GLUCOSE OXIDATION IN HEART-TYPE FATTY ACID BINDING PROTEIN NULL MICE

A Thesis

by

SEAN ADHIKARI

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

MASTER OF SCIENCE

August 2006

Major Subject: Genetics

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Approved by:

Chair of Committee,	Bert Binas
Committee Members,	Gale Wagner
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ABSTRACT

Glucose Oxidation in Heart-Type Fatty Acid Binding Protein Null Mice. (August 2006)

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Chair of Advisory Committee: Dr. Bert Binas

Heart-type fatty acid binding protein (H-FABP) is a major fatty acid binding factor in skeletal muscles. Genetic lack of H-FABP severely impairs the esterification and oxidation of exogenous fatty acids in soleus muscles isolated from chow-fed mice (CHOW-solei) and high fat diet-fed mice (HFD-solei), and prevents the HFD-induced accumulation of muscle triglycerides. Here, we examined the impact of H-FABP deficiency on the relationship between fatty acid utilization and glucose oxidation. Glucose oxidation was measured in isolated soleus muscles in the presence or absence of 1 mM palmitate (simple protocol) or in the absence of fatty acid after preincubation with 1 mM palmitate (complex protocol). With the simple protocol, the mutation slightly reduced glucose oxidation in CHOW-muscles, but markedly increased it in HFD-muscles; unexpectedly, this pattern was not altered by the addition of palmitate, which reduced glucose oxidation in both CHOW- and HFD-solei irrespective of the mutation. In the complex protocol, the mutation first inhibited the synthesis and accumulation of triglycerides and then their mobilization; with this protocol, the mutation increased glucose oxidation in both CHOW- and HFD-solei. We conclude: (i) H-FABP mediates a non-acute inhibition of muscle glucose oxidation by fatty acids, likely by enabling both the accumulation and mobilization of a critical mass of muscle triglycerides; (ii) H-FABP

does not mediate the acute inhibitory effect of extracellular fatty acids on muscle glucose oxidation; (iii) H-FABP affects muscle glucose oxidation in opposing ways, with inhibition prevailing at high muscle triglyceride contents.

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INTRODUCTION

History and background

The cytosolic Fatty Acid Binding Proteins (FABPs) were discovered in 1972 [40,44] and are now known to be encoded by a family of homologous genes [50] with complex, often overlapping expression patterns [18]. The ligand-binding properties [15] and three-dimensional structures [28,16] of many FABPs have been studied; the ability of FABPs to promote long chain fatty acid (LCFA) diffusion, sequestration, and transport has been demonstrated in cell-free systems [57]. Transfection studies have demonstrated the ability of at least some FABPs to affect cellular LCFA uptake, metabolism, lipid composition, and LCFA-dependent gene regulation [39,65,66,58,21]. In its entirety, this work leads to the hypothesis that FABPs are crucial in mediating and integrating the diverse effects of intracellular LCFA and some related compounds. Testing this hypothesis is of obvious clinical interest, since altered fatty acid metabolism and fatty acid signaling appear to be central to a number of pathological conditions, most notably the metabolic syndrome (e.g., [60,54]). However, cell-free, transfection, and expression studies cannot reveal the quantitative contributions of FABPs to lipid functions in vivo. To obtain such information, it is necessary to selectively alter FABP levels or functions in the native context, i.e., in vivo or in freshly isolated tissues.

Although it might become possible to target FABPs pharmacologically [29], the effective and selective manipulation of FABP function in intact tissues currently requires genetic means.

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One limitation of the genetic approach is that it is inherently slow which makes it difficult to distinguish direct/acute from indirect/chronic effects of altered FABP levels. Further, the information that can be gained from the genetic manipulation can be limited by the simultaneous expression of a given FABP in several organs, redundant expression of different FABPs in the same organ, or compensatory activation of normally silent FABP genes. Indeed, the FABPs are generally expressed rather broadly and with redundancy. Luckily, however, they are expressed differentially and with very little overlap or redundancy in those organs that are also the principal contributors to whole body fuel metabolism, especially muscle, heart, liver, fat, and gut. Therefore, genetic manipulation of FABPs in these tissues may be expected to create models of regionally disturbed lipid metabolism that can be used to study principles of fuel selection, interactions between LCFA metabolism and other pathways (especially glucose metabolism), pathophysiological roles of regional lipid oversupply, and principles of metabolic homeostasis. It is also worth mentioning that animal models with genetically manipulated FABP levels may be directly relevant for human disease, since allelic variants of FABP genes appear to modulate human metabolic disease and its treatment [64,6].

Several genetic models of altered FABP expression have been created in the last few years. Heart-type FABP [3] and keratinocyte-type FABP [19] have been over-expressed in transgenic mice, and endogenous FABP genes have been inactivated in embryonic stem cells that were then used to create “knockout” mice lacking either adipocyte-type FABP [22,20], heart-type FABP [4], intestinal FABP [61], keratinocyte-type FABP [45,35] or liver-type FABP [37,42].

Despite the limitations noted above, the mouse genetics approach has been quite useful in illuminating various aspects of FABP biology, as reviewed previously [18,13,14,17,36]. The current project describes the fuel-metabolic phenotype of mice that lack H-FABP (H-FABP null), a focus that has not previously been made. This FABP-deficient model has three advantages that make it especially attractive for studying fuel metabolism: First, the primarily affected tissues (heart and skeletal muscle) play major roles in normal whole body fuel metabolism as well as in the development of the metabolic syndrome; second, normal FABP expression in these tissues is high yet not redundant; third, little or no compensatory increases of other cytosolic fatty acid binding proteins appear to occur in these tissues when the original FABPs are deleted.

Reduced fatty acid binding in H-FABP null cytosols

H-FABP was discovered by incubating high speed supernatants ("cytosols") of tissue homogenates with labeled fatty acids, followed by size-exclusion chromatography [44,40]. These separations revealed a dominant peak in the 13-15 kDa region, the size of the later-purified FABPs. When cytosols from hearts and soleus muscles [49,5] of H-FABP null mice were incubated with ^{3}H-palmitate and ^{3}H-oleate, respectively, the radioactive peaks in that region essentially disappeared in all cases. No compensatory expression of related FABPs in hearts of H-FABP null mice was detected by northern blotting [4,42]. Taken together, the above results demonstrate (i) dominant roles for myocellular H-FABP in cytosolic LCFA binding and (ii) lack of adequate compensation at the level of the primary defect.

Reduced LCFA uptake in H-FABP null

The word "uptake" in the literature simply denotes the cellular accumulation of a label, including both its movement into the cell and its metabolic fixation. That is, uptake measured over more than a minute usually has a metabolic component, whereas initial uptake (measured within seconds) precedes metabolism. A massively decreased cardiac LCFA uptake in resting H-FABP null mice, measured over 30 minutes, was first demonstrated with the non-beta-oxidizable but esterifiable fatty acid analog, ^{125}I-BMIPP [4]. Also in resting mice and by using a more quantitative method, the cardiac uptakes (over 10 minutes) of palmitic acid and arachidonic acid were later found to be decreased by 40% and 50%, respectively [41]. Shearer et al. [52] then found that during forced (treadmill) physical exercise, the uptake of ^{125}I-BMIPP (over 30 minutes) was reduced ~12 fold in H-FABP null heart and 4-5 fold in null soleus muscle, as compared to wild type controls. Under this condition, whole body fatty acid utilization was reduced ~4 fold by the mutation. In addition, in both organs the fatty acid uptake was linearly correlated with the number of H-FABP alleles, i.e., gene dosage (0, 1, and 2). Cell-free experiments with giant vesicles [32] isolated from null skeletal muscles showed that H-FABP is required for maximal LCFA uptake. However, in contrast to the in vivo results of Shearer et al. [52], uptake by vesicles was not decreased when only one allele was deleted. This discrepancy would suggest that metabolism (that is lacking in the vesicles) synergizes with H-FABP in facilitating LCFA transport. A markedly decreased initial uptake of palmitate was also seen in isolated null soleus muscle [5] and cardiomyocytes isolated from H-/- hearts [49]. The fatty acid uptake in null mice is strongly suggestive of reduced LCFA metabolism.

LCFA metabolism and lipid levels in H-FABP null cells

Mitochondrial fatty acid oxidation. Fatty acid oxidation in individual organs cannot be easily measured in living mice. However, it can be measured under near-physiological conditions in freshly isolated organs and cells. Palmitate (0.4 mM) oxidation was decreased by 45% in H-FABP null vs. wild type cardiomyocyte suspensions and was, unlike in wild type cells, not significantly increased by electrical stimulation [49]. Resting soleus muscles isolated from null mice also exhibited substantial (~70%) decreases of palmitate oxidation at 0.5 mM [5] or 0.1 mM [12] palmitate, but unlike in the cardiomyocytes, the "residual" oxidation by null soleus could be significantly stimulated electrically [5]. In summary, when tested under conditions with a known physiological correlate, H-FABP (muscle, heart) is required in order to maintain normal rates of mitochondrial LCFA oxidation in cardiac and skeletal muscle cells.

Fatty acid esterification and lipid composition. In resting null vs. wild type mice, substantially reduced cardiac incorporation of palmitic acid into neutral lipids but not phospholipids was observed, while incorporation of arachidonic acid was reduced in both pools [40]. In isolated resting soleus muscle, palmitate incorporation into triglycerides and phospholipids was reduced, but in contracting muscles palmitate incorporation into phospholipids remained at wild type rates [5]. Overall, in H-FABP null cells, the observed reduction of fatty acid esterification into triglycerides was comparable in scale with that of uptake (section 3), while rates of phospholipid synthesis were affected less.

Based on these facts, it is not surprising that lipid composition of FABP null tissues was affected. These changes are now being studied in detail [42,38,39,41,1]; here

we highlight only triglycerides since they are particularly relevant for fuel metabolism. Although triglyceride levels in skeletal muscles [5] and cardiomyocytes [49] from chow-fed mice were not affected by the null mutation, the decrease of triglyceride levels observed in wild type soleus muscles upon incubation was blunted in null muscles [5]; further, the increase of triglyceride levels in skeletal muscle and heart that occurs normally under a chronic high fat diet was prevented in the null mice ([12], and Fig.1). Thus, H-FABP facilitates physiological mobilization and deposition of triglycerides, but the net effect on the triglyceride levels appear to be sensitive to timing and physiological circumstance. It should also be kept in mind that H-FABP in other organs, such as heart, may affect the net accumulation of fat.

Taken together, the above results clearly demonstrate that in heart and skeletal muscle, H-FABP is an important determinant of LCFA metabolism and lipid composition. In view of the close relationship between lipid and glucose metabolism, this predicts that glucose metabolism in H-FABP null mice is also altered. In fact, the prospect of using the null mice for the study of fuel selection was a driving force in creating these mice.

Altered glucose uptake and metabolism in H-FABP null mice

A markedly increased cardiac uptake of glucose was first observed in resting null mice injected with the non-metabolizable glucose analog, 2-deoxyglucose [4]. These experiments were recently confirmed and extended with a more quantitative approach that revealed a 6-7 fold increase of cardiac, and a 1.7 fold increase of soleus muscle, deoxyglucose uptakes in null versus wild type mice during exercise. Moreover, deoxyglucose uptake in these organs correlated linearly with the number of H-FABP

alleles, resulting in a reciprocal pattern of fatty acid and glucose uptakes [52]. In vitro experiments with isolated cells and tissue supported these results and added further details. Glucose oxidation in freshly isolated null cardiomyocytes was increased by ~80% compared to wild type cardiomyocytes but, unlike in wild type cells, was not increased by electrical stimulation [49]; it remains to be seen whether this reflects an in vitro limitation. The results with isolated soleus muscles were, in part, surprising. On one hand, when measured at physiological insulin levels, the insulin-caused increment of deoxyglucose uptake into null soleus muscles from mice fed a standard diet was more than doubled as compared to the increment in wild type muscles. In addition, the mutation partially prevented the decrease of insulin responsiveness of deoxyglucose uptake seen in wild type soleus muscles isolated from mice subjected to high fat diet [12]. These two effects are in line with genotypically reduced insulin levels under both diets [12] and with the observation (made in a new H-FABP deficient mouse model) that a 50% reduction of H-FABP levels is sufficient to prevent fat-diet induced hyperglycemia [53]. These results demonstrate that H-FABP defines a pool of fatty acids that counteracts insulin action. Moreover, since in vitro, the improved insulin-dependent glucose uptakes were seen in the absence of added fatty acid, they were not caused by substrate competition, in line with current thinking on fatty acid regulation of insulin action [54]. On the other hand, we found that basal (non-insulin-dependent) glucose oxidation [5] and deoxyglucose uptake [12] were decreased by ~25-30% in isolated null soleus muscle. The net effect of the mutation, in any case, seems to be increased whole body glucose utilization. This is suggested by decreased glucose levels under resting conditions [4,12]; more directly, during exercise whole body glucose usage was increased

1.5 fold [52]. Decreased skeletal muscle [5,12] and increased cardiac [49] glycogen levels in null mice are consistent with this interpretation and in line with the known fact that cardiac and skeletal muscle glycogen contents change in a reciprocal manner [10].

Mechanisms of altered fuel metabolism

A priori, FABPs may modulate fuel metabolism through primary and secondary mechanisms. The primary effects may be classified by whether they involve interactions with other (non-FABP) proteins, and by whether they are exerted on a substrate/product or regulatory level.

First, in what can be called a "milieu function", cytosolic FABP levels as high as 1.2 mM in heart (calculated after [26]), which exceeds the aqueous solubility of LCFA, should raise the cytosolic levels and the cytosolic fraction of LCFA. Addition of FABPs to membranes indeed increases fatty acid distribution towards the aqueous phase [23,34]. Further, the equilibrium fraction of LCFA in the aqueous phase correlates with LCFA diffusion rate [34], and LCFA transport in membrane model systems is stimulated by addition of FABPs [56]. Thus, FABPs may increase the cytoplasmic levels, fractions, and diffusional mobilities of LCFA and LCFA-CoA without contacting other proteins. As a result, the availability of LCFA/LCFA-CoA as substrates and their removal (if they are products) may be facilitated by the milieu function (Fig. 2A). Deletion of these functions in FABP null tissues would be expected to reduce rates of LCFA diffusion and metabolism, which is in line with the observed reductions of LCFA oxidation, esterification, and ester hydrolysis (sections 2-5). In addition, as illustrated in Fig. 2B, the milieu function could determine the availability of FABP ligands for regulatory proteins that sense unbound fatty acid levels. In this case, the effects of FABP deletion

may be more difficult to predict, but one might surmise that impaired fatty acid sensing would trigger attempts to re-establish cellular fatty acid homeostasis.

A second type of primary FABP effects, which may be called "targeting", could derive from physical interactions between FABPs and other proteins. Again, these interactions might modulate the availability of FABP ligands for enzymatic conversion (Fig. 2A) or fatty acid sensing (Fig. 2B). The best-documented specific interaction of a non-FABP with H-FABP concerns the PPAR transcription factor family, in which transfected H-FABP was shown to activate PPAR-alpha [57] although physical contact was not demonstrated. Since PPAR-alpha is required for a high LCFA-oxidative capacity of heart [63], the activation by H-FABP would predict a reduced capacity for LCFA oxidation in H-FABP null cells. However, at least under physiological conditions that favor high rates of LCFA oxidation, PPAR-alpha-dependent transcription, or generally transcription of genes relevant for LCFA oxidation, does not appear to be altered in null hearts. This is suggested by the findings that the levels of PPAR-alpha-dependent mRNAs as well as the oxidation of albumin-bound LCFAs remained normal in homogenates of null hearts [11,4,49,42]. Furthermore, oxidation of the medium chain fatty acid octanoate (that is not bound by FABPs) remained normal in null cardiomyocytes, while it was reduced in PPAR-alpha-/- hearts [63,31,11].

In addition to the demonstrated effects on LCFA oxidation and esterification, further primary effects (via milieu and/or targeting) of H-FABP on fuel metabolism may be predicted from the literature. For example, hexokinase is inhibited by LCFA [56], glucose-6-phosphatase is inhibited by polyunsaturated fatty acids [9], and glucokinase [59] and acetyl CoA carboxylase [43] are inhibited by LCFA-CoA. Assuming that H-

FABP facilitates the availability of these lipids, we would expect in FABP null, as compared to wild type cells, increased rates of glycolysis, glycogen synthesis, lipogenesis and (in a PUFA-rich environment) gluconeogenesis. These predictions remain all to be tested.

Secondary effects of H-FABP on fuel metabolism may act in similar ways as the primary effects (notably, a secondary reduction of LCFA-CoA would affect its availability) or in qualitatively new ways. For example, LCFA oxidation is known to increase gluconeogenesis [27,62] and reduce glycolysis [47] through several mechanisms involving metabolites such as acetyl CoA, NADH, xylulose-6-phosphate, and citrate. Therefore, one can expect that the reduced LCFA oxidation increases glycolysis in FABP null muscle and heart. Further, accumulation of LCFA esters has been linked with insulin resistance in skeletal muscle [54]. Therefore, one can expect improved insulin action in FABP null tissues under high fat diet; as indeed observed in H-FABP (see section 5). In contrast, the situation is less predictable during normal diet when lipid levels are not much changed by the mutation, although insulin action was improved under this condition as well [12].

Present study of glucose metabolism in H-FABP null muscles

The present study focuses on the question of whether and how H-FABP affects skeletal muscle glucose oxidation. In freshly isolated cardiac muscle cells, genetic H-FABP deficiency has been shown to markedly decrease fatty acid oxidation, and at the same time, glucose oxidation was markedly increased [49]. Since glucose and fatty acid are, within limits, interchangeable fuels, we have interpreted the increased glucose oxidation as a compensation that avoids fuel shortage. Using isolated skeletal muscles,

we later found that just like H-FABP deficient isolated cardiomyocytes, H-FABP deficient isolated skeletal muscles show a pronounced decrease of fatty acid oxidation [5, 12]. Unlike in cardiomyocytes, however, increased glucose oxidation was not observed in H-FABP deficient skeletal muscles; in fact, glucose oxidation was even slightly reduced, regardless of whether the muscles were resting or stimulated electrically to contract [5].

OBJECTIVES

The present study was inspired by the belief that regardless of the paradoxical decrease of basal glucose oxidation in H-FABP null soleus muscles [12], fatty acids can antagonize skeletal muscle glucose oxidation in wild-type muscles under more appropriate conditions. In view of the markedly reduced plasma glucose level of H-FABP deficient mice [4, 12], we surmised that the lack of increased skeletal muscle glucose oxidation results from some systemic fuel-sparing mechanism favoring cardiac over skeletal muscle needs, rather than from the relief of intramuscular effects of fatty acids on muscle glucose oxidation. We therefore felt that in a more appropriate experimental protocol, H-FABP may still mediate local, antagonistic effects of fatty acids on muscle glucose oxidation, as required by the concept of the glucose-fatty acid cycle [47]. Thus, at the outset of this project, our central hypothesis was that because it impairs muscle fatty acid oxidation, H-FABP deficiency stimulates muscle glucose oxidation in all situations when fatty acids are normally oxidized. More specifically, we distinguish here two situations. After their uptake by muscle cells, fatty acids that are initially present in the medium are either immediately oxidized or first esterified; in the latter case, there will be a time delay that may extend beyond the actual presence of fatty acids in the medium. Accordingly, the present project studied the impact of H-FABP deficiency both on the acute effect of fatty acid on muscle glucose oxidation and the impact of H-FABP deficiency on muscle glucose oxidation in situations when intramuscular triglycerides are mobilized for oxidation. Accordingly, we can reformulate the above hypothesis for each of these situations as follows:

Hypothesis 1: The acute effect of exogenous fatty acids on muscle glucose oxidation is impaired or abolished in H-FABP deficient muscles.

To test this hypothesis, we incubated soleus muscles from wild type and H-FABP null mice in the presence and absence of 1mM palmitate, a fatty acid concentration at which hydrolysis of triglycerides is minimal. Since according to Randle [47], fatty acid oxidation impairs glycolysis and glucose uptake, we measured not only glucose oxidation, but also glycolysis, glucose uptake, and glycogen synthesis.

Hypothesis 2: H-FABP deficiency impairs the mobilization of muscle triglycerides and consequently ameliorates the inhibitory effect of endogenous fatty acids on glucose oxidation.

To test this hypothesis, we analyzed soleus muscles from wild type and H-FABP null mice that were subjected to two treatments. First, mice were fed a high fat diet (HFD) for four weeks and then their soleus muscles were isolated and assayed for glucose oxidation. Our laboratory has previously shown that HFD increases muscle triglyceride levels, and that this increase is prevented by H-FABP deficiency [12]. Thus, muscles from HFD-fed H-FABP null mice cannot mobilize as much triglyceride for oxidation as wild type muscle, regardless of any effect of H-FABP deficiency on triglyceride mobilization itself. Second, we aimed to increase triglyceride levels in vitro by incubation with 1 mM palmitate, which was followed by a measurement of glucose oxdidation in the absence of fatty acid, which maximizes triglyceride hydrolysis.

In order to assess triglyceride degradation, one muscle from each pair per animal was removed and shock frozen before and after incubation in the final step, and triglycerides were measured using an enzymatic method or a thin layer chromatography

approach (TLC). In order to demonstrate the oxidation of internal triglycerides, 3H-palmitic acid was present in the first step, non-incorporated 3H-palmitic acid was washed out in the second step, and triglyceride oxidation was measured from the release of tritiated water in the final step. In parallel, 14C-glucose was present in the final step in order to measure glucose oxidation.

MATERIALS AND METHODS

Animals

An animal use protocol (AUP) was approved by the University Laboratory Animal Care Committee (ULACC) of Texas A&M University. For anesthesia, mice were injected with 1.2% tribromoethanol (Avertin) at 20 μL/g body weight before sacrifice. All mice used in this study were male or female strain C57BL/6, between ages 3-5 months old. Mice were either null, heterozygous, or wild-type for the H-FABP gene locus in a genetically fixed background. Mice received a standard chow (no. 8604, Harlan Teklad) or a high-fat diet [no. TD 93075, Harlan Teklad; main digestibles in g/kg: 289 protein, 207 starch, 90 sucrose, 274 shortening (Primex), 16 cellulose] for ~4 wk, transported to the lab on the previous day before the experiment, and sacrificed the following morning.

Preparation of BSA with fatty acids [33]

To make a stock solution of 125 mM palmitic acid, 801mg of palmitic acid was dissolved to a final volume of 25 mL absolute ethanol, by slight warming and periodically vortexing. For the control without fatty acids, absolute ethanol was used. Into 15 mL polypropylene tubes, 4 mL 37.5 mM KCl was added for the control and 4 mL 37.5 mM KOH was added for the palmitic acid solution. Into the KCl, 0.8 mL absolute ethanol was added and into the KOH, 0.8 mL ethanol with 125 mM palmitic acid was added to make potassium palmitate (K-palmitate) and both were mixed. The ethanol was evaporated from each tube by gassing with N_2 for 30 minutes and occasionally vortexing during evaporation.

Meanwhile, 20% fatty acid-free bovine serum albumin (BSA) was prepared by dissolving 10 grams fatty acid-free BSA in distilled water to a final volume of 50 mL. After the volumes of the KCl and KOH were 4 mL each again, 4 mL of the KCl was added to 16 mL 20% BSA to make control BSA and 4 mL K-palmitate was added dropwise to 16 mL 20% BSA in a beaker under constant warming and stirring with a Teflon stirring bar to make 5 mM K-palmitate in BSA. Both the control and K-palmitate were aliquotted into 1.5 mL Epindorff tubes and stored at -20°C.

Measurement of fatty acid oxidation [12]

Incubations for each muscle or pair of muscles were performed under constant gassing with carbogen (95% O_2 and 5% CO_2) in Krebs-Henseleit-Buffer (119 mM $NaCl_2$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 25 mM $NaHCO_3$) supplemented with 5 mM glucose, 4% bovine serum albumin (fatty-acid free), 0.1 mM or 1 mM palmitic acid, and 1 uCi/mL tritiated palmitic acid as a radioactive tracer. Mice were anesthetized with avertin (2,2,2 tribromoethanol, Fluka cat. No. 90710), and solei from the hind limbs were rapidly removed and immersed into 1.5 mL of the media under constant gassing for 1 hour. After incubation, solei were blotted on paper, trimmed, weighed, and shock frozen for esterification analysis.

Oxidation was measured by output of tritiated water, the by-product of fatty acid oxidation. 1 mL of media was extracted with 5 mL of 2:1 chloroform-methanol, acidified with 2M KCl/HCl, centrifuged, and an aliquot of the upper aqueous phase was counted in Fisher Scintiverse scintillation liquid.

Example (For 3 mice and 1 blank, 1.5 mL each = 6 mL, so make 7 mL to avoid pipetting losses):

- Prewarm shaking water bath to 30°C.
- Combine 4.6 mL water, 0.7 mL 10x KHB, 0.196 mL 7.5% $NaHCO_3$, and 35 uL 1M glucose in a 15 mL polypropylene tube.
- Gas (by bubbling) with a Pasteur pipette for 15 minutes with carbogen.
- Add 70 uL 250 mM $CaCl_2$ and 1.4 mL 20% BSA with palmitic acid (see Preparation of BSA with fatty acids). Add 7 uL 3H-palmitic acid from Moravek, to make 1 uCi/mL.
- Mix well and distribute 1.5 mL aliquots of media to 20 mL scintillation vials.
- Begin gassing and shaking vials at least 10 minutes before incubation.
- Inject each mouse with avertin (0.5 mL/25 g body weight) and use 8 minute intervals between incubations. After anesthesia, rapidly remove solei from hind limbs and immerse into vial containing 1.5 mL prewarmed and pregassed solution.
- After 60 minutes incubation, remove muscles from solution, blot on paper, trim, weigh, and shock freeze for esterification analysis.
- Aliquot 5 mL of 2:1 chloroform:methanol solution into 15 mL polypropylene tubes.
- Aliquot 1 mL of blank and each sample of media in which muscles were incubated into each tube containing chloroform:methanol.
- Vortex and shake each tube on rotating shaker for 10 minutes.
- Add 2 mL 2M HCl/KCl to each tube, vortex, and shake for another 10 minutes.

- Centrifuge tubes at 3000 rpm for 5 minutes.
- Aliquot 0.5 mL of aqueous (upper) phase in duplicates into 7 mL scintillation vials and add 5 mL scintillation cocktail. Shake well and count for 5 minutes each.

Measurement of 3H-triglyceride and phospholipid content [12]

Muscles were homogenized using a Wheaton glass homogenizer in 1mL 1:1 chloroform-methanol, aliquotted into 1.5 mL tubes, and centrifuged at 2000 rpm at 4°C for 10 minutes. The supernatant was then transferred to a new tube, 0.4 mL water was added, and the tubes were shaken for 10 minutes. After centrifugation again under the same conditions, the lower organic phase was transferred with a Pasteur pipette to a new tube and evaporated with N_2 air. The remaining lipid was then redissolved in 100 μL 2:1 chloroform-methanol and 20 μL of each sample was spotted on an oven-dried silica gel plate for thin layer chromatography (TLC) analysis. The silica plates were placed in a closed container with a 60:40:3 heptane-isopropyl ether-acetic acid solution with the fluid level just below the spots, allowing bands of triglycerides and phospholipids to rise to the top. The bands were then scraped into 20mL scintillation vials and counted in 4 mL Fisher ScintiSafe scintillation liquid per vial.

Measurement of glucose uptake [12]

The same conditions as the measurement of fatty acid oxidation were used, except that three different incubation media were used in order to measure glucose uptake, in the following order:

Solution 1: Krebs-Henseleit buffer (KHB) with 8 mM glucose and 32 mM mannitol, incubated 40 minutes

Solution 2: KHB with 40 mM mannitol, incubated 10 minutes

Solution 3: KHB with 1 mM 2-deoxy-glucose, 39 mM mannitol, and 1 μCi/mL 3-H labeled 2-deoxy-glucose, incubated 20 minutes

4% fatty acid-free bovine serum albumin (BSA) was present throughout all incubations, as well as 1 mM palmitic acid, for those muscles incubated in the presence of fatty-acids. Each mouse was anesthetized with Avertin, solei were rapidly removed, one muscle was incubated in media solely containing the above reagents, and the other muscle was incubated in media containing the above reagents supplemented with either 1 mM palmitic acid, 2 mU/mL insulin (Humulin R), 2 mM AICAR, 0.5 mM 2,4-dinitrophenol (DNP), or a combination of these.

After incubation, muscles were blotted on paper, trimmed, weighed, and dissolved in 1M KOH. After neutralization with an equal volume of 1M HCl, aliquots were counted in Fisher ScintiVerse scintillation liquid to measure total deoxy-glucose uptake. Example (for 3 mice = 6 muscles, 1.5 mL per muscle, 9 mL per solution = 27 mL, 30 mL was prepared):

- Combine 19.86 mL water, 3 mL 10x KHB, and 0.84 mL 7.5% $NaHCO_3$ and mix well.
- Gas (by bubbling) with a Pasteur pipette for 15 minutes with carbogen.
- Add 0.3 mL 250 mM $CaCl_2$ and mix well.
- Split solution into 2 aliquots of 12 mL each and add 3 mL control BSA to one and 3 mL BSA with K-palmitate to the other (total 15 mL each).

- Aliquot 5 mL control solution each into 3 tubes (1a, 2a, 3a) and aliquot 5 mL K-palmitate solution each into 3 tubes (1b, 2b, 3b).
- Into tube 1a, add 40 μL 1M glucose and 0.32 mL 0.5M mannitol, and add the same to 1b.
- Into tube 2a, add 0.4 mL 0.5M mannitol, and add the same to 2b.
- Into tube 3a, add 0.39 mL 0.5M mannitol, 50 μL 0.1M 2-deoxy-glucose, 7.5 μCi (7.5 μL) 3H-2DG, and 1.5 μCi (15 μL) 14C-mannitol, and add the same to 3b.
- Mix all tubes well. Aliquot 1.5 mL each of solution 1 into a set of vials, and do the same for the other solutions. Place racks in shaking bath and gas for 10 minutes before incubation.
- Inject each mouse with avertin (0.5 mL/25 g body weight) and use 8 minute intervals between incubations. After anesthesia, rapidly remove solei from hind limbs and immerse one muscle into vial containing 1.5 mL prewarmed and pregassed fatty-acid free media, and the other muscle into media with 1 mM palmitic acid.
- Incubate all muscles in solutions 1, 2, and 3 for 40, 10, and 20 minutes, respectively.
- After incubation, remove each muscle, blot on paper, trim, weigh, and place in 1.5 mL Epindorff tube.
- Add 0.5 mL 1M KOH to each tube and dissolve muscles by heating at 80°C for 30 minutes. Vortex each tube and neutralize by adding 0.5 mL 1M KCl and mixing.

- Aliquot 0.3 mL in duplicates into 7 mL scintillation vials and count in 5 mL scintillation liquid.

Measurement of glucose utilization

The same conditions as the measurement of fatty acid oxidation and glucose uptake were used, except that the measurement of glucose utilization involved only a two-step incubation procedure, and that Krebs-Henseleit buffer (KHB) was supplemented with 10 mM HEPES acid for all incubations. Muscles were pre-incubated for 30 minutes in a wash solution containing Krebs-Henseleit buffer (KHB) with 5 mM glucose, followed by a 90-minute incubation in a second medium containing the same reagents, plus 0.5 μCi/mL 14-C glucose and 1 μCi/mL 3-H glucose. For each animal, one muscle was incubated in media containing solely the reagents just stated, and the other muscle was incubated in media containing the reagents plus either 1 mM palmitic acid, 2 mU/mL insulin, 2 mM AICAR, 0.5 mM 2,4-dinitrophenol (DNP), or a combination of these. After the muscles were placed in the incubation medium, they were gassed for 1-2 minutes and sealed with a rubber septum during the 90-minute incubation period. At the end of the incubation, the vials were placed on ice for 5 minutes, the muscles were removed and shock frozen, a tube containing 1M KOH was placed in the vial, the vial was sealed again with a rubber septum, and perchloric acid was injected into the media to liberate the 14-C CO_2. After a 90-minute collection period, aliquots of the KOH were counted for 14-C CO_2 content in Fisher ScintiVerse scintillation liquid, in order to measure glucose oxidation.

Glycolysis was measured by placing an open tube with 0.5 mL non-radioactive water in vials with metabolized media, sealing the vials, and incubating them at 37°C for

72 hours. This allowed the separation of tritiated water, the by-product of glycolysis, from tritiated glucose, as the tritiated water came into equilibrium with the non-radioactive water in the tube. Duplicate aliquots of 0.15 mL were then counted in 5 mL scintillation liquid.

Glycogen synthesis was measured by adding 0.2 mL 30% KOH to each Epindorff tube containing a muscle (~10 mg) and incubating them at 97°C for 15 minutes. Afterwards, 20 μL of glycogen (25 g/L) and 1 mL cold ethanol was added, and the samples were incubated at -20°C overnight to allow precipitation of glycogen. The samples were then centrifuged at 10,000 rcf at 4°C for 5 minutes, the supernatant was aspirated and discarded, and the pellet was washed twice by the addition of cold ethanol, centrifugation, and removal of the supernatant as just described. The pellet was then dried by heating at 97°C for 1-2 minutes, cooled, and then re-dissolved in 0.6 mL water. Aliquots of 0.25 mL in duplicates were then counted in 5 mL scintillation liquid for 14-C glycogen content.

Measurement of pyruvate dehydrogenase (PDH) activity [55]

The following solutions (stored in 1 mL aliquots at -20°C) were used in the measurement of the total (both non-phosphorylated and phosphorylated form) and actual (phosphorylated) PDH activity:

Homogenization buffer: 250 mM sucrose, 2 mM EDTA, 10 mM Tris-HCl pH 7.4, 50 U/mL Heparin; added freshly: 0.1 mM PMSF

Assay cocktail 1: 100 mM Tris, 0.5 mM EDTA, 2 mM $MgCl_2$, 0.5 mM NAD^+, 1 mM TPP, 2 mM CoA, 0.08 mM Cytochrome C, 5 mM L-carnitine pH 7.4; Added freshly: 10 μL/mL NADHCR

Assay cocktail 2: Same as Solution 1, except containing 2 mM Ca and 4 mM Mg

The following procedure was used for the assay:

- Preheat shaking water bath at 37C.
- Add 10 μL NADHCR suspension to both tubes of assay cocktail solutions.
- Add 20 μL 100 mM $CaCl_2$ and 3.33 mL 600 mM $MgCl_2$ to one of the assay cocktail solutions to make assay cocktail 2, which will be used in the measurement of the total PDH activity.
- Add 0.4 mL of each assay cocktail to 20 mL scintillation vials and place in water bath. Place 2 mL Epindorff tubes with lids cut off with 0.5 mL 1M KOH in vials.
- Add 1 μL 0.1M PMSF to homogenization buffer to make 0.1 mM PMSF.
- Weigh and grind tissues with pestle over liquid nitrogen.
- Homogenize with homogenization buffer at 20% w/v.
- Aliquot homogenized tissues into Epindorff tubes. Freeze in liquid N_2 and thaw for 3 cycles.
- For total activity, add 50 μL homogenate to assay cocktail 2 and preincubate for 10 min at 37C before starting the reaction. Meanwhile, place homogenate stock on ice and add 50 μL to assay 1 for actual activity upon starting incubation.
- Upon starting the reaction, add 50 μL radioactive pyruvate to reaction, seal with rubber septum, and incubate for 20 minutes. After 20 minutes, inject 0.2 mL 3M Perchloric acid into the assay cocktail to stop the reaction and liberate the $^{14}CO_2$.
- Shake for 90 minutes to collect $^{14}CO_2$ in the KOH and then count 0.2 mL aliquots in duplicates in 5 mL scintillation vials.

Measurement of internal triglyceride oxidation

For the measurement of internal triglyceride oxidation, the same conditions and solutions as the measurement of glucose utilization were used, except that three separate phases were used during incubation, each for 1 hour:

Step 1 (Pulse): Incuabation in KHB with 5 mM glucose, 1mM palmitic acid, and 5 μCi/mL 3-H palmitic acid in order to label internal triglycerides

Step 2 (Wash): Incubation in KHB with 5 mM glucose and 1 mM palmitic acid without radioactivity, to wash out non-incorporated labeled fatty-acids

Step 3 (Chase): Incubation in KHB with 5 mM glucose, without fatty-acids, in order to ensure that only internal triglycerides are used for fatty-acid oxidation

For each animal, a pair of muscles were incubated with a quick rinse (~30 sec) before and after each phase. The first rinse was to remove excess blood before labeling, the second rinse before the wash phase prevented excess radioactive solution from being carried over by the muscle, and the third rinse prevented excess fatty acids from being carried over into the Chase phase. From each pair, one muscle was removed before and after the Chase phase, blotted on paper, trimmed, weighed, and shock frozen in order to analyze triglyceride degradation, by means of TLC analysis using the "Measurement of radiolabeled triglyceride content" method [12]. Triglyceride oxidation was measured in the vials from the Chase phase by measuring the output of tritiated water, using the extraction method under "Measurement of fatty acid oxidation."

Measurement of total internal triglyceride content [32]

Frozen soleus muscle tissue (~10 mg) was placed in a glass homogenizer and homogenized with 0.5 mL 4:1 chloroform:methanol. The homogenate was transferred to

a round bottom 2 mL tube and shaken for 3-4 hours at 22°C. Afterwards, phases were separated by adding 0.2 mL 1M H_2SO_4 and vortexing. For the blank reaction, 0.5 mL 4:1 chloroform:methanol was simply combined with 0.2 mL 1M H_2SO_4 and processed the same way as the other samples. The tubes were then centrifuged at 1,000 rpm for 10 minutes at room temperature and the bottom organic phase (~0.2 mL) was transferred to a new tube. The organic phase was evaporated with N_2 and the remaining triglyceride was re-dissolved in 40 μL isopropanol. Triglyceride reagents from Wako Diagnostics were used for the quantification of triglyceride content. 0.26 mL of Reagent R1 was mixed with 3 μL of the sample and incubated at 37°C for 5 minutes. 0.13 mL of Reagent R2 was added and mixed with the reaction and incubated at 37°C for another 5 minutes. The reaction mixture was then transferred to a 0.6 mL ultraviolet (UV) cuvette and the absorbance was measured at OD 600nm with a reference wavelength set at 700nm. The differences in OD readings were then normalized to muscle weight.

RESULTS

External fatty acid oxidation and esterification

In order to determine the conditions of the greatest genotypic difference with respect to fatty acid oxidation, we developed a gene dosage curve of exogenous fatty acid oxidation, using null, heterozygous, and wild-type mice in each experiment, in the presence of 0.15 mM or 1.5 mM palmitic acid (Figure 1). The greatest genotypic difference was observed at 1.5 mM palmitic acid, therefore we used this concentration in all subsequent experiments. Previously, fatty acid utilization in vivo in exercising mice was assessed, and a strictly linear relationship was demonstrated among genotypes to demonstrate the gene dosage effect [52]. In other words, the heterozygous genotype (+/-) was exactly midway between knockouts and wild-types in terms of ability to oxidize fatty acids. Oxidation was also assessed by means of vesicle transport [32], and the relationship was found to be strictly qualitative (the heterozygote performs at the same level as the homozygous wild-type). We wanted to verify which model was correct by using the isolated muscle incubation method. At 0.1 mM palmitic acid, the relationship was neither strictly linear nor was the heterozygote the same as true-breeding wild-type, but the relationship indicated a "bent" curve. In other words, the presence of one gene allows performance up to 60-70%, compared with normal physiological conditions. At 1 mM palmitic acid, the relationship appeared more linear, as the heterozygote performed at 50-60% of the wild-type. With respect to triglyceride synthesis, there was no significant difference between heterozygous and wild-type muscles.

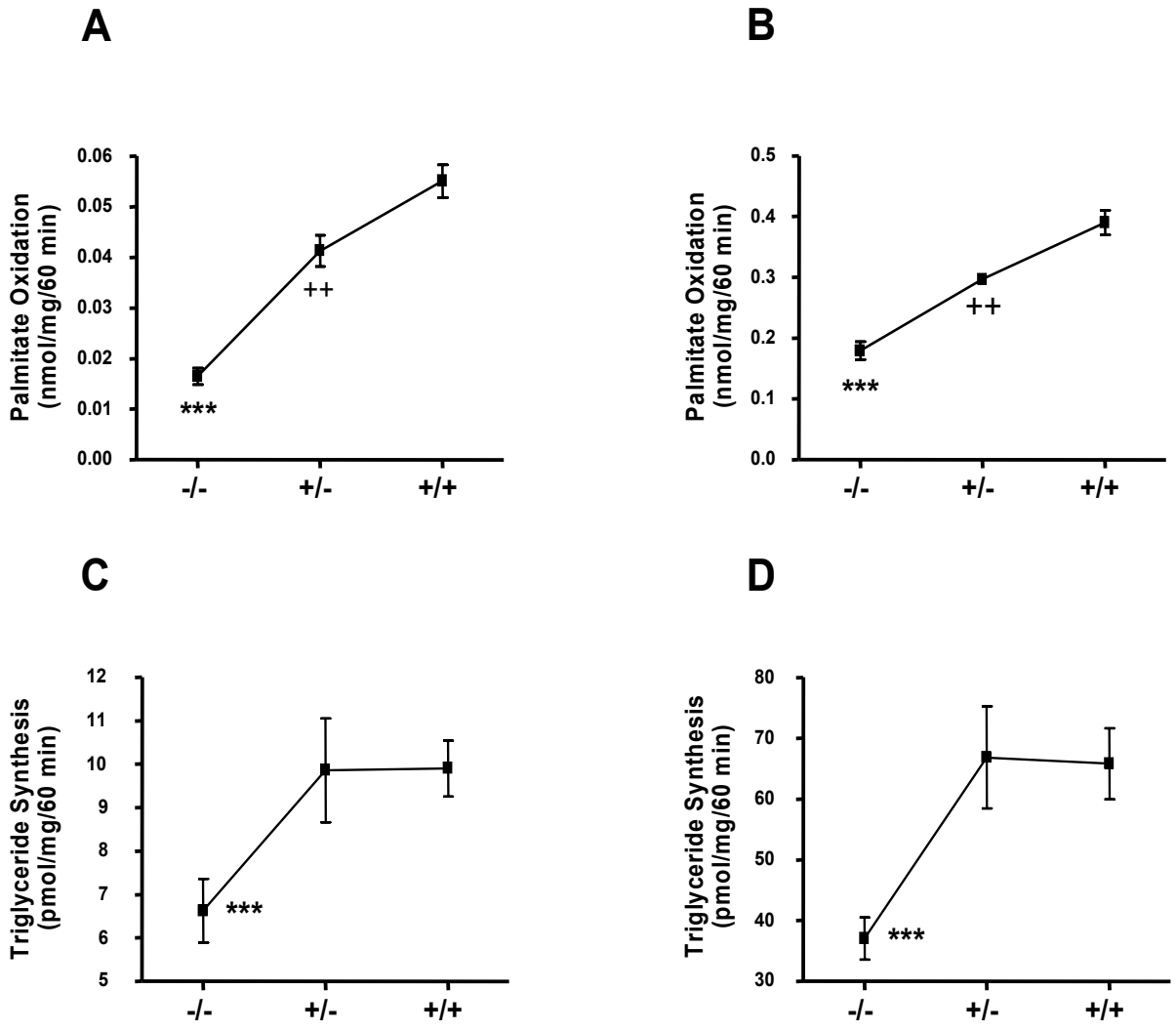

Figure 1. Palmitate oxidation. Oxidation was measured at 0.15 mM (A) and 1.5 mM (B) palmitate and triglyceride synthesis at 0.15 mM (C) and 1.5 mM (D) palmitate with H-FABP null (-/-), heterozygous (+/-) and wild-type (+/+) mice. Each point represents 5-6 muscles from male mice studied in 5-6 independent experiments. Significant differences were observed between null and wild-type (*) and between heterozygous and wild-type (+) with double symbols representing $P<0.01$ and triple symbols representing $P<0.001$.

Measurement of 2-deoxy-glucose uptake into soleus muscle

We have previously assessed glucose uptake in the absence of fatty acids [12] after standard chow and high-fat diets. As a result, we have shown that insulin-independent (basal) glucose uptake was moderately reduced in the null muscle regardless of the diet, whereas no significant genotypic difference existed in the presence of 2 mU/mL insulin after standard chow diet. In contrast, insulin-dependent glucose uptake was significantly decreased in wild-type muscles after high-fat diet (HFD) in the presence of 2mU/mL of insulin, indicating a non-acute effect of the diet, which was absent in the null phenotype. Hence, glucose uptake in the null muscle at 2 mU/mL insulin was considerably higher than wild-type after HFD.

Next, we wondered whether or not the acute presence of fatty acids would affect the genotypic pattern in the absence and presence of insulin (Figure 2). In order to determine whether or not the previously described Randle cycle applied to the situation of the null muscle, we incubated pairs of knockout and wild-type muscles from each animal, one in the absence and one in the presence of 1 mM palmitic acid. Surprisingly, in both the absence and presence of insulin, palmitic acid suppressed glucose uptake in the null muscle to the same extent as in the wild-type.

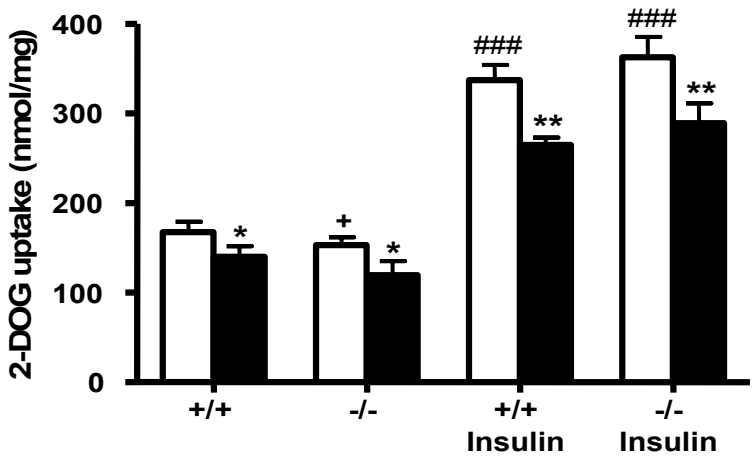

Figure 2. 2-deoxyglucose uptake. Soleus muscles are from wild-type (+/+) and null (-/-) mice without and with insulin in the absence and presence of 1 mM palmitate. Open bars represent muscles incubated without fatty acids and closed bars represent incubation in the presence of palmitate. Each column represents 5-7 muscles from female mice studied in 3-4 independent experiments. Significant differences were observed between absence and presence of palmitate (*), between genotypes (+), and between absence and presence of insulin (#) with single symbols representing $P<0.05$, double $P<0.01$, and triple $P<0.001$.

Measurement of glucose oxidation

We measured glucose oxidation in order to determine whether or not the patterns observed in glucose uptake were the same as oxidation (Figure 3). As with glucose uptake, we examined the acute effect of fatty acids, and found that palmitic acid inhibited glucose oxidation in both null and wild-type muscles to the same extent, regardless of whether or not insulin was present. We wondered whether or not the same genotypic patterns in the absence and presence of palmitate would be observed after a 4-week high-fat diet (HFD) feeding. After HFD, the genotypic pattern between wild-type and H-FABP null muscles was now completely different. Both in the absence and presence of insulin, glucose oxidation was considerably increased in the null muscle, compared to the wild-type. However, what remained similar to the observations with chow fed mice was that the acute addition of 1 mM palmitate reduced glucose oxidation in both wild-type and null muscles to a comparable degree, arguing that some non-acute effect of the diet causes the genotypic change in glucose oxidation.

In order to understand the nature of this change in genotypic pattern, we clarified whether this change was due to an increase in oxidation of the null or rather a decrease in the wild-type. In each incubation, a mouse fed standard chow was used for each genotype as a control in order to observe the changes between the diets. We observed that the high-fat diet caused a dramatic decline in glucose oxidation of the wild-type, which was prevented in the null. Since we have previously shown that HFD caused a dramatic accumulation of triglycerides in the wild-type, but not the null, we wondered whether or not internal triglycerides would play a role in counteracting glucose oxidation.

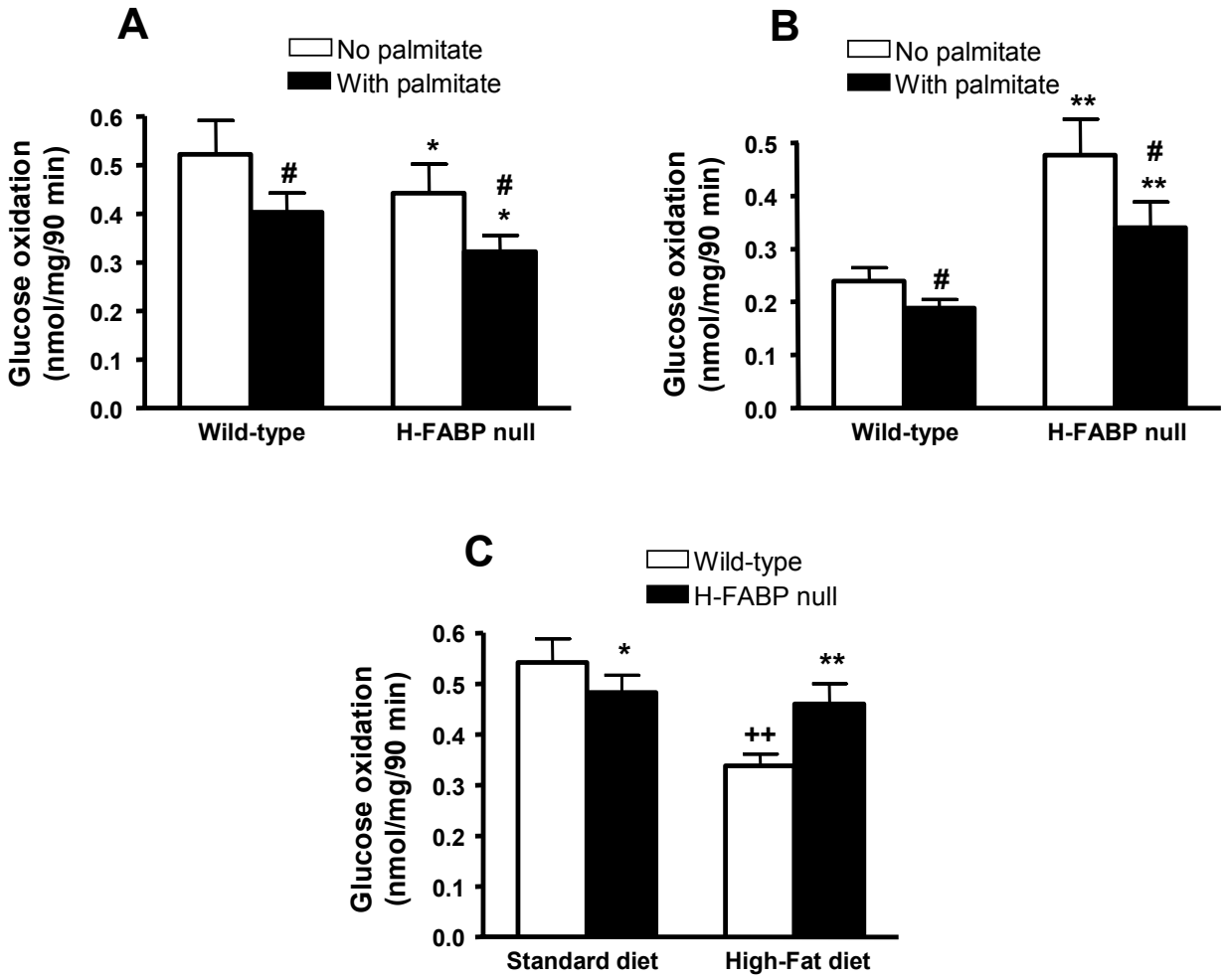

Figure 3. Glucose oxidation. The effect of H-FABP deficiency on glucose oxidation depends on diet, but not on the acute presence of fatty acids. Soleus muscles were isolated from wild-type or null mice fed standard chow (A), high-fat (B), or both in parallel (C). Muscles were incubated with or without 1 mM palmitate. Each column represents muscles from 5-10 mice studied in 3-5 independent experiments. Comparisons were made between genotypes (*), between absence or presence of palmitate (#), and between diets (+). Single symbols (*, #) indicate $P<0.05$ and double symbols (**, ++) indicate $P<0.01$.

Measurement of endogenous triglyceride mobilization

After observing the changes in glucose oxidation between standard and high-fat diets, we investigated whether or not the synthesis and degradation of intramyocellular triglycerides were altered between wild-type and null muscles (Figure 4). Previously, it has been demonstrated that no difference exists in triglyceride levels between freshly isolated knockout and wild-type muscles and that the H-FABP null muscle exhibits impaired degradation of triglycerides [5]. It has also been shown that after HFD in freshly isolated muscles, wild-type muscles have accumulated enormously large levels of triglycerides, whereas this was prevented in the knockout [12]. Therefore, after HFD, wild-type muscles had considerably higher levels of triglycerides than the null muscles. We hypothesized that this was the explanation for the altered pattern of glucose oxidation, as if wild-type muscles contain higher triglyceride levels and if those triglycerides are degraded and oxidized at a greater rate, the Randle effect would occur at a greater intensity, and would inhibit glucose oxidation in the wild-type, but not the knockout. The first method to measure triglyceride levels involved enzymatic reactions, using reagents supplied by the manufacturer to measure total triglyceride levels. The second was a Pulse-Chase method, in which 3H-palmitate was used as a radioactive tracer in order to demonstrate the synthesis and breakdown of triglycerides, after isolation from thin layer chromatography (TLC). Using both methods, we demonstrated that both triglyceride synthesis and degradation occurred rapidly in the wild-type, but were impaired in the knockout.

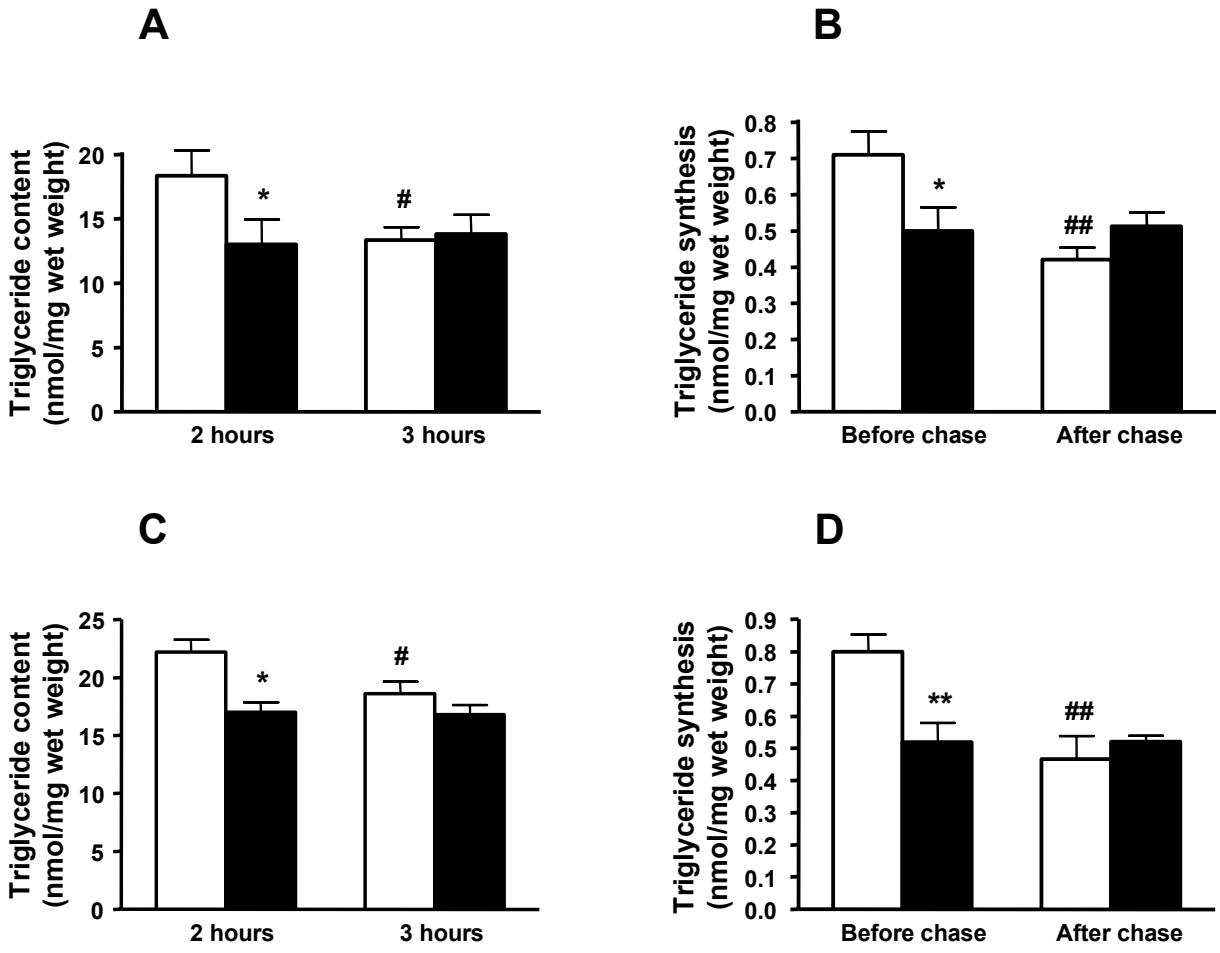

Figure 4. Triglyceride synthesis and degradation. Muscles are from mice fed standard chow (**A, B**) and high-fat diet (**C, D**), using enzymatic (**A, C**) and radioactive TLC (**B, D**) approaches. Open bars represent wild-types and closed bars represent null muscles. Each column represents muscles from 5-6 mice, males or females, studied in 3-5 independent experiments. Comparisons were made between genotypes (*) and between time intervals or phases (#) with single symbols (*, #) representing $P<0.05$ and double symbols (**, ##) representing $P<0.01$.

Reciprocal effects on triglyceride and glucose oxidation

Considering the patterns observed with triglyceride synthesis and mobilization between the genotypes, we believed that at least a part of the mobilized triglycerides were oxidized, and therefore the null muscle should exhibit reduced triglyceride oxidation, in spite of the nature of the FABP mutation. To verify this, we measured triglyceride oxidation (Figure 5) by counting the output of tritiated water (as previously described under Measurement of fatty acid oxidation in the methods [12]) in the Chase phase. As a result, triglyceride oxidation was indeed considerably reduced in the null muscle, indicating that H-FABP is also required for efficient oxidation of internal triglycerides.

Using the same setup, we wanted to clarify whether or not glucose oxidation was influenced by our conditions (Figure 5). As a result of pre-incubation for 2 hours in 1 mM palmitate, the stimulatory effect on glucose oxidation in the null muscle that was observed after high-fat diet was demonstrated again even with mice fed standard chow diet. Considering the pattern of triglyceride synthesis and mobilization noted earlier, we believe that we were able to mimic the observed effect of the high-fat diet, due to the increased synthesis, followed by the increased mobilization and oxidation. We repeated the experiments with high-fat fed mice and obtained similar results, indicating the impaired synthesis, degradation, and oxidation of triglycerides in the null muscle.

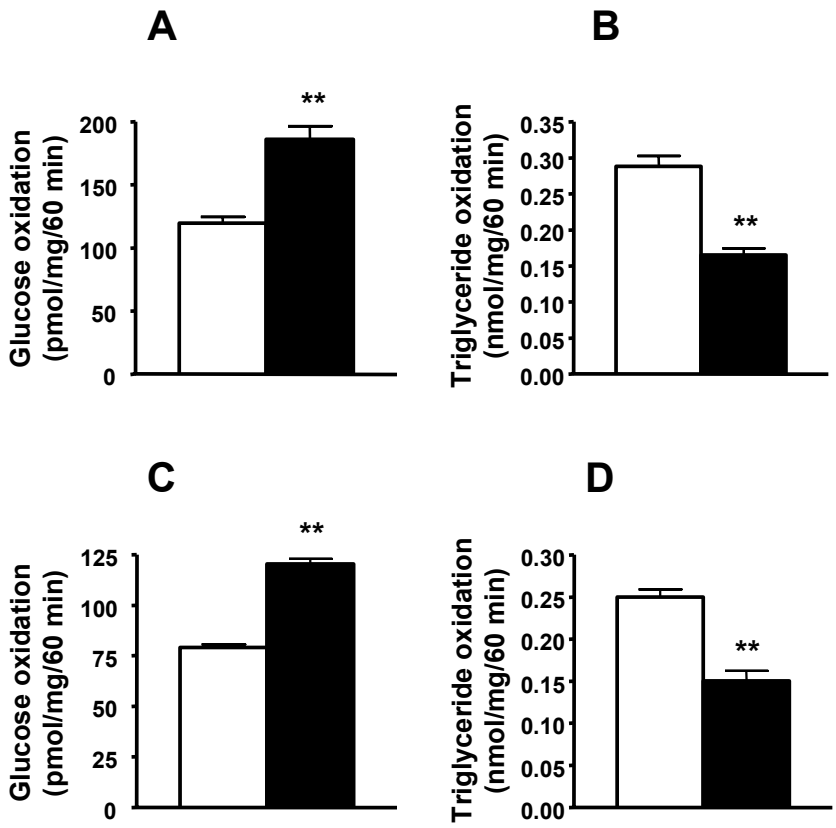

Figure 5. Triglyceride oxidation and glucose oxidation. Open bars represent wild-types and closed bars represent null muscles. Each column represents muscles from 5-6 mice, males or females, studied in 3-5 independent experiments. Comparisons were made between genotypes (**) with $P<0.01$.

Glycolysis and glycogen synthesis

In the absence of insulin, the same genotypic pattern was observed with glycolysis (Figure 6) as with glucose oxidation, as the H-FABP null phenotype exhibited a slight decrease with respect to wild-type. However, unlike oxidation, there was no significant difference between muscles incubated in the absence or in the presence of 1 mM palmitate, indicating no acute effect of fatty acids. In the presence of insulin, there was no genotypic difference in the absence of fatty acids, as was observed with uptake and oxidation. However, unlike in the basal state, we did observe a significant reduction in the presence of 1 mM palmitate, indicating that the Randle cycle was observed only in the presence of insulin.

We measured glycogen synthesis (Figure 6) by counting the total 3H and 14C content of each muscle and normalizing to wet muscle weight. We verified that insulin produces a much stronger effect on glycogen synthesis (3-4 fold) than on glycolysis or oxidation (1.5-2 fold), which was previously demonstrated in the literature. However, there was no significant difference between genotypes or between muscles incubated in the absence or presence of palmitate, indicating that the Randle cycle does not operate on glycogen synthesis.

After high-fat diet (HFD), the null phenotype had significantly higher rates of glycolysis than the wild-type, both in the absence and presence of palmitate, as observed with glucose oxidation. However, we did not observe an acute effect of palmitate in the absence or presence of insulin. The results on glycogen synthesis after HFD were similar to those obtained after standard chow, as the dramatic effect of insulin remained, yet no acute effect of palmitate was observed.

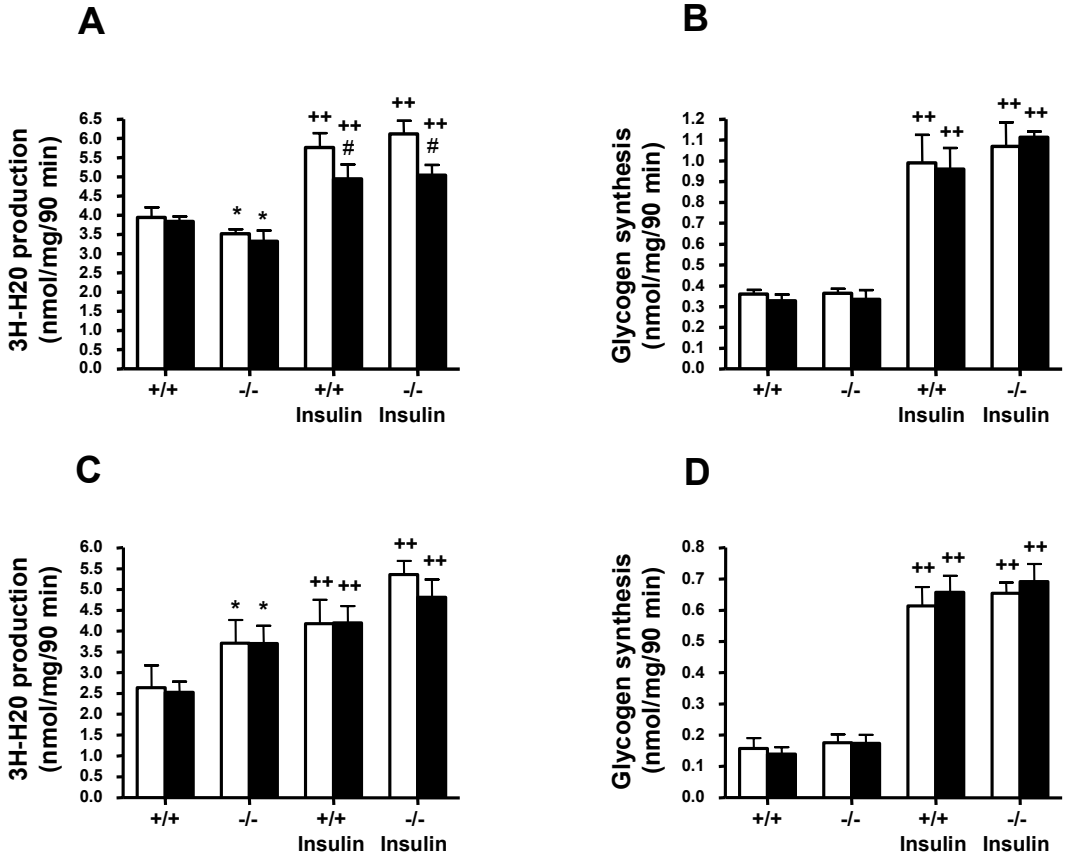

Figure 6. Glycolysis and glycogen synthesis. Results are from standard chow (**A, B**) and high-fat diets (**C, D**) using wild-type (+/+) and H-FABP null (-/-) mice. Each column represents muscles from 5-8 male mice, studied in 4-6 independent experiments. Open bars represent muscles incubated in the absence of fatty acids and closed bars represent incubation in the presence of 1 mM palmitate. Comparisons were made between genotypes (*), between absence and presence of palmitate (#), and between absence and presence of insulin (+). Single symbols (*, #) represent $P<0.05$ and double symbols (++) represent $P<0.01$.

Measurement of glucose oxidation with 2,4-dinitrophenol

In order to determine whether or not the slight reduction of basal glucose oxidation in the null phenotype was due to a reduction in the oxidative capacity of the mitochondria, 2,4-dinitrophenol (DNP), an uncoupler of the electron transport chain known to maximize glucose oxidation to its full capacity, was used (Figure 7). We verified the moderate decrease of oxidation in the null muscle, but in the presence of DNP, there was no longer any genotypic difference. Hence, we concluded that the oxidative capacity of the mitochondria is not changed due to the mutation.

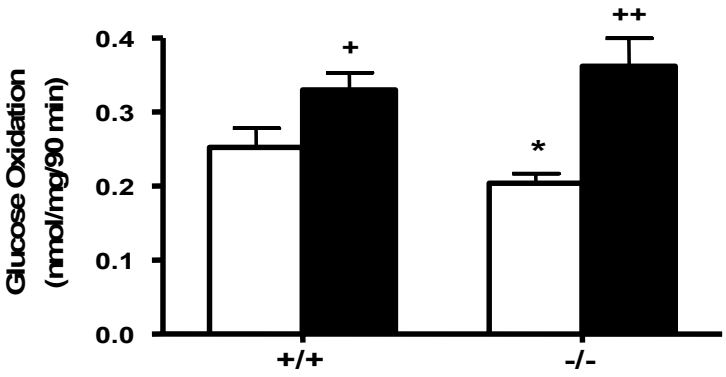

Figure 7. Glucose oxidation with 2,4-dinitrophenol. Comparison of wild-type (+/+) and H-FABP null (-/-) muscles in the absence (open bars) and presence of 2,4-dinitrophenol (closed bars). Each column represents muscles from 5-6 male mice studied in 3-5 independent experiments. Comparisons were made between genotypes (*) and between absence and presence of DNP (+), with single symbols (*, +) representing $P<0.05$ and double symbols (++) representing $P<0.01$.

Measurement of pyruvate dehydrogenase (PDH) activity

It has been previously shown that an increase in fatty acid oxidation causes a decrease in glucose oxidation [47] via the inactivation (phosphorylation) of the pyruvate dehydrogenase (PDH) complex. We did not obtain an increase in glucose oxidation after standard chow diet, but it was later obtained with high-fat diet. We therefore hypothesized that since H-FABP null muscles after HFD exhibit dramatically reduced fatty acid oxidation and dramatically increased glucose oxidation, the in vivo activity of PDH should be significantly increased in the null as well. Unexpectedly, however, we did not obtain a significant difference between the total or actual PDH activities between genotypes (Figure 8).

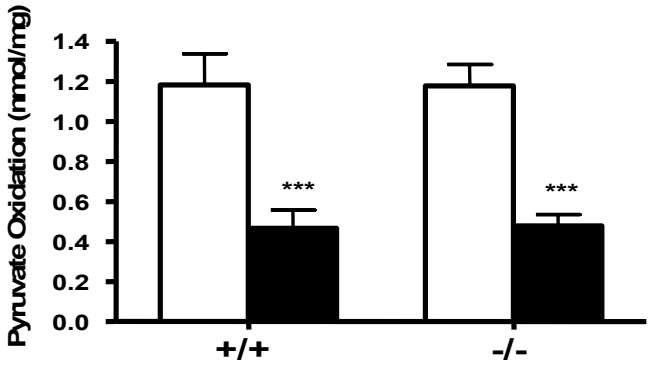

Figure 8. Total and actual PDH activity. The total (open bars) and actual (closed bars) PDH activity of wild-type (+/+) and H-FABP null (-/-) muscles. Each column represents muscles from 5 male mice studied in 5 independent experiments. Comparisons were made between total and actual activities (***) with $P<0.001$.

DISCUSSION

Our results provide us with new insights into the relationship between the regulation of fatty acid and glucose metabolism, as well as the role of H-FABP in their processes. We have demonstrated that the acute effect of 1 mM palmitic acid inhibits glucose uptake and oxidation to a comparable degree regardless of whether or not H-FABP was present. This surprising discovery contradicted our initial hypothesis because the absence of H-FABP resulted in a massive (>50%) reduction in fatty acid oxidation. According to the Randle cycle, an increase in the oxidation of free fatty acids (FFA) inhibits glucose oxidation [47], presumably via the inactivation of pyruvate dehydrogenase (PDH). Since fatty acid oxidation was significantly impaired in the null muscle, we would not have expected an inhibition of glucose oxidation to occur in the null muscle. We have measured fatty acid oxidation at low and high concentrations (0.15 and 1.5 mM palmitate) and the percent inhibition of oxidation due to the mutation was not significantly different between the two concentrations. When comparing absolute values, however, both wild-type and null muscles exhibited increased fatty acid oxidation between 0.15 and 1.5 mM palmitic acid by a comparable degree, which may provide an explanation for why the acute effect on glucose oxidation was not different. Alternatively, a threshold concentration of palmitic acid may exist, at which the addition of more fatty acids will not affect glucose oxidation beyond a certain limit. This hypothesis would require us to further examine the relationship between fatty acid concentration and glucose oxidation. The palmitate-mediated decrease of glucose oxidation may also not require the entry of palmitate into cells, as fatty acid regulation through various cell surface receptors has been demonstrated in other tissues. Since we

have also shown the acute inhibitory effect of fatty acids on glucose uptake with respect to both the wild-type and null, this mechanism is likely. Nevertheless, we have demonstrated an effect of fatty acids on glucose metabolism that appears to operate independently of the H-FABP protein.

Secondly, we have demonstrated that an increase in glucose oxidation in the null muscle is associated with the impaired synthesis and mobilization of internal triglycerides. Freshly isolated muscles from wild-type and null mice fed standard chow diet have been shown not to be significantly different with respect to triglyceride levels [5], yet impaired degradation in the null has been demonstrated during incubation at 0.5 mM palmitic acid, a concentration known to allow triglyceride degradation. Therefore, if triglyceride mobilization and oxidation is higher in wild-type muscles even after standard chow diet, one may ask why the Randle effect on glucose oxidation did not cause the switch in pattern between null and wild-type with respect to glucose oxidation in this situation. We believe that a certain threshold amount of triglycerides is required to vastly suppress glucose oxidation in the wild-type, after its subsequent degradation and oxidation. If there is a direct relationship between triglyceride content and rate of mobilization, then we can justify that the higher the triglyceride contect, the greater the rate of subsequent degradation and oxidation, and hence the greater the impact will be on glucose oxidation. Furthermore, our measurements of total triglyceride levels before and after incubation without fatty acids are in excellent agreement with the rates of synthesis and breakdown, according to the radioactive tracer measurements. Our results suggest that the inhibition of glucose oxidation through increased triglyceride oxidation is the principle mechanism of fatty acid regulation on glucose metabolism.

We have also observed how the long-term (4 weeks) effects of administering a high-fat diet can be mimicked within such a short period of time (2 hours) after incubation in a high concentration of fatty acids. If glucose metabolism is rapidly responsive to triglyceride levels, mobilization, and oxidation, we may have been able to obtain similar results after the administration of the high-fat diet for no more than a week, as plasma fatty acid concentrations are known to rise to high-fat steady state levels within a few days. Furthermore, we have observed that isolated muscles from high-fat mice exhibited similar patterns in triglyceride accumulation, mobilization, and oxidation, compared to muscles isolated from mice fed standard chow. We have also shown that the acute presence of palmitate suppresses glucose oxidation equally in both wild-type and null muscles, similar to the pattern observed after standard chow diet. This indicates that the principal relationship between fatty acid and glucose metabolism remains the same, regardless of the diet.

Finally, the present study confirms our older result that unlike in heart where H-FABP deficiency causes a marked increase in basal glucose oxidation, there is a moderate decrease of basal muscle glucose oxidation due to H-FABP deficiency after chow feeding. It will require further experiments to determine the conditions that are optimal for either the inhibitory or the stimulatory effect of H-FABP on glucose oxidation and whether these effects can be completely dissociated. We hypothesize that under conditions of high fuel competition and low triglyceride levels, the inhibitory effect of H-FABP deficiency on muscle glucose oxidation prevails, while at nutritional abundance and high triglyceride levels, the stimulatory effect prevails. In future experiments, we will need to determine how to vary the levels of fuel competition and triglycerides in

order to test our hypothesis. We have also previously demonstrated that the stimulatory effect of H-FABP on glucose metabolism after high-fat diet is observed with glucose oxidation as well as glycolysis, but not glycogen synthesis. Nevertheless, the total and actual pyruvate dehydrogenase (PDH) activity was not changed (Adhikari and Binas, unpubl. results). This provides the suggestion that the mechanism of suppression acts on glucose oxidation, and that the result is not simply due to increased or decreased glucose uptake, as glycogen synthesis was not altered by the diet.

CONCLUSION

In summary, it is now clear that H-FABP deficiency affects muscle glucose oxidation both by reducing basal glucose oxidation and by increasing it (present study), with the balance depending on physiological circumstances. Specifically, our results allow the hypothesis that H-FABP dependent muscle triglyceride mobilization, perhaps the oxidation of triglyceride-derived fatty acids, is a main factor counteracting muscle glucose oxidation. The same H-FABP dependent relationship between glucose oxidation and triglyceride mobilization appears to be maintained under a chronic high-fat diet and thus contributes to the metabolic pattern that characterizes the pathogenesis of obesity and insulin resistance. Finally, the present study also showed that the acute inhibitory effect of exogenous palmitate on skeletal muscle glucose oxidation is not disturbed by H-FABP deficiency and hence fatty acid-dependent mechanisms exist that regulate muscle glucose oxidation without the involvement of H-FABP.

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