABP) (Fournier et al., 1978) were the first of the family being characterized. Despite the relevant structural similarities among all of them, each member presents distinct features, particularly in the way they bind the ligand in these tissues. FA flux is substantial in all above-mentioned organs and thus it was proposed that FABPs are necessary for intracellular binding and transport of LCFAs. There are some other proposed functions for FABPs such as, (a) modulation of specific enzymes of lipid (Storch and Thumser, 2000) and maintenance of low FA concentrations in metabolic pathways, either anabolic or catabolic (Kaikaus et al., 1990); (b) maintenance of cellular membrane FA levels (Glatz et al., 1995); and (c) regulation of the expression of FA-responsive genes (Glatz and Van der Vusse, 1996). Within these categories, numereous more specific functional roles may be assigned to these proteins. For instance, FABPs may modulate lipid metabolism via an involvement in the FA uptake or export process, by regulation of substrate and/or product

Table I.1: The mammalian fatty acid binding protein family (modified from Storch and Thumser, 2000).

Name	**Previous/other names**	**Tissue localization**
L-FABP	Z Protein, heme-binding protein, hepatic FABP	Liver, small intestine
I-FABP	Gut FABP	Small intestine
H-FABP	Muscle FABP	Cardiac and skeletal muscle, brain, mammary gland, kidney, adrenals, ovaries, testis
A-FABP	aP2, ALBP	Adipocyte, monocyte
E-FABP	K-FABP, Mal1-1, KLBP, skin-FABP, psoriasis-associated-FABP	Epidermis, adipocyte, mammary tissue, tongue epithelia, testis
B-FABP	BLBP	Brain, central nervous system
M-FABP	Myelin P2	Peripheral nervous system

concentrations in the cytosolic compartments as a whole or more locally near particular enzymes. In addition, FABPs can exert their action with more specific FABP-membrane interactions at particular membrane sites. Finally, the FABPs might regulate gene expression either by a passive maintenance of unbound FA levels as determined by ligand partitioning, or by a more direct role in cellular targeting of FAs, for example via direct interaction with nuclear hormone receptors.

Although studies of binding properties do not directly address a role in cellular FA trafficking, they nevertheless provide indirect support for such a function by demonstrating the specificity and affinity of FABPs for FAs. Equilibrium binding analyses and structural studies clearly demonstrate that most FABPs bind LCFAs (C16-C20) with high affinity and a molar stoichiometry of 1:1 (Sacchettini et al., 1989; Sacchettini et al., 1992; Cistola et al., 1989). The exception is L-FABP, which binds not only LCFAs but also other acyl ligands such as bind other hydrophobic ligands, including acyl coenzyme A, lysophospholipids, heme and bile salts. (Haunerland et al., 1984; Vincent and Muller-Eberhard, 1985) The binding charecteristics of L-FABP are also unique among the FABPs, as this protein can bind a molar ratio of two LCFAs (Richieri et al., 1994).

The hydrophobic nature of LCFAs complicates the determination of binding affinities. Therefore, a wide range of different techniques have been utilized to measure ligand binding. Collectively, these studies are in general agreement concerning the ligand specificity and binding stoichiometry, but offer widely differing absolute values for binding affinities. Briefly, methods such as Lipidex and liposome assays have

generally yielded affinities for LCFAs in the μM range (Glatz and Veerkamp, 1983; Brecher et al., 1984). More recently, the sensitivity of florescence spectroscopy has led to the development of several florescence-based assay. As determined by this method, binding affinities differ for FABP and FA type, with Kd values ranging from 2 to 1000 nM, and in general affinities are higher for saturated vs. polyunsaturated LCFAs (Richieri et al, 1994). Using the ADIFAB assay, Kd values of H-FABP, L-FABP and I-FABP for oleate of 10, 9 and 39 nM, respectively are obtained (Richieri et al, 1998). Affinities of most of the FABPs are generally highest for palmitate, oleate and stereate, and increased water solubility of polyunsaturated LCFAs is reflected by higher Kd values (Richieri et al., 1994).

A number of general correlations between the extent of lipid metabolic activity and the amount of intracellular FABP have also been demonstrated, providing additional, albeit indirect, support for a LCFA transport and /or trafficking function of FABPs. For example, there is a correlation between H-FABP content and muscle development, i.e. a requirement for mitochondrial beta-oxidation (Haunerland et al., 1993), and induction of peroximal beta-oxidation correlates well with L-FABP levels (Veerkamp and Moerkerk, 1993).

Among the most generally accepted functions for the FABPs is their participation in the utilization of dietary lipids. Tissues with high targets of FA metabolism and uptake/storage, e.g. intestine, liver, adipocyte and muscle, have increased FABP levels which parallel FA flux and utilization (Glatz and Van der Vussa, 1996). Furthermore, the intestinal distribution of L-FABP and I-FABP, from duodenum

to colon and crypt to villus tip, correlates well with the known distribution of dietary lipid uptake and intracellular processing (Cohn et al., 1992). In addition to increasing L-FABP content, the feeding of high fat diets to rodents results in peroxisomal proliferation and increased levels of acyl coenzyme-A oxidase, cytochrome P-450 4A1 and peroxisomal beta-oxidation (Isseman et al., 1992).

The numerous links between cellular lipid metabolism and FABP levels, including PPAR activation, have clearly demonstrated a role for the FABPs in mediating diverse aspects of lipid utilization, quite possibly via transport mechanisms. To further understand these roles and to enable the nutritional and/or pharmacological modulation of FABP function, it is necessary to establish the fundamental mechanisms by which FA trafficking in cells is modulated by the FABPs.

In addition to the many indirect approaches to an FABP transport function, several direct approaches have been taken in recent years. In an *in vitro* system that measures the flux of oleate through a lipid-water interface was found to increase three fold (Weisiger et al., 1989). Similarly, L-FABP and H-FABP were reported to increase the amount and rate of movement radiolabeled fatty acid between two compartments, although a substantial loss of label to the surface of the apparatus was not accounted for (Peeters et al., 1989).

PPAR-alpha is predominantly expressed in tissues with high fatty acid catabolic rates, such as the liver, kidney, heart and muscle (Kliewer et al., 1999), and ligands for PPAR- alpha include LCFAs and FA derivatives such as leukotriene B4, as well as the hyperlipidemic fibrates (Kaikaus et al., 1993). PPAR- alpha activation stimulates FA catabolism by inducing mitochondrial and peroxisomal beta-oxidation levels, while also

inducing L-FABP expression (Kaikaus et al., 1993; Isseman et al., 1992). Since intracellular FA accumulation leads to increased levels of L-FABP, it can therefore be inferred that L-FABP is probably associated with mitochondrial and peroxisomal beta-oxidation. It is hypothesized that FABPs promote the effects of LCFAs on gene transcription and cell growth/differentiation by trafficking ligands directly to the PPARs, in a manner similar to that proposed for cellular retinoic acid-binding protein (CRABP) and the retinoic acid receptor (RAR)(Isseman and Green, 1990; Kaikaus et al., 1990). Such a mechanism implies direct protein-protein interactions between FABPs and PPARs, which have yet to be demonstrated. Nevertheless, Schachtrup et al., (2004) speculated that PPARalpha mRNA was absent in the alveolar macrophages although liver-type FABP was expressed, indicating that gene expression of liver-type FABP was independent of PPARalpha.

The importance of FABPs in lipid metabolism has also been explored in a more direct ways by creating knock-out mice models. It has been found that H-FABP null mice have severe defect LCFA cellular transport and beta-oxidation in cardiac myocytes (Binas et al., 1999; Schaap et al., 1999). This defect was manifested in the H-FABP null mice by excercise intolerance, localized cardiac hyperthrophy, and increased myocardial glucose uptake.

Creation of L-FABP null mice also showed that this protein is important in the LCFA flux into the cytoplasm, as it is shown that lipogenesis is decreased in the L-FABP null mice (Martin et al., 2003)

Studies of I-FABP null mice demonstrated that I-FABP is not required for normal development or dietary fat absorbtion, but there was significant sexual

dimorphism in the phenotype (Vassileva et al., 2000). Nevertheless, it should be noted that mice lacking I-FABP is confounded by substantial compensatory upregulation of epidermis-FABP (E-FABP), which is normally minimally expressed in wild-type adipose tissue (Hotamisligil et al., 1996).

FABPs and their relation to LCFAs and glucose metabolism

"The last decade has seen a resurgence of interest in effects of fatty acids on glucose metabolism" (Randle, 1998). Lipid and glucose metabolism are linked via substrate competition, hormone signaling, and gene expression (Randle, 1998; Finck et al., 2002). Patients with uncontrolled diabetes typically have abnormally high levels of circulating lipids, especially non-esterified fatty acids (NEFA) (Saltiel, 2001). How elevated NEFAs contribute to the dysregulation of glucose homeostasis, directly impair insulin sensitivity remains uncertain, but evidence is emerging that muscle might be one of the direct targets.

Insulin resistance (IR), the failure to respond to normal circulating concentrations of insulin, is a common state associated with obesity, aging, sedentary lifestyle, and genetic predisposition. The failure of insulin to stimulate glucose uptake by muscle appears to be a primary defect. Also, in certain fat depots, subsequent resistance to antilipolytic effects of insulin causes increased lipolysis and fatty acid release. These fatty acids attenuate the ability of insulin to suppress glucose production, but allow a continual increase in insulin-stimulated fatty acid synthesis. Thus, the dysregulation of carbohydrate and lipid metabolism accelerates the progression of IR. Beta-cells of the pancreas normally compensate for the insulin resistant state by increasing basal and postprandial insulin secretion. This consequently, further aggravates IR. At some point,

beta-cells can no longer compensate, failing to respond appropriately to glucose. This ultimately leads to the deterioration of glucose homeostasis and the development of glucose intolerance, the inability to properly dispose of glucose. Adipose and liver cells produce more FAs, the liver produces more glucose (gluconeogenesis) in an unregulated fashion, and the beta-cells undergo progressive decompensation, resulting in the late stages of disease, where high doses of insulin is required. Approximately, 5-10% of glucose-intolerant patients progress to frank diabetes.

There are several mechanisms by which plasma fatty acid inhibits glucose transport activity, leading to the FA-induced IR. Increased plasma free fatty acid concentrations are typically associated with many IR states, including obesity and type 2 diabetes mellitus (Reaven et al., 1988; Boden et al., 1994; McGarry, 1992). In a cross-sectional study of young, normal-weight offspring of type 2 diabetic patients, it has been found an inverse relationship between fasting plasma fatty acid concentrations and insulin sensitivity, consistent with the hypothesis that altered fatty acid metabolism contributes to insulin resistance in patients with type 2 diabetes (Perseghin et al., 1997).

About 40 years ago, Randle et al. (1963) demonstrated that fatty acids compete with glucose for substrate oxidation in isolated rat heart muscle and rat diaphragm muscle (Figure I.2). They hypothesized that increased fat oxidation causes the insulin resistance associated with obesity. The mechanism they proposed to explain the insulin resistance was that an increase in fatty acids caused an increase in the intramitochondrial acetyl CoA/CoA and NADH/NAD^+ ratios, with subsequent inactivation of pyruvate dehydrogenase. This in turn would cause intracellular citrate concentrations to increase,

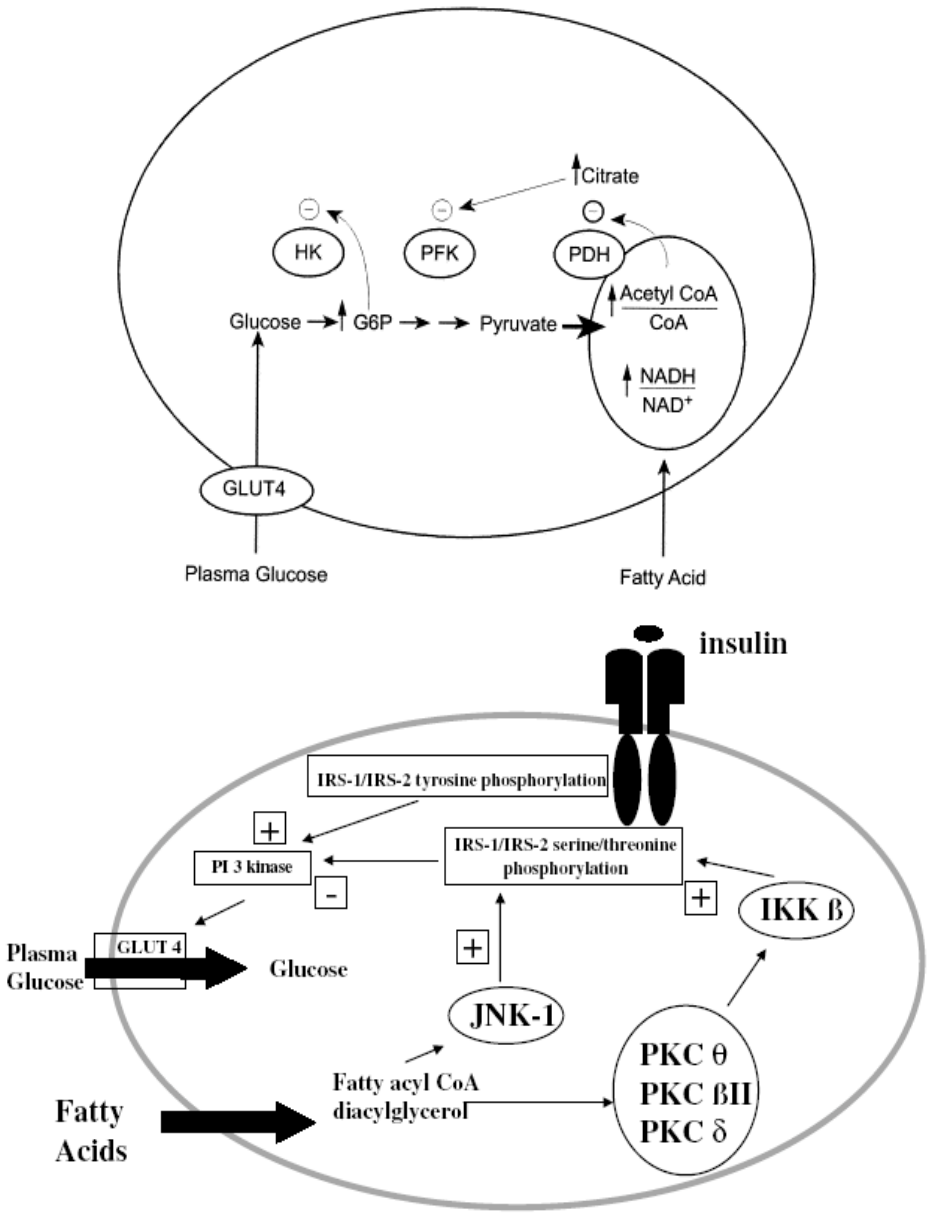

Figure I.2: Mechanism of fatty acid–induced insulin resistance in skeletal muscle (as proposed by Randle et al.,1963) (top). Potential mechanisms by which plasma fatty acid inhibits glucose transport activity as proposed by Shulman (bottom). G6P = glucose-6-phosphate; HK = hexokinase; NAD^+ = nicotinamide-adenine dinucleotide; NADH = reduced nicotinamide-adenine dinucleotide; PDH = pyruvate dehydrogenase; PFK = phosphofructokinase. (From Shulman G.I. 2000; Perseghin et al., 2003).

leading to inhibition of phosphofructokinase, a key rate-controlling enzyme in glycolysis. Subsequent accumulation of glucose-6-phosphate would inhibit hexokinase II activity, resulting in an increase in intracellular glucose concentrations and decreased glucose uptake.

A recent series of work by Shulman group has challenged this conventional hypothesis (Roden et al., 1996; Dresner et al.,1999) (Figure I.2). In the first study, the authors used ^{13}C and ^{31}P NMR spectroscopy to measure skeletal muscle glycogen and glucose-6-phosphate concentrations in healthy subjects. The subjects were maintained in euglycemic, hyperinsulinemic conditions with either low or high levels of plasma fatty acids (Roden et al.,1996). Increasing the plasma fatty acid concentration for 5 hours caused a reduction of approximately 50% in insulin-stimulated rates of muscle glycogen synthesis and whole-body glucose oxidation compared to controls. In contrast to the results from the model of Randle and coworkers, which predicted that fat-induced insulin resistance would result in an increase in intramuscular glucose-6-phosphate, they found that the drop in muscle glycogen synthesis was preceded by a fall in intramuscular glucose-6-phosphate. These data suggest that increases in plasma fatty acid concentrations initially induce insulin resistance by inhibiting glucose transport or phosphorylation activity, and that the reduction in muscle glycogen synthesis and glucose oxidation follows. The reduction in insulin-activated glucose transport and phosphorylation activity in normal subjects maintained at high plasma fatty acid levels is similar to that seen in obese individuals (Petersen et al., 1998) and patients with type 2 diabetes (Rothman et al., 1992). Moreover, fatty acids seem to interfere with a very early step in insulin stimulation of GLUT4 transporter activity or hexokinase II activity.

This conclusion is at odds with the mechanism proposed by Randle et al. (1963) which predicts an increase in intramuscular glucose-6-phosphate concentrations resulting from inhibitory effects of fatty acid on phosphofructokinase activity (due to an increase in intracellular citrate concentration).

Much research has been published on the effects of LCFA on insulin action. Increased extracellular fatty acid load can cause acute (Roden et al., 1996) and chronic (Kim et al., 2001) skeletal muscle IR (reviewed by Petersen and Shulman, 2002). Likewise, chronically reduced oxidative disposal of fatty acid has been shown to cause muscle IR (Dobbins et al., 2001), and IR in obesity is associated with reduced oxidative capacity (reviewed by Kelley and Mandarino, 2000; Kelley et al., 2002). These situations have in common that they favor the accumulation of lipids such as TGs, fatty acyl CoA, and DGs. Of these, the latter two are likely, but not certainly, critical in inhibiting insulin-dependent glucose uptake (Yu et al., 2002). The mechanism of fatty acid-induced IR is still not causally established and may involve also more than one mechanism (Thompson et al., 2000; Schmitz-Peiffer, 2002); this is especially true for the chronic situation. The “lipid oversupply” hypothesis (Kraegen et al., 2001) explains chronic (skeletal muscle) IR with an accumulation on long chain acyl CoA (LCACoA) or complex LCFA esters that inhibit insulin signalling (Shulman, 2000) or metabolic enzymes (Thompson and Cooney, 2000). It is based on the findings that muscle triglyceride (TG) and LCACoA levels are correlated with IR and that various manipulations of LCFA metabolism that increase overall lipid supply and/or esterification produce insulin resistance (Krssak et al., 1999; Dobbins et al., 2001; Kim et al., 2001).

Although these situations all favor the accumulation of lipids such as triglycerides and fatty acyl CoA (Cooney et al., 2002; Kelley et al., 2002), the levels of triglycerides and LCACoA in muscles are not always correlated with insulin sensitivity (Straczkowski et al., 2001; Goodpaster et al., 2001; Bruce et al., 2003; An et al., 2004) and the exact pathways of fatty acid-induced insulin resistance are still being elucidated (Shulman, 2000; Thompson et al., 2000; Schmitz-Peiffer, 2000; An et al., 2004). In addition, sometimes (Storlien et al., 1991; Wilkes et al., 1998), but by far not always (Kim et al., 2000), it has also been found that basal (insulin-independent) glucose uptake can be increased due to fat diet, and basal skeletal muscle glucose uptake was increased in obese patients (Kelley et al., 2002). Voshol et al. (2001) found normal skeletal muscle, and increased cardiac insulin sensitivity while TG levels were increased in both organs; nevertheless, other intracellulars were not measured. Skeletal muscle TG levels were unchanged in rats made insulin-resistant with high-sucrose and high-starch diets but increased in athletes with increased insulin sensitivity (Straczkowski et al., 2001). Moreover, PPAR-alpha knockout mice showing decreased FFA oxidation and accumulation of TG are protected from fat-induced insulin resistance (Guerre-Millo et al., 2001), although PPAR-alpha agonistics improve insulin sensitivity (Tordjman et al., 2001)

One of the proposed reasons for muscle IR is an inbalance between the fatty acid load onto the cell and the cellular capacity to oxidize the load. In otherwise healthy subjects, both increased extracellular fatty acid concentrations (review, Petersen and Shulman, 2002) as well as a decreased oxidative capacity of muscle cells (review, Kelley et al., 2002) can cause IR.

Another modifier of insulin sensitivity is muscle contraction (review, Tomas et al., 2002). Although muscle contraction directly increases availability of GLUT4 transporters, it has a separate effect on insulin-dependent recruitment of GLUT4 transporters. However, the mechanism of this effect is less understood than in case of LCFA-induced insulin resistance.

In summary, the links between LCFA and glucose metabolism remain incompletely understood, especially with regard to LCFA oxidation. Sometimes (chronically) decreased LCFA oxidation favors IR, sometimes not. Thus, the oversupply hypothesis needs additional evidence, ideally through genetic, i.e., causal approaches and lipid analysis beyond TGs. With H-FABP deficient mice as the model, we rely on a protein whose expression under normal circumstances is essentially restricted to heart and skeletal muscle, thus, avoiding difficulties in interpretations that are inherent in transgenic models (Febbraio et al., 1999) with wider expression patterns. This model should provide further arguments for or against the over/undersupply hypothesis; specifically for the role of the ratio of LCFA esterification to oxidation and new insights into the functions of H-FABP in skeletal muscle LCFA metabolism on glucose metabolism, to causally relate them with glucose metabolism and insulin action.

The overall objective of this dissertation is to establish the role of H-FABP in the lipid and glucose metabolism and the role of L-FABP in the LCFA oxidation and relation to PPAR-alpha in knock-out mice models. No single treatment, pharmacological or genetic, will cause only one change. For these reasons, it is important to compare models of altered fatty acid usage with different primary lesions, and to identify the common denominators. To this end, the dissertation records two primary steps. The first

chapter documents uses H-FABP mice model to see the effects of H-FABP in the lipid oxidation and esterification both in the standart diet and high fat diet. Moreover, insulin action is evaluated in H-FABP deficient mice kept under healthy (normal diet) and disease conditions (high fat diet), the latter known to lead to severe whole body and isolated muscle IR in wild type mice. The second chapter discusses the importance of L-FABP in hepatic LCFA oxidation *in vivo*. In addition, this chapter records the requirement of L-FABP for the action of the transcription factor PPAR-alpha, a master onswitch for fatty acid oxidation and a potential target of L-FABP under the conditions of intense hepatic fatty acid oxidation.

CHAPTER II

NON-ACUTE EFFECTS OF H-FABP DEFICIENCY ON SKELETAL MUSCLE GLUCOSE UPTAKE IN VITRO*

Introduction

"Fatty acid overload" due to increased extracellular fatty acid levels (Randle, 1998; Straczkowski et al., 2001; Kim et al., 2001) or pharmacologically reduced fatty acid oxidation (Dyck and Bonen, 2001) can impair insulin action on skeletal muscle (review, Petersen and Shulman, 2002), and insulin resistance in obesity was associated with reduced muscle oxidative capacity (reviews, Kelley and Mandarino, 2000; Kelley et al., 2002). These situations all favor the accumulation of lipids such as triglycerides and fatty acyl CoA (Cooney et al., 2002; Kelley et al., 2002). Conversely, genetic interference with LCFA uptake was shown to prevent fat-induced accumulation of muscle triglyceride and fatty acyl CoA as well as muscle insulin resistance (Kim et al., 2004). However, the levels of triglycerides and LCACoA in muscles are not always correlated with insulin sensitivity (Straczkowski et al., 2001; Goodpaster et al., 2001; Bruce et al., 2003; An et al., 2004; Han et al., 2004) and the exact pathways of fatty acid-induced insulin resistance are still being elucidated (Shulman, 2000; Thompson et

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al., 2000; Schimitz-Peiffer, 2000, An et al., 2004). In addition, in some cases (Straczkowski et al., 2001, Wilkes et al., 1998) it has also been found that basal (insulin-independent) glucose uptake can be increased due to fat diet, and basal skeletal muscle glucose uptake was increased in obese patients (Kelley et al., 2002). The LCFA-induced reduction of insulin sensitive muscle glucose uptake is non-acute, since it is maintained for a while in the absence of LCFA (e.g., Kim et al., 2000; Thompson et al., 2000); similarly, any stimulatory effects of LCFA on basal muscle glucose uptake would be expected to be non-acute, since LCFA oxidation is well known to acutely reduce glucose oxidation in skeletal muscle (Randle, 1998), an effect that is unlikely to be of pathogenetic significance (Kelley and Mandarino, 2000; Shulman, 2000).

Since no pharmacological or genetic treatment can be expected to act at only one site, it is important to compare models of altered fatty acid usage with different primary lesions in order to identify the common denominators of glucose uptake. Here we examined the role of heart-type fatty acid binding protein (H-FABP) in the non-acute regulation of basal and insulin-stimulated glucose uptake. H-FABP is an important, if not dominant, LCFA binding protein in heart and skeletal muscle cytosol and is required for high levels of skeletal muscle LCFA oxidation and esterification at least under standard diet (Binas et al., 2003). Thus, we were interested to know whether the pool of LCFA associated with H-FABP would be relevant for the non-acute regulation of muscle glucose uptake. Accordingly, we measured the glucose uptake into soleus muscles in vitro after subjecting wild type and H-FABP null mice (Binas et al., 1999) to either standard or a high fat diet.

Materials and methods

Mice. Mice lacking the H-FABP gene were originally produced on the 129/Balb/c background (Binas et al., 1999). For the experiments reported here, they were backcrossed onto the C57/Bl6 background for at least 7 generations, and F1 offspring of heterozygous parents or F1 offspring of null X null and wild type X wild type crosses were used. If not indicated otherwise, males were used. Mice were genotyped with a reliable single-tube PCR assay as described (Binas et al., 2003). The experiments were approved by the University Laboratory Animal Care Committee.

Diets and starvation. Mice received a standard chow (Harlan Teklad, #8604) or a high fat diet (Harlan Teklad, #TD 93075, main digestibles in g/kg: 289 protein, 207 starch, 90 sucrose, 274 shortening [Primex], 16 cellulose) for about 4 weeks, or they were starved overnight (14-16 hours) in cages with fresh bedding.

Blood metabolite levels. Blood was drawn with heparinized (for plasma) or non-heparinized (for serum) glass capillaries from tails of overnight-fasted mice. It was centrifuged immediately to remove blood cells, and the resulting plasma or serum was kept on ice for metabolite analysis within the next 24 hours. Glucose levels were measured (Sigma Diagnostics #315) in plasma, and free fatty acid levels were measured (WACO, #994-75409E) in serum. Before determination of triglyceride (Sigma Diagnostics #336) and insulin (Chrystal Chem. IL., kit #90060) levels, plasma was kept frozen (-70^0C).

Glucose tolerance tests. Mice were starved overnight and injected i.p. with glucose (20%, 2 mg /g body weight) for determination of tail blood glucose and insulin at the indicated time points.

Tissue metabolite levels. Muscle samples were shock frozen and stored at –80^{o}C. Muscle glycogen concentration was determined essentially as previously described (Passonneau and Lauderdale, 1974) by digestion in 0.5 ml 1 N KOH, neutralization with 1 N HCl, treatment of an aliquot (0.1 ml) with 0.5 mg/ml of amyloglucosidase (Sigma, #A7420), and measurement of the released glucose with Sigma's kit # 315. Muscle triglyceride levels were measured with a modification (Luiken et al., 2003) of a previously described method (Frayn and Maycock, 1980; Storlien et al., 1991). Levels of LCACoA were determined in shock-frozen tissue samples as described (Neschen et al., 2002; Yu et al., 2003).

Deoxyglucose uptake by isolated muscle. Overnight-fasted mice were anesthetized with Avertin. Soleus muscles were individually incubated in 1.5 ml of buffer (composition see below) in continuously gassed (5% CO_2/95% O_2) 20 ml plastic scintillation vials in a shaking water bath (29ºC). Following the procedure of Etgen et al. (1999), three-step incubations (each step in a new vial) were performed in Krebs-Henseleit buffer (KHB)/0.1% bovine serum albumin with the following supplements and durations: Step 1, 8 mM glucose/32 mM mannitol, 40 minutes; step 2, 40 mM mannitol, 10 min; step 3, 1 mM [^{3}H-]2-deoxyglucose at 0.5 mikroCi/ml (NEN #NET328)/ 39 mM [U-^{14}C]-mannitol at 0.1 mikroCi/ml (NEN #NEC314), 20 minutes. Insulin (Humulin, Eli Lilly, #0002-8215-01) was present throughout the incubations at the concentrations indicated in the main text. Muscles were then blotted on filter paper, trimmed, weighed and dissolved in 0.1 ml hyamine hydroxide at 60^{0}C overnight. 30 mikroliter were counted in a dual-label liquid scintillation spectrophotometer. Glucose-transport activity (expressed in micromoles/20 min/g muscle) was calculated by subtracting the

extracellular from intracellular [^{3}H]-2-deoxyglucose by using the extracellular marker [U-^{14}C]-mannitol.

Fatty acid metabolism in isolated soleus muscle. The same setup, but another incubation medium and time schedule as for the glucose uptake experiments was used. The incubation medium consisted of KHB, 2% bovine serum albumin (fatty acid-free, Sigma), 0.1 mM palmitic acid (Sigma), 1 mikroCi/ml of either [^{3}H]-palmitic acid (NEN #NET043) or [^{3}H]-oleic acid (#NET289), and insulin as indicated. Palmitic acid (125 mM stock in ethanol) was mixed with warmed albumin-containing medium until completely clear. Freshly excised soleus muscles were incubated for 60 minutes at 29^{0}C and freeze-clamped at the end. Fatty acid oxidation was measured through production of tritiated water (Dyck and Bonen, 1998) in 1 ml of incubation medium, using a vial incubated without muscle as blank. Fatty acid esterification into triglycerides was quantified by homogenization of the muscles with a glass homogenizer in 1 ml ice-cold chloroform-methanol (1:1), followed by lipid extraction and thin layer chromatography (8). Bands of main lipid classes were visualized with iodine vapor, scraped out and counted in 5 ml scintillation liquid (Scintisafe, Fisher Scientific).

Statistical analysis. Data are shown as means ± SE. They were calculated using GraphPad Prism Statistical software program and analyzed using one way ANOVA (Bonferroni's multiple comparison test) and two-tailed Student's *t* test, if not otherwise indicated. Comparisons were made between H-FABP null and wild type mice (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$), between standard diet and high fat/high sugar diet (+=$p<0.05$, ++=$p<0.01$, +++=$p<0.001$), and between incubations in presence or absence of insulin (#=$p<0.05$, ## =$p<0.01$, ###=$p<0.001$).

Results

LCFA oxidation in isolated soleus muscles. In the absence of insulin, oxidation of palmitate was decreased by 53% (Fig. II.1A) in mutant muscles from non-fasted, chow-fed mice, while oleate oxidation was reduced by 56% ($P<0.01$, n=5 per genotype). With this diet, insulin reduced the rate of palmitate oxidation in wild type soleus muscle, but did not significantly reduce the already lowered rate in H-FABP null muscle (Fig. II.1A, B). Accordingly, the decrement caused by the mutation was significantly ($P<0.05$) larger in the absence of insulin than in its presence. A similar result was found with oleic acid (not shown). With the high fat diet, the genotype-specific reduction was 67% for palmitic acid in the absence of insulin (Fig. II.1A), and similar results were obtained from skeletal muscles of fasted mice (Fig. II.1B) and females (not shown). With this diet, the insulin-caused reduction of wild type muscle fatty acid oxidation became non-significant.

LCFA esterification in isolated soleus muscles. In the absence of insulin, esterification of palmitate into triglycerides was lower by 26% in null as compared to wild type muscles from non-fasted, chow-fed mice (Fig. II.1C), while esterification of oleate was reduced by 38% ($P<0.05$, n=4 per genotype). With this diet, insulin moderately but significantly ($P<0.05$) stimulated palmitate esterification in both genotypes. With high fat diet, esterification of palmitate was reduced by 33% in null as compared to wild type muscles (Fig. II.1C); this genotypic reduction occurred on a lowered level, as the high fat diet reduced the esterification rates in both wild type (-27%) and null (-34%) muscles. Similar results were obtained with muscles from fasted

(Fig. II.1D) and female (not shown) mice. Again, insulin moderately stimulated palmitate esterification in both genotypes (Fig. II.1C, D).

Basal deoxyglucose uptake in isolated soleus muscles. Without insulin, deoxyglucose uptake was lower by ~28% ($P<0.05$) in isolated soleus muscle from H-FABP null as compared to wild type mice, regardless of whether the mice had been fed chow or high fat diet (Fig. II.2A); similar results were seen in female mice (-29% and –27%, $P<0.05$) and with extensor digotorum longus (EDL) muscle (-19%, $P<0.05$) (data not shown).

Insulin-stimulated deoxyglucose uptake in isolated soleus muscles. With standard chow, high levels of insulin (2 mU/ml) increased deoxyglucose uptakes by 4.1 mikromol/mg/20 min (109%) in wild type, and by 4.7 mikromol/mg/20 min (+178.3%) in H-FABP null samples (Fig. II.2A), but the difference between these increments was not statistically significant. A similar result was obtained with female mice (data not shown). The slightly (but not significantly) larger increment in H-FABP null muscles prompted us to develop a dose response curve. This curve was significantly steeper in H-FABP null as compared to wild type soleus muscle (Fig. II.2B). As a result, insulin-dependent deoxyglucose uptake at 0.02 mU/ml insulin was significantly higher in null vs. wild type soleus muscle (+141%) and approached plateau levels already at 0.2 mU insulin/ml, but the plateau levels were similar in both genotypes. With the high fat diet, total deoxyglucose uptake at 2 mU/ml insulin was only non-significantly (+30%) increased in H-FABP null versus wild type muscle (Fig. II.2A); however, the genotypic difference of the insulin-dependent increments (Fig. II.2B) was much larger (+190%) and clearly significant ($P<0.01$). In the presence of the high insulin concentration, the

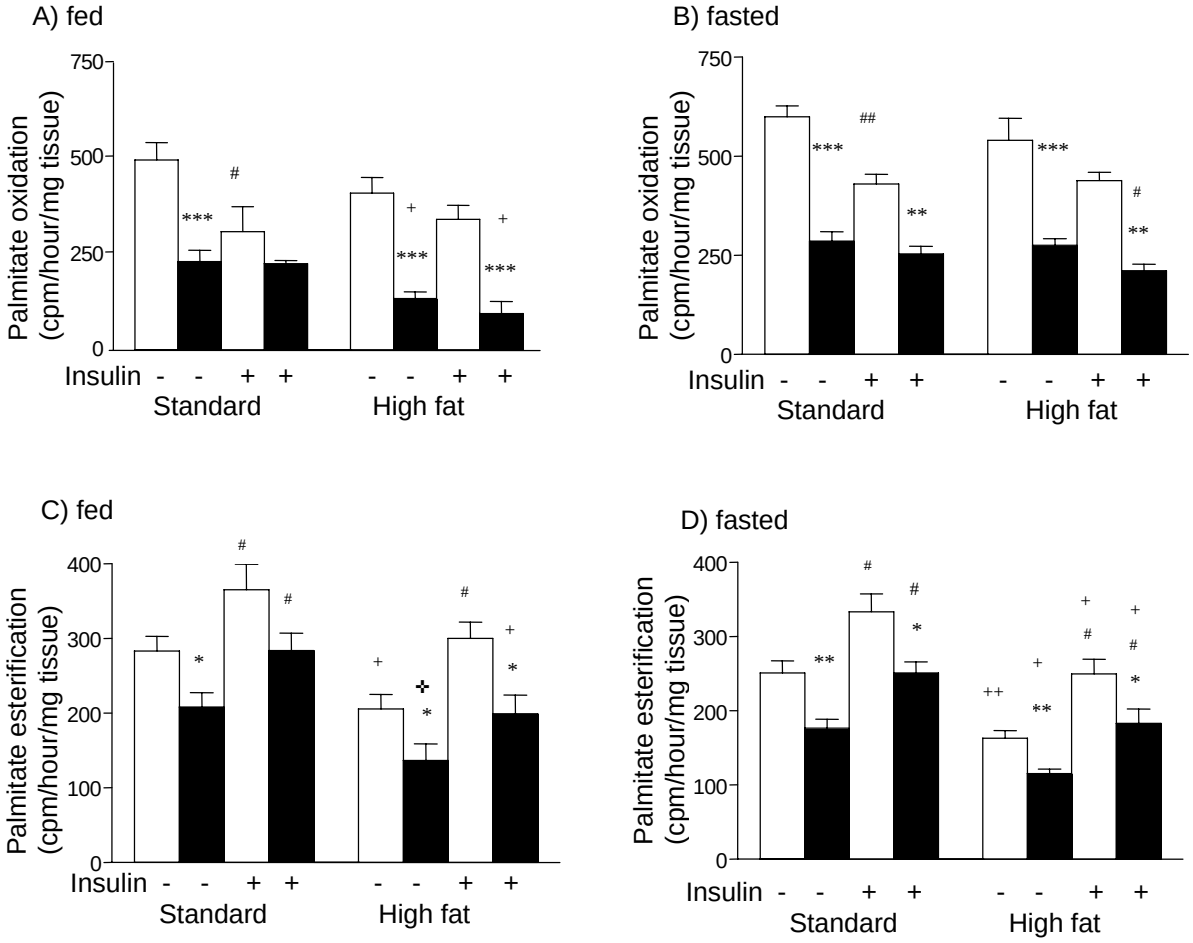

Figure II.1: Skeletal muscle LCFA oxidation and esterification are impaired in H-FABP null mice under both standard diet and high fat diet. Isolated soleus muscles from wild type (white columns) or H-FABP null (black columns) male mice maintained on diets as indicated were incubated with labeled palmitic acid in the absence (-) or presence (+) of 2 mu/ml insulin as indicated below the columns. ***A***, palmitic acid oxidation in muscle from mice fed ad libitum; ***B***, same as A but mice were fasted overnight before sacrifice; ***C***, esterification of palmitate into triglyceride fraction, mice fed ad libitum; ***D***, same as C but mice were fasted overnight. Each column represent 6-13 mice. Comparisons were made using one way ANOVA (Bonferroni's multiple comparison test) between H-FABP null and wild type mice (*P<0.05, **P<0.01, ***P<0.001), between standard diet and high fat/high sugar diet (+=P<0.05, ++=P<0.01, +++=P<0.001), and between incubations in presence or absence of insulin(#P<0.05, ##P<0.01, ###P<0.001).

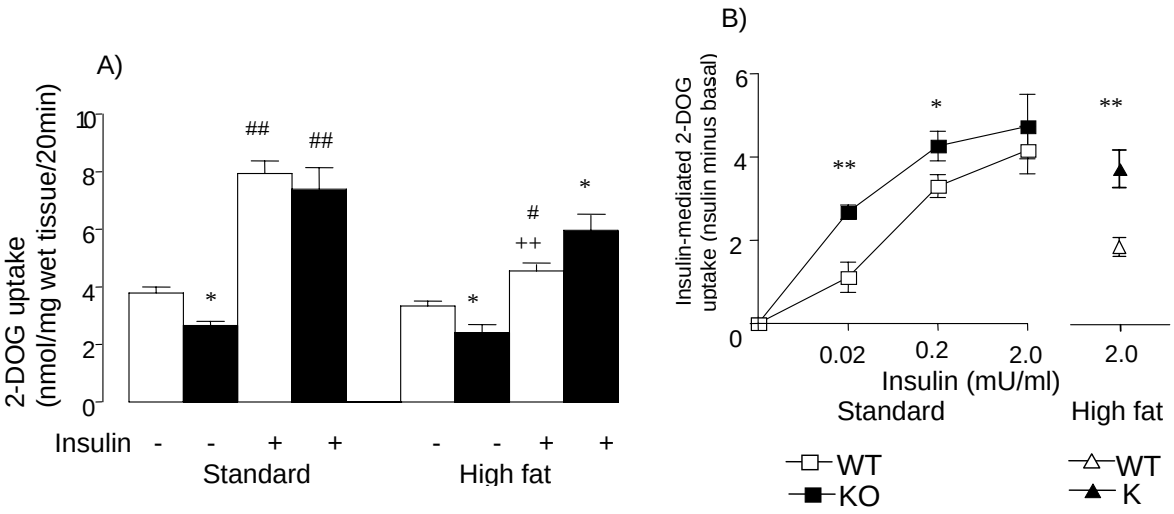

Figure II.2: Improved insulin action, but reduced basal glucose uptake in isolated H-FABP null skeletal muscle. *A*, Male wild type (white columns) and H-FABP null (black columns) mice were maintained on standard or high fat diet as indicated below columns, fasted overnight, and then used for isolated soleus muscle incubation in presence of radiolabeled deoxyglucose and absence (-) or presence (+) of 2 mU/ml insulin. Each column represents 6-10 mice. The unit is 2-dog uptake (nmol/20min/gr). *B*, Insulin dose response of insulin-dependent glucose uptake (total minus basal). Male wild type (squares) or H-FABP null (triangles) mice were used for measurement of deoxyglucose uptake as under *A*, except that variable insulin concentrations were added to muscles from mice treated with standard diet. For mice maintained under high fat diet, only the insulin-dependent uptake at 2 mu/ml is given. Each data point represents 6-10 mice. *P<0.05, **P<0.01, comparisons between H-FABP null and wild type mice; #P<0.05, ###P<0.001, comparisons between incubations in presence or absence of insulin; +++P<0.001, comparison between standard diet and high fat/high sugar diet.

total rate of deoxyglucose uptake by wild type soleus muscle was significantly reduced (-43%) by high fat diet, whereas the corresponding reduction with H-FABP null muscle was smaller (-24%) and not significant at $P<0.05$ (Fig.II.2A).

Lipid levels in vivo. With standard diet, the mutation doubled (+97%) plasma fatty acid levels, but did not affect plasma triglyceride levels (Table II.1), similar to the phenotype before the backcross (Binas et al., 1999). With high fat diet, circulating fatty acid levels more than doubled (+117%) in wild type mice, but increased less (+36%) in H-FABP null mice due to the higher starting level, although the free fatty acid level remained higher (+24%) than in wild type mice (Table II.1). No genotype-specific differences were observed in plasma triglyceride levels of mice on a high fat diet, although triglyceride levels were increased in both genotypes (Table II.1).

Muscle triglyceride levels remained normal in the H-FABP null mice kept with the standard diet, but the H-FABP null mutation effectively prevented the almost threefold increase of muscle triglycerides caused by high fat diet (Fig. II.3A). Total and individual LCACoA levels in gastrocnemius muscle were not affected by the mutation with the standard diet, but with the high fat diet, they were moderately reduced (Table II.2). Statistical significance at $P<0.05$ was reached for C18:3 (-43%) with the two-tailed t-test, and for C16:0 (-39%), C18:3 (-43%), and total LCACoA (-26%) with the one-tailed t-test (Table II.2). LCACoA were also measured in soleus muscles, but statistics could not be applied because solei had to be pooled (1 muscle per mouse, 7 mice per pool). The results (2 pools per genotype) showed total LCACoA levels of 23.3 and 27.6 (male wild type) versus 21.5 and 21.2 (male H-FABP null) nmol/g, and 21.1 and 20.1 (female wild type) versus 21.3 and 20.0 (female H-FABP null) nmol/g. Thus, with high

fat diet there was moderate decrease of muscle LCACoA levels due to the mutation in males.

Carbohydrate and insulin levels in vivo. With the standard diet, fasting glucose levels were decreased by ~25% in male and female H-FABP null vs. wild type mice (Table II.3). With the high fat diet, glucose levels increased in both wild type (males, +110%; females, +73%) and H-FABP null (males, +127%; females, +87%) mice, but remained lower in null vs. wild type mice (males, -19.5%; females, -18%) (Table II.3). Skeletal muscle glycogen levels were decreased in H-FABP null skeletal muscles by 25% under standard diet and by 26.5% under high fat diet (Fig. II.3B). Mice maintained on standard diet did not exhibit a genotype-specific difference of glucose tolerance, but with the high fat diet (that significantly impaired glucose tolerance in both genotypes), we observed a moderate yet significant improvement of glucose tolerance in H-FABP null versus wild type mice 15 minutes after injection, but not at the later time points (Fig. II.4A). With the standard diet, fasting insulin levels in H-FABP null mice were significantly decreased (-66%)compared with wild type levels (Table II.3) (in females the decrease (-30%) was not significant), but after glucose injection, insulin levels increased faster in H-FABP null mice than in wild type mice, resulting in identical levels by the end of the assay (Fig. II.4B). With the fat diet, fasting insulin plasma levels increased by a comparable factor in wild type (males, +446%; females, +803%) and H-FABP null (males, +469%; females, +549%) mice, meaning that the insulin levels remained substantially lower than in the H-FABP null mice (Table II.3). However, unlike after standard diet, the insulin levels did not increase further during the glucose tolerance test (Fig. II.4B).

Table II.1. Blood levels of free fatty acids and triglycerides in H-FABP null (-/-) and wild type (+/+) control mice (maintained under either standard chow or 4 weeks of high fat diet). Mice were fasted overnight before blood sampling. Data are means ± SEM (n). $^{*}P<0.05$, $^{**}P<0.01$, comparisons between genotypes. $^{+}P<0.05$, $^{++}P<0.01$, comparisons between diets.

	FFA (mEq/L)	TG (mM)	FFA (mEq/L)	TG (mM)
	Standard diet		High fat diet	
+/+	0.37 ± 0.02 (6)	0.10 ± 0.01 (5)	0.80±0.02^{++} (6)	0.13±0.01^{+} (6)
-/-	0.73 ± 0.04** (6)	0.11 ± 0.01 (5)	0.99±0.06$^{*,+}$ (6)	0.14±0.01^{+}(6)

Table II.2. LCACoA contents (in nmol/g wet tissue) of H-FABP null (-/-) and wild type control (+/+) gastrocnemius muscles (from mice maintained under either standard chow or 4 weeks of high fat diet). Data are means ± SD. In brackets, P values are shown for differences between wild type and H-FABP null samples that are considered statistically significant according to one tailed (*) and two-tailed (**) t-tests. Only genotypes, but not diets should be compared, since the diet groups differed by age and pretreatment (fasted, standard diet; fed, fat diet).

	C16:0	C18:0	C18:1	C18:2	C18:3	Total
Standard diet						
Males +/+ n=5	1.70±0.17	0.98±0.05	4.74±0.40	2.90±0.30	1.83±0.23	14.10±1.30
Males -/- n=5	1.75±0.10	1.16±0.08	5.68±0.31	3.20±0.38	1.73±0.17	15.21±1.01
High fat diet						
Males +/+ n=7	1.13± 0.2	1.63±0.14	4.52±0.47	2.26±0.28	1.81±0.28	11.94±1.48
Males -/- n=7	0.69±0.11	1.51±0.09	3.50±0.27	1.59±0.16	1.03±0.11	8.84±0.73
Females +/+ n=6	1.11±0.20	1.78±0.15	4.23±0.41	2.15±0.26	2.18±0.53	11.87±1.75
Females -/- n=6	0.81±0.09	1.85±0.09	4.01±0.26	1.99±0.18	1.71±0.24	10.94±0.84

Table II.3. Blood levels of glucose and insulin in H-FABP null (-/-) and wild type (+/+) control mice (maintained under either standard chow or 4 weeks of high fat diet). Mice were fasted overnight before blood sampling. Data are means ± SEM (n). $^{*}P<0.05$, $^{**}P<0.01$, comparisons between genotypes. $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$comparisons between diets.

	Glucose (mM)	Insulin (ng/ml)	Glucose (mM)	Insulin (ng/ml)
	Standard diet		High fat diet	
Males +/+	7.57±0.32 (7)	0.29 ± 0.06 (11)	15.92±0.8^{+++} (6)	1.58 ± 0.15^{+++} (9)
Males -/-	5.66±0.27* (7)	0.10 ± 0.03* (9)	12.82±0.6$^{*,+++}$ (6)	0.57 ± 0.15$^{**,++}$ (8)
Females +/+	6.86 ± 0.37 (8)	0.18 ± 0.03 (10)	11.84 ± 0.7^{+++} (6)	1.59 ± 0.49^{++} (9)
Females -/-	5.20 ± 0.39* (8)	0.12 ± 0.04 (11)	9.71 ± 0.50$^{*,+++}$ (6)	0.81 ± 0.28^{+} (11)

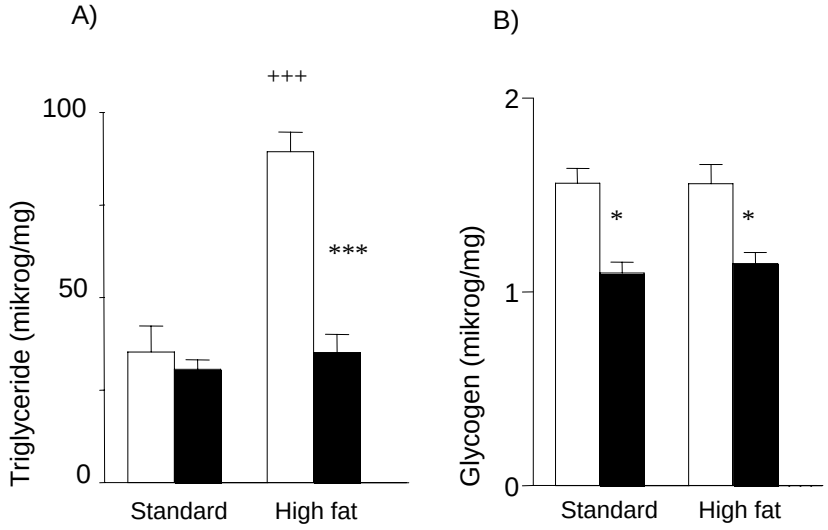

Figure II.3. Triglyceride and glycogen levels in gastrocnemius muscle. Male wild type (white columns) and H-FABP null (black columns) null mice fed ad libitum either standard or high fat diet were used (n=6-8 [triglyceride] or 8-10 [glucogen] per column). Results are given as mean ± SEM. +++=P<0.001, comparisons between diets; P<0.05**,*****P<0.001, comparison between genotypes.

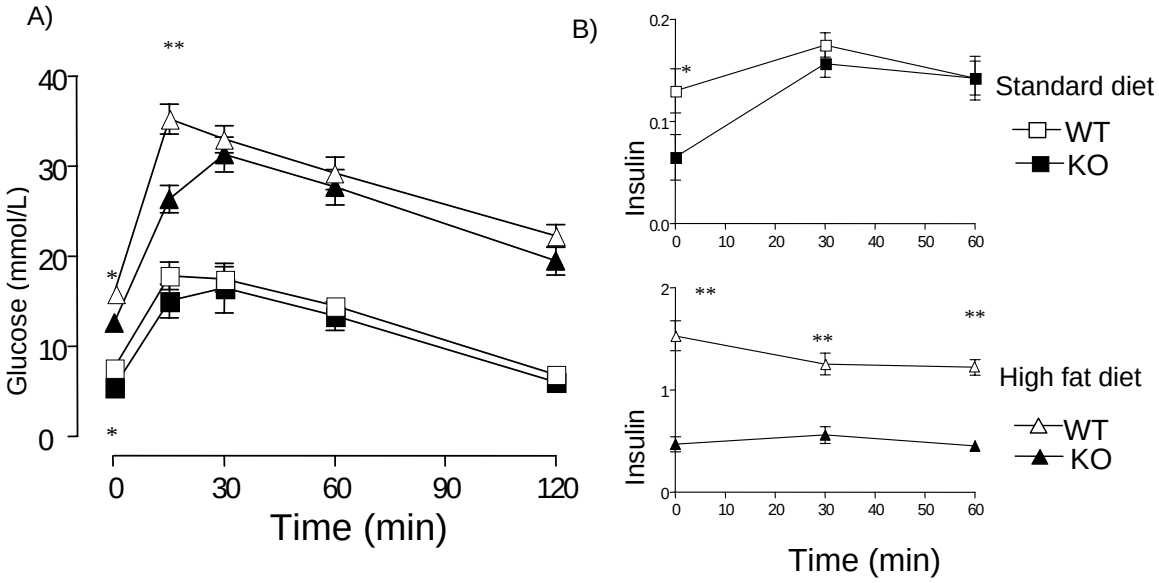

Fig. II.4. Glucose tolerance and insulin levels. *A,* Male wild type (triangles) and H-FABP null (squares) mice maintained under standard diet (empty symbols) or high fat diet (filled symbols) were fasted overnight and subjected to intraperitoneal glucose injection (n=8-10 per data point). *B,* Acute insulin response of male wild type (triangles) and H-FABP null (squares) mice during glucose tolerance test following standard diet (empty symbols) and high fat diet (filled symbols). N=4 per data point for 30 and 60 minutes, but n= 8-11 for zero time (see table 3). Results are given as mean ± SEM. *P<0.05, **P<0.01, comparisons between genotypes.

Discussion

The present study provides further support for an important role of H-FABP in muscle LCFA and glucose metabolism.

First, our data extend our previous result that muscle H-FABP is a major determinant of myocellular uptake and metabolic availability of palmitate during standard diet (Binas et al., 2003, Luiken et al., 2003) by adding another fatty acid (oleate), another diet (high fat diet), and comparing fasting with feeding. At least with standard chow, LCFA metabolism was reduced without a decrease of total and individual LCACoA levels, similar to reduced LCFA oxidation, but normal LCACoA levels, in livers of mice lacking liver FABP (Erol et al., 2004). Further, under nutritional stress (high fat diet), lack of H-FABP completely prevented the three-fold accumulation of triglycerides (while reducing LCACoA levels only moderately), adding to our previous finding that this mutation blunts the contraction-caused decrease of muscle triglyceride levels (Binas et al., 2003). Thus, H-FABP is a limiting factor for muscle LCFA metabolism regardless of physiological situation, but more work is needed to clarify the mechanism leading to altered triglyceride levels in some (contraction, fat diet) but not other (standard chow) conditions.

Second, we showed here that FABP is an important mediator of at least some of the fatty acid effects that reduce muscle insulin sensitivity or responsiveness. Current thinking (Shulman, 2000; Schimitz-Peiffer, 2000; Petersen and Shulman, 2002) holds that relatively slow effects of non-oxidative LCFA metabolites are important in opposing insulin action. In agreement with this notion, we found that H-FABP null muscles not only showed increased insulin sensitivity (standard chow) and

responsiveness (fat diet), but these effects were seen in the absence of added LCFA (during both preincubation and labeling), making rapid substrate-level competition between glucose and LCFA oxidation (Randle, 1998) an unlikely explanation. Our results vary this notion by showing that insulin sensitivity of glucose uptake can be increased by reduced LCFA binding/metabolism while maintaining normal insulin responsiveness. It is also of note that LCACoA levels were not affected by the mutation under this condition (standard chow). With the high fat diet, when insulin-dependent deoxyglucose uptake by isolated soleus muscle was significantly stimulated by the H-FABP null mutation even at high (2 mU/ml) levels, we saw only a trend toward decreased cellular LCACoA levels. Therefore, we suggest that the metabolic availability of LCACoA (indirectly determined by H-FABP) may be more relevant for insulin sensitivity or resistance than cellular LCACoA levels. This would fit various recent observations that a higher insulin sensitivity can be associated with moderate (Houmard et al., 2002) or no (Bruce et al., 2003; An et al, 2004) decrease of myocellular LCACoA levels. The H-FABP null mice are a useful model with which to study whether the metabolic availability of LCACoA or another aspect of LCFA metabolism regulate insulin-dependent glucose transport.

Third, we observed that in vitro soleus muscle deoxyglucose uptake was reduced by the mutation in the absence of insulin, regardless of the nature of prior diet. Thus, at least in vitro and without added fatty acid, the decreased basal uptake antagonized the increased insulin sensitivity/responsiveness, thereby reducing the net change of skeletal muscle glucose uptake. These results resemble our previous observation that glucose oxidation (measured in the absence of insulin but presence of fatty acid) was decreased

in soleus muscle isolated from H-FABP null mice fed a standard diet (Binas et al, 2003), and the decreased basal glycogen synthesis rates (measured in absence of fatty acid) in isolated soleus muscle from mice lacking the plasma membrane LCFA transporter CD36, a mutation that at the same time increases the insulin responsiveness of glycogen synthesis (Hajri et al., 2002).

Fourth, we found here that blood LCFA levels were increased, and blood glucose and insulin levels were decreased, in H-FABP null mice. We speculate that hormonal and substrate conditions in vivo dampen the non-acute genotype-specific changes of muscle glucose uptake that we observed in vitro. For example, the reduced plasma insulin levels might limit the effect of the non-acutely increased insulin sensitivity/responsiveness, while the presence of LCFA might, through the glucose-fatty acid cycle (Randle, 1998), reduce glucose uptake in wild type muscle more than in H-FABP null muscle (that oxidize LCFA less efficiently) and thus offset the non-acutely reduced basal glucose uptake. It may not surprise, then, that glucose tolerance was not (chow) or only slightly (fat diet) improved in H-FABP null vs. wild type mice. Thus, both the acute and the non-acute effects of substrate and hormone concentrations will have to be studied in order to fully understand the role of H-FABP in muscle glucose uptake in vivo. Such information will also be helpful to understand the energetic impact of reduced LCFA oxidation in vivo that involves reduced soleus muscle ATP and phosphocreatine levels, but not a reduced mitochondrial enzyme capacity (Binas et al., 2003).

In conclusion, we have described here two non-acute and opposite effects of H-FABP on muscle glucose uptake. Our results are compatible with the view that lipid

pools determined by H-FABP are more important for regulating basal and insulin-dependent skeletal muscle glucose uptake than total cellular lipid levels.

CHAPTER III

LIVER FATTY ACID BINDING PROTEIN IS REQUIRED FOR HIGH RATES OF FATTY ACID OXIDATION BUT NOT FOR THE ACTION OF PPAR-ALPHA IN FASTING MICE*

Introduction

Liver fatty acid binding protein (L-FABP), a member of the genetically related cytosolic FABP family (Mc Arthur et al., 1999; Hertzel and Bernlohr, 2000; Storch and Thumser, 2000), is expressed in the liver, and together with other FABPs in intestine and kidney. Its main known molecular function is the reversible binding of hydrophobic ligands, including long-chain fatty acids (LCFA), LCFA-CoA, phospholipids, peroxisome L proliferators, and others (Mc Arthur et al., 1999; Hertzel and Bernlohr, 2000; Storch and Thumser, 2000; Haunerland et al., 1984; Wolfrum et al., 2000). The abundance (reaching 2.5% of cytosolic protein) and correlation of its levels with the extent of LCFA metabolism in vivo (Ockner et al., 1979; Hung et al., 2003) suggest that L-FABP is important in hepatic LCFA metabolism. In line with this, numerous alterations of LCFA metabolism have been observed in cell lines transfected with L-FABP cDNA (Mc Arthur et al., 1999), notably in the context of the present study

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increases in LCFA uptake (Murphy et al., 1996) and oxidation (Linden et al., 2002), whereas an L-FABP antisense cDNA inhibited LCFA uptake (Wolfrum et al., 1999). However, because these cell lines were poorly differentiated, it is not clear whether the results apply to the metabolically highly active, fully differentiated hepatocyte. Moreover, the mechanisms underlying the potential roles of L-FABP in LCFA metabolism remain unclear.

Several observations are consistent with a substrate-level role of L-FABP. Displacement of the fluorescent LCFA analog NBD-stearate from L-FABP by alpha-bromopalmitate was accompanied by reduced cytoplasmic diffusion of NBD-stearate (Luxon, 1996). Cytoplasmic diffusion of NBD-stearate was also reduced in permeabilized hepatocytes, but restored by addition of L-FABP (Kaikaus et al., 1993). Furthermore, L-FABP modulated ligand transfer and metabolism in cell-free systems (Mc Arthur et al., 1999; Storch and Thumser, 2000). Finally, hepatic L-FABP levels are boosted by peroxisome proliferators (Kaikaus et al., 1993; Lee et al., 1995) and reduced in peroxisome proliferator-activated receptor alpha (PPAR-alpha) null mice (Kersten et al., 1999), consistent with the idea that L-FABP itself might be part of the lipid oxidation machinery. Thus, L-FABP might modulate the availability of its ligands for metabolism by raising their cytosolic concentration, sequestering them, and facilitating their intracellular movement. More recently, the possibility that L-FABP affects LCFA metabolism by increasing the activity or level of the transcription factor PPAR-α has caused great interest. L-FABP and PPAR-alpha exhibit a similar ligand binding spectrum (compare ref Corton et al., 2000 with ref Wolfrum et al., 2000 and references therein). LFABP can be found in the nucleus (Bordewick et al., 1989) and physically

interacts with PPAR-alpha (Wolfrum et al., 2001). L-FABP ligands added to cultured hepatoma cells stimulate transcription of a reporter gene carrying a peroxisome proliferator-responsive element, in strict correlation with the endogenous content of L-FABP (Wolfrum et al., 2001). Finally, overexpression of L-FABP in L cells increases nuclear fatty acid uptake (Huang et al., 2002). Thus, L-FABP might stimulate the intrinsic activity of PPAR-alpha Moreover, it might increase PPAR-alpha levels, because transfection of L-FABP cDNA into hepatoma cells increased the levels of PPAR-alpha mRNA (Linden et al., 2002). It was suggested (Wolfrum et al., 2001) that L-FABP is the gateway of hydrophobic compounds to gene transcription, and this concept was most recently extended to three other members of the FABP gene family, heart-type, adipocyte, and keratinocyte FABP (Huang et al., 2002).

Taken together, the literature suggests substantial roles for L-FABP in LCFA metabolism both by modulating availability of substrates as well as by increasing enzymatic capacity throughactivation of PPAR-alpha and possibly other transcription factors. Here, we used the recently created L-FABP null mice (Martin et al., 2003) to study the role of L-FABP in hepatic LCFA oxidation and the potential involvement of PPAR-alpha.

Materials and methods

Mice. The creation of mice lacking the L-FABP gene (L-FABP null mice) has been described previously (Martin et al., 2003). The mutation was backcrossed onto the C57/Bl6 (Harlan, Madison, WI) background for two to three (Fig. 1.3, Table 2) or four generations (Table 1). PPAR- alpha null mice (Lee et al., 1995) were backcrossed for six generations onto the C57/Bl6 (Jackson, Bar Harbor, ME) background. Experimental L-

FABP null and PPAR- alpha null mice and their respective wild-type controls were derived from heterozygous intercrosses. Mice were used at the age of 3.6 months (Fig. 1-3, Table 2) or 2-3 months (Table 1). In each physiological situation represented in a given figure or table, null and wild-type mice of the same sex were exactly matched for backcross generation and age (less than 2 weeks difference). However, animal groups studied under different physiological situations differed by age and backcross within the segments given above. In comparisons of PPAR- alpha null and L-FABP null mice, all mice (including the wild-type controls) were treated identically and simultaneously. Animal experiments were approved by the University Lab Animal Care Committee.

Diets and starvation. Mice were fed either a standard chow (#8604, Harlan Teklad; 3.24 kcal/g [57.6% from CHO,12.2% from fat, 30.2% from protein]), a ketogenic diet for 3 days (#TD 96355, Harlan Teklad; 6.69 kcal/g [0.3% from CHO, 90.7% from fat, 9.0% from protein]; main digestibles in g/kg: 586 shortening [Crisco], 86 corn oil, 151 protein, 5.4 carbohydrate), or a diabetogenic diet for ~4 weeks (#TD 93075, Harlan Teklad; 4.80 kcal/g [24.0% from CHO, 55.0% from fat, 21.0% from protein]; main digestibles in g/kg: 289 protein, 207 starch, 90 sucrose, 274 shortening [Primex], 16 cellulose). Alternatively, mice were starved overnight (14-16 h) in cages with fresh bedding.

Determination of blood metabolites. Tail blood was drawn from conscious mice in most cases, but in a few cases, blood was obtained by cardiac puncture under Avertin. Serum was kept at 4°C and used within 24 hours for measurement of nonesterified fatty acids (NEFA) (#994-75409E, Wako Diagnostics, Richmond VA),

triacylglycerol (#336, Sigma, St. Louis, MO), β-hydroxybutyrate (BHB; #310UV, Sigma), and glucose (#315, Sigma).

Octanoate challenge. Sodium octanoate (500 mM, in 0.9% NaCl) was injected intraperitoneally at 6 mikroliter per gram of body weight into mice starved overnight. At the indicated times, aliquots of 10 mikroliter of tail blood were withdrawn for analysis of BHB.

Determination of long-chain acyl CoA. Liver pieces from mice starved overnigh were shock-frozen with liquid nitrogen, weighed, powdered, and processed as previously described (Neschen et al., 2002).

Measurement of fatty acid oxidation in isolated hepatocytes. Primary hepatocytes were obtained by an established method (Madden et al., 2000) with modifications. In brief, mice were anesthetized with Avertin, and each liver was perfused through the V. cava inferior with two consecutive solutions (prewarmed to 37°C): first, with Ca/Mg-free Earle.s balanced salt solution/0.5 mM EGTA (perfused for 4.6 min at 7.8 ml/min), and second with HEPES-buffered Hanks balanced salt solution (#24020-117, Gibco-BRL, Gaithersburg, MD) containing 0.3 mg/ml collagenase D (#17103-011, Gibco-BRL) (perfused for 6.8 min at 8.10 ml/min). Hepatocytes were released into Dulbecco.s modified Eagle.s medium (DMEM), filtered, and centrifuged at 600 rpm for 2 minutes, followed by centrifugation in 50 vol/% each of DMEM (with 10% FBS) and of Percoll (with Hanks salts). The pellet was washed two times with serum-free DMEM. Cell viability, determined by trypan blue exclusion, always exceeded 95%. Hepatocytes (500,000 cells in a final vol of 1 ml) were incubated (duplicate for each mouse) for 1 h at 37°C with agitation under an atmosphere of 5%

CO2/95% O2 in sealed glass vials in Krebs-Henseleit bicarbonate buffer supplemented with 1 mM carnitine (Sigma), 2% (w/v) fatty acid-free bovine serum albumin (BSA) (Sigma), 1 mM palmitate (Sigma), and 0.5 mikroCi/ml [1-14C]-palmitate (#P- 8575, Sigma). The fatty acid (0.4 M stock in ethanol) was completely dissolved by prewarming the buffer at 37°C before adding the cells. At the end of incubation, 30 µl of the suspension were removed onto ice for later enzymatic measurement of BHB, a tube with 0.2 ml hyamine (ICN Radiochemicals, Irvine, CA) was inserted, the vial was sealed again, and 0.3 ml of 3 M perchloric acid was injected into the cell suspension. After incubation for 90 minutes at 4°C, the acid incubation mixture was centrifuged for 5 minutes at 6000 rpm and the supernatant as well as the hyamine sample were subjected to liquid scintillation counting (Scintisafe, Fisher Scientific, Pittsburgh, PA). The rates of palmitate oxidation were calculated as the sum of trapped 14CO2 (hyamine sample) and 14C-labeled acid soluble products (supernatant). BHB was determined with Autokit 3HB (Wako Diagnostics, Richmond, VA) after removing the cells (4°C, 2 minutes, 5000 rpm).

Measurement of fatty acid oxidation in liver homogenates. Guided by a published protocol (Veerkamp et al., 1986), we determined palmitate oxidation rates in duplicates per mouse. In brief, fresh liver pieces were homogenized at 5% (wt/vol) in ice-cold buffer (10 Mm Tris-HCl, pH 7.4, 2 mM Na2-EDTA, 0.25 M sucrose) with a tight-fitting Dounce homogenizer. 25 mikroliter of liver homogenate were added to 475 µl of assay buffer containing 75 mM Tris-HCl (pH 7.2), 10 mM K2HPO4, 1 mM EDTA, 5 mM MgCl2, 0.5 mM L-carnitine, 25 mikroM cytochrome c, 0.1 mM CoA, 25 mM sucrose, 5 mM ATP, 0.5 mM malate, and 1 mM NAD. After preincubation for 30

minutes at 37°C and agitation, palmitate was added to a final concentration of 1 mM (1 mikroCi/ml) palmitate/2% fatty acid-free BSA, and incubation was continued for another 30 minutes. BHB and radiolabeled oxidation products were determined as described for the hepatocyte incubations.

Northern blotting. Total liver RNA was isolated with RNAwiz (Ambion, Austin, TX) and separated at 20 mikrogram/lane on 1% agarose/formaldehyde gels. Northern blotting was performed according to standard methods (Sambrook and Russell, 2001), using BrightStar-Plus membranes, ULTRAhyb buffer, and the Strip-EZ DNA kit (Ambion). Manufacturer.s instructions were followed except that washing was performed at 60°C. The probes (26.29) used were cDNAs encoding medium-chain acyl CoA dehydrogenase (MCAD), liver carnitine palmitoyltransferase 1 (L-CPT1), cytochrome P4504A3 (CYP4A3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PPAR-alpha (26), long-chain acyl CoA dehydrogenase (LCAD) (27), mitochondrial hydroxymethyl glutaryl (HMG) CoA synthase (28), and L-FABP (29). Signals were visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), quantified with ImageQuant software, and normalized to the signal of GAPDH mRNA.

Data evaluation. Data were evaluated with statistics software (GraphPad Prizm, San Diego, CA). Error bars represent the standard errors of the mean. Differences between wild-type and null groups were analyzed by Student’s t test at the 95% confidence level.

Results

The ketone body BHB is an end product of hepatic fatty acid oxidation (McGarry and Foster, 1980), and its plasma levels are often used to assess hepatic fatty acid oxidation in vivo (see Kersten et al., 1999 and Leone et al., 1999). Accordingly, we measured plasma hydroxybutyrate levels in L-FABP null mice in order to obtain an indication for potentially altered LCFA oxidation. Wild-type and L-FABP null mice were subjected to standard diet (a nonketogenic condition), to starvation (a ketogenic condition), to a short term high-fat, low-sugar diet (another ketogenic condition), and to a chronic highfat/ sugar (diabetogenic) diet, followed or not followed by starvation. Body weights were not affected by the genotype under these nutritional conditions at the time point of metabolic assays (results not shown). As shown in Figure III.1A, steady-state levels of blood BHB were significantly reduced in null vs. wild-type female mice under all ketogenic conditions (ketogenic diet and starvation irrespective of type of prior diet), whereas under nonstarving conditions (standard or diabetogenic diet), no significant ketogenesis occurred in either genotype. Similar results were obtained in male mice, but unlike in females, the genotypic difference in BHB levels was further increased by long-term diabetogenic diet before the starvation. Figure III.1B shows that in contrast to BHB levels, levels of circulating NEFA were not reduced under any ketogenic or nonketogenic condition. Rather, NEFA levels tended to increase; this was not statistically significant during starvation but reached significance under all other

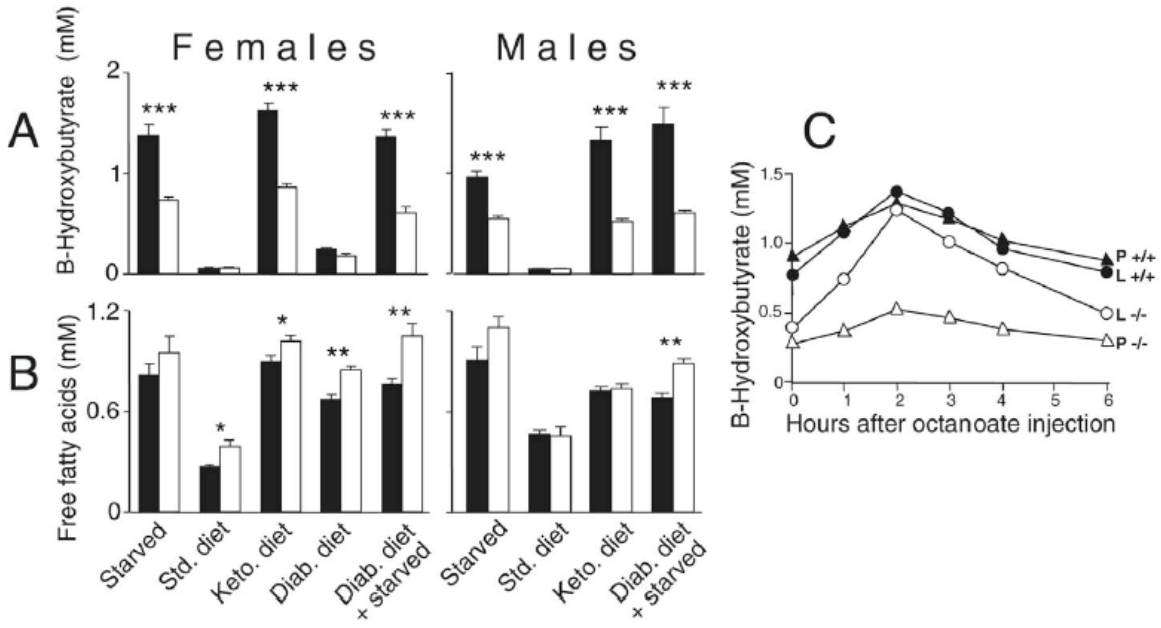

Figure III.1. A liver-intrinsic defect impairs hepatic long chain, but not medium chain, fatty acid oxidation in LFABP null mice. Serum levels of beta-hydroxybutyrate (***A***) and NEFA (***B***) were measured in female (left panels) and male (right panels) mice subjected to starvation (overnight), standard diet, ketogenic diet (3 days), or diabetogenic diet (4 wk). Black columns, wild type; white columns, L-FABP null. Each column pair represents mice that were matched for age, sex, and backcross generation, but mice represented by different column pairs may slightly differ by age and backcross generation. Each column includes 3 experiments with 2 mice each per genotype (n=6 total per genotype), except for free fatty acid measurements in starved and standard diet-fed mice where each column represents 2 experiments with 3 mice each (females, n=6 per genotype total) or 3 and 2 mice each (males, n=5 per genotype total). *P<0.05; **P<0.01, ***P<0.001; comparisons between genotypes. ***C)*** Octanoate injection restores ketogenesis in L-FABP null, but not PPARa null, mice. L-FABP null mice (L–/–, empty circles; n=5) and their wild-type litter mates (L+/+, filled circles; n=5) as well as PPARa null mice (P–/–, empty triangles; n=4) and their wild-type litter mates (P+/+, filled triangles; n=4) were injected with octanoic acid, and plasma beta-hydroxybuturate levels were determined at the indicated time points. All mice were females. Error bars (SE) did not exceed symbol sizes.

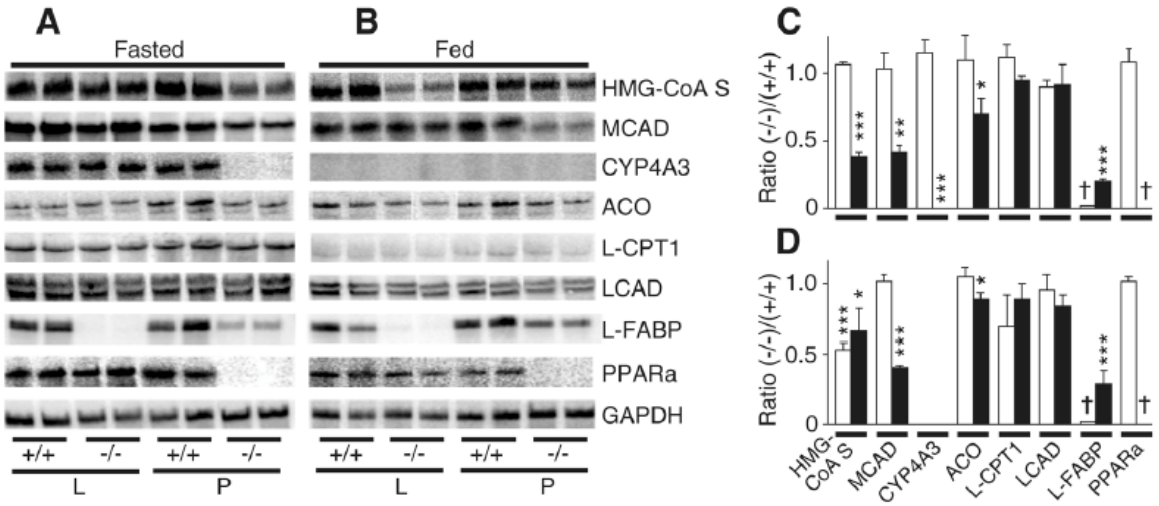

Figure III.2. Expression of key genes of lipid oxidation in L-FABP null and PPARa null livers**.** Shown are representative Northern blots prepared from mice starved overnight (14–16 h) (***A***) and mice fed standard chow (***B***). L, LFABP; P, PPARa; +/+, wild type; –/–, null. Separate matched wild-type controls were used for the L and P mice. For each genotype, two samples from two different mice were applied to neighboring lanes on the gel. All mice were females. ***A***, ***B)*** Representation of two blots that were each hybridized multiple times. Identical results were obtained with a second pair of Northern blots not shown that were prepared with RNA samples from additional mice, resulting in 4 analyzed mice for each of the 4 groups of mice. ***C)*** Quantification of expression levels in starved mice. ***D)*** Quantification of expression levels in fed mice (except for CYP4A3 because of low levels). Signals of all bands of all blots were normalized to the respective GAPDH signals, and the resulting numbers from null mice were divided by those from wild-type control mice. White columns, (L–/–)/(L+/+) ratio; black columns, (P–/–)/(P+/+) ratio. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; comparison between null and wild type. †Ratio equals zero by definition.

conditions in females. In males, the increase was seen only after long-term diabetogenic diet (Fig. III.1-B). The above results suggest that hepatic fatty acid oxidation is reduced in L-FABP null mice and that this reduction is not due to decreased substrate provision to the liver. To determine conclusively whether the decreased BHB levels are due to an intrahepatic defect, we isolated primary hepatocytes and incubated them with palmitate. Table III.1 shows that freshly isolated hepatocytes from L-FABP null mice produced significantly less BHB than those from wild-type mice. In parallel, total fatty acid oxidation was determined as the sum of radiolabeled carbon dioxide and radiolabeled acid-soluble products released. Again, a reduction was seen in the L-FABP null variant, similar in extent to the reduction of BHB (Table III.1).

Taken together, the above results demonstrate that hepatic fatty acid oxidation is impaired in the absence of L-FABP and that the reduction is due, at least in part, to an intrahepatic defect. A potential explanation for the reduced fatty acid oxidation and ketogenesis in L-FABP null mice might be decreased intracellular substrate levels. We therefore measured levels of LCFA-CoA, the direct substrates for β oxidation. As shown in Table III.2, a trend for increased stearoyl-CoA (C18:0) and decreased linoleoyl-CoA (C18:2) levels was seen in both females and males, but only for stearoyl-CoA in females was statistical significance reached at $P<0.05$. However, levels of palmitoyl-CoA (C16:0) were not changed despite the significant reduction in palmitic acid oxidation; moreover, total LCFA-CoA levels, defined here as the sum of all C16 and C18 LCFACoAs, were clearly unaltered. The essentially normal LCFA-CoA levels do not rule out their reduced availability. However, because L-FABP is able to increase the

Table III.1. Fatty acid oxidation in isolated liver tissue.

a: For each experiment, a wild-type (+/+) and an L-FABP null (–/–) mouse (both female) were starved overnight and then used to obtain either intact hepatocytes (six independent experiments total) or liver homogenates (four independent experiments total). After incubating duplicate aliquots of the cells or homogenates with 1 mM radiolabeled palmitate, we determined labeled oxidation products (nmol/h/500,000 cells or nmol/min/g wet liver) and beta-hydroxybutyrate concentrations (mikromolar). ASP, acid-soluble products. For experimental details, see Materials and Methods. Results are given as mean ± SE. **P* < 0.05; ***P* < 0.003 null vs. wild type. Comparisons were made by Student's *t* test.

	+/+	–/–
Intact hepatocytes		
^{14}C-CO_2 release	3.45 ± 0.53	3.80 ± 0.46
^{14}C-labeled ASP	43.45 ± 5.63	26.93 ± 4.51*
Total oxidation (CO_2 + ASP)	46.9 ± 6.16	30.73 ± 4.97*
Beta-hydroxybutyrate	96.81 ± 6.45	62.46 ± 5.90**
Liver homogenates		
^{14}C-CO_2 release	11.55 ± 1.44	12.67 ± 0.76
^{14}C-labeled ASP	483.5 ± 70.56	458.4 ± 68.50
Total oxidation (CO_2 + ASP)	495.05 ± 72.0	471.07 ± 69.26
Beta-hydroxybutyrate	13.72 ± 1.66	14.42 ± 2.12

Table III.2. Profile of LCFA-CoA in livers of wild-type and L-FABP null mice.
Female (F) and male (M) wild-type (+/+) and L-FABP null (–/–) mice were starved overnight. LCFA CoA levels (nmol/g) are given as mean ± SE, numbers (*n*) of analyzed mice are given in brackets. **P* < 0.05 null vs. wild type.

	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	Total
F +/+ (*n*=7)	45.4 ± 8.8	16.1 ± 5.6	45.4 ± 5.6	87.8 ± 16	66.4 ± 13	15.3 ± 3	280.9 ± 50
F –/– (*n*=7)	43.3 ± 8.3	20.4 ± 4	57.9 ± 14.2*	92.2 ± 20	55.2 ± 9.1	12.79 ± 2	278.1 ± 55
M +/+ (*n*=7)	48.25 ± 7.8	24.4 ± 5.5	31 ± 5.1	88.3 ± 15	85.6 ± 14	18.6 ± 3.2	296.2 ± 50
M –/– (*n*=9)	48.1 ± 9	26.3 ± 5	38.3 ± 6.2	90.1 ± 17	74.8 ± 13	16.4 ± 3.5	292.2 ± 53

activity (Wolfrum et al., 2001) and levels (Linden et al., 2002) of PPAR-alpha, a transcription factor that is required for a high capacity of ketogenesis (Kersten et al., 1999; Leone et al., 1999; Le May et al., 2000), it seemed possible that the capacity for ketogenesis was reduced in L-FABP null mice. Three approaches were taken to assess the capacity for fatty acid oxidation and ketogenesis.

First, we tested whether ketogenesis in L-FABP null mice can be restored with octanoic acid. This fatty acid is a good substrate for ketogenesis in wild-type hepatocytes, but it is not a physiologically significant primary substrate of ketogenesis, not a ligand of L-FABP, and not efficiently metabolized by isolated PPAR-α null hepatocytes (Le May et al., 2000). Octanoic acid was injected intraperitoneally into starved wild-type, L-FABP null, and PPAR-alpha null mice, and ketogenesis was followed over 6 h thereafter. Figure III.1C shows that after octanoate injection, a massive increase in ketogenesis occurred in both wild-type and L-FABP null mice. The resulting peak BHB levels were comparable between wild-type and L-FABP null mice even though the null mice had started from a much lower level and were much higher than achievable in any physiological condition, indicating similar capacities for ketogenesis. In contrast, the PPAR-alpha null mice that also exhibited low starting BHB levels were unable to raise these levels significantly after octanoic acid injection (Fig. III.1C). These results suggest that the effect of LFABP deficiency on ketogenesis is restricted to primary substrates that are ligands of L-FABP.

Second, as a composite enzymatic assay that assesses all steps of LCFA oxidation except substrate provision, we measured the oxidation of albumin-bound palmitic acid in liver homogenates. As shown in Table III.1, no difference in either BHB

production or radioactive palmitate oxidation was seen between L-FABP null and wild-type homogenates. This suggests that the effect of L-FABP deficiency on LCFA oxidation originates on the level of intracellular substrate availability rather then oxidative capacity.

Third, the expression of key genes of lipid oxidation was compared between L-FABP null, PPAR- alpha null, and wild-type mice. Figure III.2A shows that compared with wild-type litter mates, starving PPAR- alpha null mice showed significantly reduced levels of mRNAs encoding CYP4A3, MCAD, ACO, mitochondrial HMG CoA synthase, and L-FABP, whereas mRNAs encoding LCPT1 and LCAD showed little or no reduction. These results are quantified in Figure III.2C, giving the ratios of null to wild-type levels. The expression of LCAD has not been previously measured in the PPAR- alpha null mice, but all other results agree with published findings (Kersten et al., 1999; Leone et al., 1999; LeMay et al., 2000). The published decrease of mitochondrial HMG CoA synthase mRNA was more dramatic, however, perhaps because Le May et al. (2000) starved their mice longer (24 hours) than we did (14-16 hours). More importantly, Figure III.2A and III.2C further show that none of the above mRNAs (except L-FABP) was changed in L-FABP null vs. wild-type livers; moreover, absence of L-FABP did not affect levels of PPAR- alpha mRNA.When the Northern blotting experiments were repeated under feeding (standard diet), a similar tendency to reduced gene expression was seen in PPAR- alpha null mice (Fig. III.2B, 2D), again in agreement with literature, although we found a somewhat smaller genotypic difference in HMG CoA synthase than Le May et al. (2000) and a larger difference in L-FABP

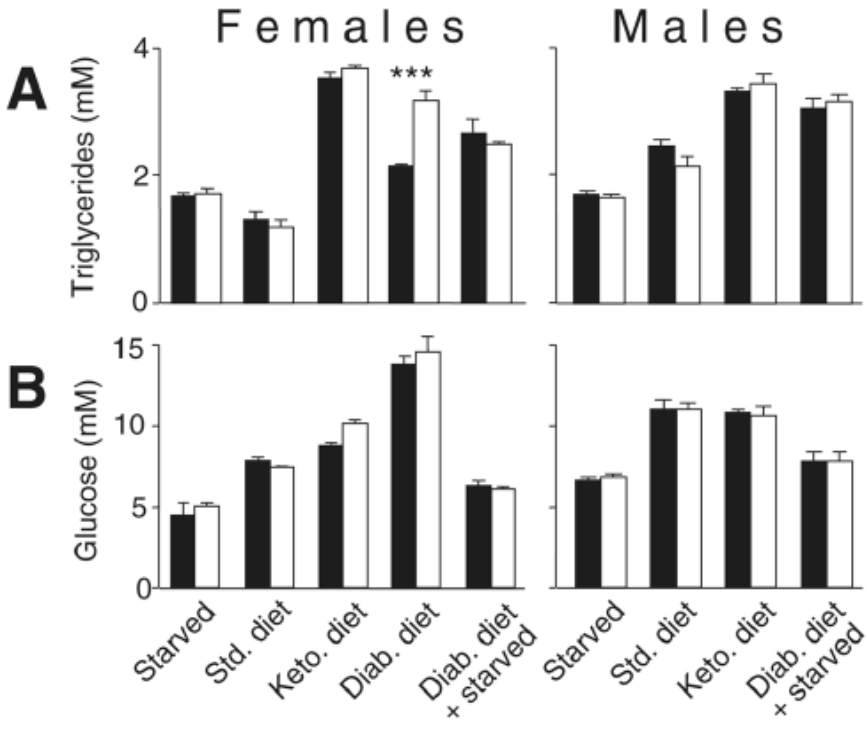

Figure III.3. Effect of L-FABP deficiency on circulating triacylglycerol and glucose levels. Blood samples were taken from mice subjected to starvation (overnight), standard diet, ketogenic diet (3 days), diabetogenic diet (4 wk), and diabetogenic diet plus starvation. Levels of triglycerides (triacylglycerols) (***A***) and glucose (***B***) were determined in female mice (left panels) and male mice (right panels). Black columns, wild type; white columns, L-FABP null. Each column includes 3 experiments with 2 mice each per genotype (n=6 total per genotype). Each column pair represents mice that were matched for age, sex, and backcross generation, but mice represented by different column pairs may slightly differ by age and backcross generation. ***P<0.001; comparisons between genotypes.

mRNAs than Kersten et al. (1999). Again, in contrast with PPAR- alpha null mice, no significant genotypic difference was seen in L-FABP null mice for most of the above mRNAs. Unexpectedly, however, a significant reduction of mitochondrial HMG CoA synthase mRNA was seen in all fed L-FABP null mice, and there was a similar tendency with L-CPT1 that did not reach significance (Fig. III.2B, 2D).

Finally, we measured two more plasma metabolites in order to probe the broader impact of the L-FABP null mutation on body fuel metabolism. As shown in Figure III.3A, plasma triacylglycerol levels remained normal under standard diet, starvation, and ketogenic diet. Interestingly, however, they were significantly increased in the plasma of L-FABP null mice after long-term diabetogenic diet (Fig. III.3A). No differences between wild-type and L-FABP plasma glucose levels were seen under any condition (Fig. III.3B).

Discussion

To assess the physiological significance of L-FABP, we recently produced L-FABP null mice. Using these mice, we demonstrated a major contribution of L-FABP to cytosolic fatty acid binding capacity of the liver; other FABPs did not compensate for this function (Martin et al., 2003). The present study was designed to answer the following two related questions. First, Is L-FABP important for hepatic LCFA oxidation in vivo? Second, Under conditions of intense hepatic fatty acid oxidation, is L-FABP required for the action of the transcription factor PPAR- alpha, a master onswitch of fatty acid oxidation (Kersten et al., 1999; Leone et al., 1999) and a potential target of L-FABP (Wolfrum et al., 2001). The results presented herein answer the first question to the affirmative, but strongly argue against the second possibility.

The present study revealed a significant role of L-FABP in hepatic LCFA oxidation, as concluded from the reduced LCFA oxidation and BHB production in isolated L-FABP null hepatocytes, which is in line with reduced BHB levels seen in blood plasma of L-FABP null mice. However, the effect did not exceed 35% in vitro (Table III.1), whereas at comparable fatty acid concentrations in vivo (Fig. III.1B), it approached and sometimes exceeded 50% (Fig. III.1A). The reasons for this discrepancy may include shortcomings of the in vitro model or extrahepatic components of the phenotype, but they are unlikely to include a reduced provision of substrate (NEFA) to the liver. Indeed, compared with wild-type mice, plasma NEFA levels were nonsignificantly increased in female L-FABP null mice during starvation following standard diet but were significantly increased under ketogenic diet and starvation after diabetogenic diet, as might be expected when fatty acid oxidation is inhibited in a major organ. Also note that the phenotype of reduced fatty acid oxidation in L-FABP null mice appeared to vary with gender.

In line with the known fact that long-term high-fat diet raises L-FABP expression in males to female levels (Kersten et al., 1999), male L-FABP null mice showed the largest decrement in ketogenesis during starvation following a long-term diabetogenic diet, whereas no such trend was seen in the females. Similarly, plasma NEFA levels of male L-FABP null vs. wild-type mice were significantly increased only under starvation following the diabetogenic diet, whereas the increase was significant in females also under most other nutritional conditions.

Taken together, our results show that the blunted fatty acid oxidation and ketogenesis is, at least in part, of intrahepatic origin, demonstrating that L-FABP is a

cell-intrinsic stimulator of LCFA oxidation. The increase in plasma fatty acid levels under some ketogenic conditions also implies that LFABP impacts whole body lipid metabolism, a conclusion that can be extended to nonketogenic conditions. Both plasma NEFA and triacylglycerols were increased in (at least female) L-FABP null mice after chronic diabetogenic diet (without starvation), conceivably due to reduced hepatic clearance. The mechanisms underlying the altered plasma levels in nonketogenic conditions clearly deserve further investigation.

The second question addressed here concerns the mechanism by which L-FABP stimulates LCFA oxidation. Although the high cytoplasmic concentration of L-FABP in fully differentiated hepatocytes is suggestive of a substrate-level function, L-FABP concentrations were recently shown to limit the activity of the lipid-dependent transcription factor PPAR- alpha in PPAR- alpha - transfected cells (Wolfrum et al., 2001). This suggested to us that transcription of PPAR- alpha target genes and consequently the capacity for fatty acid oxidation might be compromised in the absence of LFABP.

Ketogenesis from endogenous substrates is severely impaired in both PPAR-alpha null mice (Kersten et al., 1999; Leone et al., 1999 and present study) and L-FABP null mice (present study). However, we found that ketogenesis from medium-chain fatty acid (octanoate) is reduced only in PPAR- alpha null mice, whereas octanoate boosted ketogenesis in L-FABP null mice to levels higher than achievable in wild-type mice by fat diet or starvation, showing that the capacity for ketogenesis was fully maintained. This difference in ketogenic capacity can be explained by differences in gene expression.

In agreement with literature (Leone et al., 1999; Le May et al., 2000), we found that upon starvation, levels of the mRNAs encoding MCAD and mitochondrial HMG CoA synthase were reduced in the PPAR- alpha null vs. wild-type mice. In contrast, they were normal in the L-FABP null mice. Thus, at least some PPAR- alpha target genes and the associated biochemical process were not affected in starving LFABP null mice. Two other PPAR- alpha target genes, ACO and CYP4A3, representing peroxisomal and microsomal lipid oxidation, respectively, were also expressed normally, suggesting that PPAR- alpha -dependent gene transcription was generally unaffected in the starving L-FABP null mice investigated. In line with this, PPAR- alpha mRNA levels were also normal. Finally, the normal levels of the mRNAs encoding LCAD and L-CPT1 suggest that reduced hepatic LCFA oxidation in the starving L-FABP null mice was not caused by altered gene expression through PPAR- alpha -independent gene transcription either. In agreement with this conclusion, we did not find a reduced rate of palmitic acid oxidation in liver homogenates of L-FABP null mice, arguing that the levels of all enzymes involved in LCFA oxidation and ketogenesis are maintained. In fact, because the principal difference between intact isolated hepatocytes and liver homogenates is that in the latter, plasma membranes are disrupted and the oxidative substrate is bound by another carrier (albumin), the oxidation defect is most likely at the level of intracellular substrate provision.

Taken together, our data do not support a role for L-FABP in PPAR- alpha function or indeed gene expression in general under starvation conditions in vivo. In this respect, we note that similar to L-FABP null hepatocytes, H-FABP null cardiomyocytes lack compensatory FABP expression, show substantially reduced LCFA oxidation

(while maintaining normal oxidative capacity), and oxidize octanoic acid normally (Schaab et al., 1999). In contrast, octanoic acid oxidation is significantly impaired in PPAR-α null hearts (Watanabe et al., 2000).

In apparent contradiction with the above results, Wolfrum et al. (2001) and Tan et al. (2002) convincingly demonstrated that L-FABP and H-FABP are able to activate PPAR- alpha in hepatoma and COS cells, respectively. Further, Tan et al. (2002) also demonstrated activations of transfected PPAR-beta and -gamma by keratinocyte and adipocyte FABP, respectively, suggesting that PPAR activation is a general feature of the FABP family. We suspect that the solution of this contradiction may be the level of lipid flux in the cells concerned. The cell lines used by Wolfrum et al. and Tan et al. exhibit a low degree of differentiation that probably cannot support high LCFA fluxes, and low endogenous PPAR- alpha levels that were actually raised by transfection. Importantly, Tan et al. (2002) showed that transfected keratinocyte FABP activates endogenous (nontransfected) PPAR-beta only at extremely low ligand levels. Thus, in vivo, FABPs may be important for the action of cognate PPARs only under conditions of low lipid metabolism, such as during onset of differentiation.

In contrast, at least in starved differentiated hepatocytes (present study) and cardiomyocytes (Schaab et al., 1999; Binas et al., 1999), LCFA fluxes seem to remain high enough to fully activate PPAR- alpha even in the absence of FABPs. In this context, our observation of a reduction of mitochondrial HMG CoA synthase mRNA levels in the L-FABP null liver under standard diet may be of interest. This decrease occurred also in the PPAR- alpha null mice. However, under the same condition, MCAD gene expression was clearly not reduced in the L-FABP null mice, whereas it was

substantially reduced in the PPAR- alpha null mice. Thus, PPAR- alpha -independent mechanisms might contribute to the reduced expression of HMG CoA synthase under standard diet, or a differential effect of PPAR- alpha on its target genes needs to be postulated under these nonsaturating conditions. In any case, it is clear that in fed mice, the reduced (compared with starving conditions) expression of most PPAR- alpha target genes remains independent of L-FABP.

Although our results argue against a substantial transcriptional role of L-FABP in maintaining hepatic LCFA oxidation in starving mice, elucidation of the precise mechanism by which LFABP maintains high rates of LCFA oxidation in the liver will require further work. Our previous results showed that although L-FABP exerts no effects on cellular LCFA levels, it determines the cytosolic binding capacity for LCFAs (Martin et al., 2003). The same may turn out true for LCFA-CoA, the proximal enzyme substrate of LCFA metabolism and itself an L-FABP ligand (Frolov et al., 1997) whose total levels were not altered in the absence of L-FABP in the present study. Recent literature indicates that the metabolic availability of LCFA-CoAs is determined by their binding to specific proteins rather than LCFA-CoA concentration per se (Chao et al., 2003). Alternatively or in addition, the existence of several long-chain acyl CoA synthases (Coleman et al., 2002) and subcellular compartmentalization of this metabolite might lead to local differences in LCFA-CoA concentration.

In conclusion, the results presented here establish for the first time that L-FABP plays a role in hepatic LCFA oxidation in vivo. Under starving conditions, this role appears to be mainly direct, rather than through the recently proposed activation of PPAR- alpha. In contrast, under standard diet, we did see a selective effect of L-FABP

on hepatic gene expression. It thus appears that the relative importance of L-FABP in substrate provision and gene expression may vary with physiological condition, a concept that may be useful for the investigation of various members of the FABP family.

CHAPTER IV

SUMMARY

In the first part of this dissertation, the results provide support for two opposing effects of intracellular fatty acid flux on skeletal muscle glucose uptake, by decreasing insulin-dependent glucose uptake and simultaneously increasing basal glucose uptake. The results show that insulin sensitivity on glucose uptake is improved in H-FABP null muscle from mice maintained under standard diet. That is, while the maximum effect of insulin (i.e., insulin responsiveness) seen at supraphysiological insulin levels was unaltered, low physiological concentrations of insulin caused a significantly larger increase of deoxyglucose uptake by soleus muscle isolated from H-FABP null as opposed to wild type mice. This result provides further support for the general notion that fatty acids or their metabolites metabolism reduce insulin action. But, it is a novel finding of the present investigation that insulin sensitivity was altered in the absence of alterations of steady state levels of triglycerides and of both total and individual LCA CoA. Given that rates of triglyceride synthesis and breakdown are reduced in H-FABP null soleus muscle (Binas et al., 2003, and present dissertation), these results raise the question whether lipid turnover as such might be a determinant of insulin sensitivity.

Circulating insulin levels were dramatically decreased in H-FABP null mice compared with wild type. This is unlikely due to impaired beta cell function, as a glucose bolus raised insulin concentrations of H-FABP null and wild type mice to the same level. More likely and in line with the soleus muscle incubations, increased muscle insulin sensitivity is the cause of reduced insulin levels, although it may be asked

whether decreased glucose and/or increased fatty acid levels in H-FABP null mice exerting an additional influence on the pancreas (Rubi et al., 2002).

The results also suggest that in contrast to the absence of an effect of H-FABP on muscle triglyceride levels under a standard diet, the H-FABP mutation completely prevented the substantial increase of muscle triglyceride steady state levels normally caused by a chronic high fat diet. Accumulation of stromal fat (comp. Dobbins et al., 2001) is an unlikely contributor in this case because the mutation did not raise plasma fatty acid levels under high fat more than it did under the standard diet. The dramatic genotypic effect on triglyceride levels under one but not the other diet contrasts with the rather similar decrements of rates of fatty acid esterification caused by the mutation under both diets (although the high fat diet was causing an additional decrease). More work is needed in order to understand this discrepancy. Total LCA CoA levels were reduced moderately but not significantly, although some larger (but still non-significant) individual decreases, in particular linolenoyl CoA and palmitoyl CoA, were seen. These alterations in lipid levels and turnover induced by the mutation under the high fat diet were associated with an almost complete prevention of resistance of isolated soleus muscle glucose transport to supraphysiological insulin concentrations. It should be noted that the changes of LCA CoA in H-FABP null mice were more moderate than seen in other rodent models with a comparable degree of insulin resistance (Thompson et al., 2002; Yu et al., 2002). Although this has been interpreted as supporting a role of LCA CoA levels in regulating insulin sensitivity, the disparity between the modest changes of LCA CoA and the dramatic improvement of insulin sensitivity in this and the present study is striking. These results thus emphasize the need to perform more detailed studies

to clarify whether acyl CoA levels or some other aspect of LCFA metabolism, such as lipid turnover, hold the key to regulating insulin-dependent glucose transport.

The results of ex vivo incubations of isolated soleus muscle from mice kept under high fat diet are in line with whole body measurements. Although the fat diet led to impaired glucose clearance in both wild type and H-FABP null mice, the latter showed a significant improvement compared with wild type. This effect can be interpreted as a partial prevention of whole body insulin resistance. Incomplete prevention was expected, as H-FABP is not expressed in white adipose and liver tissue that also contribute to whole body insulin resistance. Furthermore, insulin levels of H-FABP null mice maintained under the high fat diet were decreased compared with wild type, although still increased compared with those on the standard diet. Unlike the standard diet, however, a bolus of glucose injected into high fat diet-fed H-FABP null mice did not increase circulating insulin to wild type levels, raising the question of whether beta cell capacity is reduced.

In summary, the present study of fatty acid metabolism and glucose uptake in H-FABP deficient skeletal muscles has provided strong evidence of the role of intracellular fatty acid transport in both insulin-dependent and basal glucose uptake. The mutation was shown to affect insulin sensitivity under standard diet and high fat diet. Reduced levels of triglycerides and possibly LCA CoA were associated with an increased maximum effect of insulin (insulin responsiveness) under the high fat diet, while unaltered triglyceride and LCFA levels were compatible with improved insulin action at lower, physiological insulin levels (insulin sensitivity) under the standard diet. These improvements of insulin action in skeletal muscle occurred despite the increased plasma

fatty acid levels seen with both diets, supporting the concept that plasma fatty acid levels are irrelevant in determining muscle insulin sensitivity and responsiveness. Reduced insulin plasma levels and improved glucose tolerance in H-FABP null mice are also indicative of improved insulin sensitivity. Our finding of increased insulin sensitivity in face of normal acyl CoA /triglyceride levels highlights the need for a more detailed analysis of the role of acyl CoA levels and lipid turnover rates.

The second part of this dissertation revealed a significant role of L-FABP in hepatic LCFA oxidation, as concluded from the reduced LCFA oxidation and BHB production in isolated L-FABP null hepatocytes. The results are consistent with the reduced BHB levels seen in blood plasma of L-FABP null mice. However, the effect did not exceed 35% in vitro, whereas at comparable fatty acid concentrations in vivo, it approached and sometimes exceeded 50%. The reasons for this discrepancy may include shortcomings of the in vitro model or extrahepatic components of the phenotype, but they are unlikely to include a reduced provision of substrate (NEFA) to the liver. Indeed, compared with wild-type mice, plasma NEFA levels were not significantly increased in female L-FABP null mice during starvation following a standard diet but were significantly increased under ketogenic diet and starvation after a diabetogenic diet, as might be expected when fatty acid oxidation is inhibited in a major organ.

Taken together, the results show that the reduced fatty acid oxidation and ketogenesis is, at least in part, of intrahepatic origin, demonstrating that L-FABP is a cell-intrinsic stimulator of LCFA oxidation. The increase in plasma fatty acid levels under some ketogenic conditions also implies that LFABP impacts whole body lipid metabolism, a conclusion that can be extended to non-ketogenic conditions. Both plasma

NEFA and triacylglycerols were increased in (at least in female) L-FABP null mice after a chronic diabetogenic diet (without starvation), conceivably due to reduced hepatic clearance. The mechanisms underlying the altered plasma levels in non-ketogenic conditions clearly deserve further investigation.

The second question addressed in this part of the dissertation concerned the mechanism by which L-FABP stimulates LCFA oxidation. Although the high cytoplasmic concentration of L-FABP in fully differentiated hepatocytes is suggestive of a substrate-level function, L-FABP concentrations were recently shown to limit the activity of the lipid-dependent transcription factor PPAR-alpha in PPAR-alpha - transfected cells (Wolfrum et al., 2001; Kersten et al., 1999; and present dissertation). This suggested that the transcription of PPAR- alpha target genes, and consequently the capacity for fatty acid oxidation, might be compromised in the absence of LFABP. Ketogenesis from endogenous substrates is severely impaired in both PPAR- alpha null mice (Wolfrum et al., 2001; Kersten et al., 1999; and present dissertation) and L-FABP null mice (present study). However, we found that ketogenesis from medium-chain fatty acid (octanoate) is reduced only in PPAR- alpha null mice, whereas octanoate boosted ketogenesis in L-FABP null mice to levels higher than achievable in wild-type mice by the fat diet or starvation, showing that the capacity for ketogenesis was fully maintained. This difference in ketogenic capacity can be explained by differences in gene expression.

In addition, following starvation, levels of the mRNAs encoding MCAD and mitochondrial HMG CoA synthase were reduced in the PPAR-alpha null vs. wild-type mice. In contrast, they were normal in the L-FABP null mice. Thus, at least some

PPAR-alpha target genes and the associated biochemical process were not affected in starving LFABP null mice. Two other PPAR- alpha target genes, ACO and CYP4A3, representing peroxisomal and microsomal lipid oxidation, respectively, were also expressed normally, suggesting that PPAR- -dependent gene transcription was generally unaffected in the starving L-FABP null mice investigated. In line with this, PPAR- alpha mRNA levels were also normal. Finally, the normal levels of the mRNAs encoding LCAD and L-CPT1 suggest that reduced hepatic LCFA oxidation in the starving L-FABP null mice was not caused by altered gene expression through PPAR- alpha -independent gene transcription either. In agreement with this conclusion, we did not find a reduced rate of palmitic acid oxidation in liver homogenates of L-FABP null mice, arguing that the levels of all enzymes involved in LCFA oxidation and ketogenesis are maintained. In fact, because the principal difference between intact isolated hepatocytes and liver homogenates is that in the latter, plasma membranes are disrupted and the oxidative substrate is bound by another carrier (albumin), the oxidation defect is most likely at the level of intracellular substrate provision.

Taken together, the results of this research do not support a role for L-FABP in PPAR- alpha function or indeed gene expression in general under starvation conditions in vivo. In this respect, I note that similar to L-FABP null hepatocytes, H-FABP null cardiomyocytes lack compensatory FABP expression, show substantially reduced LCFA oxidation (while maintaining normal oxidative capacity), and oxidize octanoic acid normally . In contrast, octanoic acid oxidation is significantly impaired in PPAR- alpha null hearts.

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Publications

1. Erol E., Kumar L., Cline G.W., Shulman G.I., Kelly D.P., Binas B. 2004. L-FABP is required for high rates of hepatic fatty acid oxidation but not for the action of PPAR alpha in genetically altered mice. FASEB J. 18(2):347-9.

2. Erol E., Cline G., Taegtmeyer M., Shulman G.I., Binas B. 2003. Opposing roles of heart-type fatty acid-binding protein (H-FABP) in skeletal muscle glucose uptake in H-FABP mutated mice. (Accepted by the American Journal of Physiology)

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