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A null mutation in H-FABP only partially inhibits skeletal muscle fatty acid metabolism

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Binas, Bert, Xiao-Xia Han, Erdal Erol, Joost J. F. P. Luiken, Jan F. C. Glatz, David J. Dyck, Rafat Motazavi, Peter J. Adihetty, David A. Hood, and Arend Bonen. A null mutation in H-FABP only partially inhibits skeletal muscle fatty acid metabolism. *Am J Physiol Endocrinol Metab* 285: E481–E489, 2003; 10.1152/ajpendo.00060.2003.—The low-molecular-mass, cytosolic heart-type fatty acid-binding protein (H-FABP) is thought to be required for shuttling FA through the cytosol. Therefore, we examined the effects of an H-FABP-null mutation on FA and carbohydrate metabolism in isolated soleus muscle at rest and during a period of increased metabolic demand (30-min contraction). There were lower concentrations of creatine phosphate (−41%), ATP (−22%), glycogen (−34%), and lactate (−31%) ($P < 0.05$) in H-FABP-null soleus muscles, but no differences in citrate synthase and β-3-hydroxyacyl-CoA dehydrogenase activities or in the intramuscular triacylglycerol (TAG) depots. There was a 43% increase in subsarcolemmal mitochondria in H-FABP-null solei. FA transport was reduced by 30% despite normal content of sarcolemmal long-chain fatty acid transporters fatty acid translocase/CD36 and plasma membrane-associated FABP transport proteins. Compared with wild-type soleus muscles, the H-FABP-null muscles at rest hydrolyzed less TAG (−22%), esterified less TAG (−49%), and oxidized less palmitate (−71%). The H-FABP-null soleus muscles retained a substantial capacity to increase FA metabolism during contraction (TAG esterification by +72%, CO_2 production by +120%), although these rates remained lower (TAG esterification −26% and CO_2 production −64%) than in contracting wild-type soleus muscles. Glycogen utilization during 30 min of contraction did not differ, whereas glucose oxidation was lower at rest (−24%) and during contraction (−32%) in H-FABP-null solei. Although these studies demonstrate that the absence of H-FABP alters rates of FA metabolism, it is also apparent that glucose oxidation is downregulated. The substantial increase in FA metabolism in contracting H-FABP-null muscle may indicate that other FABPs are also present, a possibility that we were not able to completely eliminate.

palmitate; esterification; oxidation; soleus; glucose

LONG-CHAIN FATTY ACIDS (LCFAs) are an important substrate for many tissues. LCFAs *1*) provide ATP via mitochondrial β-oxidation, *2*) serve as precursors for lipid-signaling molecules, *3*) can act as ligands for transcription factors that control cellular metabolic gene expression, *4*) provide precursors for the biosynthesis of complex membrane lipids, and *5*) are stored as triacylglycerols in the cytosol (29). Blood-borne and interstitial LCFAs, bound to albumin at multiple high-affinity binding sites, are delivered to parenchymal cells, where they are taken across the plasma membrane via protein-mediated mechanisms and passive diffusion (1, 14, 29). Because of their poor solubility in the cytosol, LCFAs are bound to small (14- to 15-kDa) cytosolic fatty acid-binding proteins (FABPs), which belong to a family of ≥13 different intracellular lipid-binding proteins (15, 16). Among these is the heart (H)-type FABP (H-FABP), which is abundantly present in heart and skeletal muscle (4, 27); other FABPs are expressed in liver, white adipose tissue, and intestine (15, 16).

It has generally been thought that H-FABP is essential for the binding of LCFAs in the cytosol and their subsequent trafficking to the appropriate destinations. Transfection studies with liver and intestinal FABPs have provided support for the LCFA transport within the cell. But in cells such as heart and skeletal muscle, in which LCFA fluxes can be rapidly altered by contractile activity, a role for H-FABP has until recently been based on correlative data. Several years ago, Binas et al. (2) created mice with an H-FABP-null mutation. These mice displayed increased levels of circulating LCFAs, either when they consumed a mixed laboratory or a ketogenic diet, or when they had been fasted (2). In a number of tissues, the uptake of the LCFA analog ^{125}I-BMIPP was reduced, whereas in the heart, but not muscle, 2-deoxy-D-glucose uptake was increased (2). Collectively, these data suggested a reduced utilization of LCFAs by H-FABP-null mice and increased utilization of glucose in the heart.

More recently, Schaap et al. (27) compared cardiac fatty acid metabolism in wild-type and H-FABP-null

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mice. Their studies demonstrated that initial rates of palmitate uptake by cardiac myocytes from H-FABP-null mice were reduced by 45%, whereas the uptake of octanoate, which is not bound by H-FABP, was not affected. Palmitate oxidation in quiescent cardiac myocytes from H-FABP-null mice was also reduced by 45% (27), but rates of palmitate esterification were not examined. When the metabolic rate of cardiac myocytes was increased by electrical stimulation, the rate of palmitate oxidation was not increased in the cardiac myocytes of the H-FABP-null mice, whereas it was increased twofold in cardiac myocytes of the wild-type mice (27). These results, and others, clearly established a key metabolic role for H-FABP in the heart.

A definitive role for H-FABP in skeletal muscle has not yet been firmly established. We (4) have previously shown that H-FABP is present in proportion to the LCFA uptake and oxidative capacities of different types of skeletal muscle. However, for unknown reasons, H-FABP is present in great excess in skeletal muscle (4). It appears that skeletal muscle and heart may be affected differently by the null mutation of H-FABP. For example, the uptake of the LCFA analog ^{125}I-BMIPP was reduced much more in the heart than in skeletal muscle, whereas, in contrast, 2-deoxy-D-glucose uptake was markedly increased in heart but was not altered in muscle (2). The exercise capacity (swimming) of H-FABP-null mice was also markedly reduced compared with wild-type mice (2). Thus, although the importance of H-FABP in cardiac LCFA metabolism has been established (27), the effects of the H-FABP-null mutation on skeletal muscle fatty acid metabolism remain to be established. Therefore, in the present studies, we have examined the capacities for LCFA transport and LCFA metabolism in skeletal muscles of wild-type and H-FABP-null mice at rest and during a metabolic challenge (muscle contraction). Except for the mitochondrial and fatty acid transport studies, in which we needed to pool mouse muscles to obtain sufficient quantities of material, all other experiments were performed in isolated soleus muscles, a highly oxidative muscle with a well-known capacity for LCFA metabolism.

METHODS

Mice with a targeted deletion of the entire H-FABP locus were created previously (2). These mice reproduce normally and exhibit no evident abnormalities. Breeding colonies for these mice are maintained at the University of Waterloo and Texas A&M University. Adult H-FABP$^{-/-}$ and the H-FABP$^{+/+}$ control mice (~6 mo of age) from these colonies were used in the present studies. The animals were housed in a controlled environment on a reversed 12:12-h light-dark cycle and fed chow ad libitum. Ethical approval for the present study was obtained from the Animal Ethics Committees at Texas A&M University, the University of Waterloo, and York University. In all experiments, animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (100 mg/100 g body mass) before muscle tissue was obtained. Once the muscle tissue had been obtained, the animals were euthanized with a second intraperitoneal injection of pentobarbital sodium (100 mg/100 g body mass).

PCR genotyping was performed with primers that reliably reveal all genotypes in a single-tube reaction. Approximately 430 bp of the wild-type allele are amplified by primers HTW-A (5′ AGC CGA GTG GAC AGG CTC AAG G 3′, 3′ end of the wild-type allele) and HTW-B (5′ CAA GGC CAC GTC ACA G 3′, 3′ flank of both alleles). Approximately 260 bp of the knockout allele are amplified by primers 703TS (5′ TAA AGC GCA TGC TCC AGA CTG CC 3′, HPTR minigene) and HTW-B. Amplifications were performed with an annealing temperature of 68°C.

Metabolic Characterization of Soleus Muscles

Soleus muscles of wild-type and H-FABP-null mice were analyzed for creatine phosphate (CP) and ATP by use of ion pair reverse-phase high-performance liquid chromatography (19). Muscle glycogen (17), lactate (13), and triacylglycerol (TAG) concentrations (31) and the activities of citrate synthase (CS) and β-3-hydroxyacyl-CoA dehydrogenase (β-HAD) (21) were determined using standard procedures.

Mitochondrial Isolation

Intermyofibrillar (IMF) and subsarcolemmal (SS) mitochondria were isolated by differential centrifugation, as described previously in detail (8, 33). Mitochondria were resuspended in 10 mM HEPES (pH 7.4), 0.25 M sucrose, 2.5 mM potassium phosphate dibasic, 10 mM succinate, 0.21 mM ADP, and 1 mM dithiothreitol, and protein concentrations were measured (5).

Fatty Acid Transport into Giant Sarcolemmal Vesicles

Giant vesicles from hindlimb skeletal muscles were isolated as previously described (3, 4, 22, 24). Briefly, hindlimb muscles were cut into thin strips (1–3 mm thick) and incubated for 1 h at 34°C in 140 mM KCl and 10 mM MOPS (pH 7.4), aprotinin (10 mg/ml), and collagenase (type VII; 150 U/ml) in a shaking water bath. At the end of the incubation, the supernatant was collected, and the remaining tissue was washed with KCl-MOPS (10 mM EDTA), which resulted in a second supernatant. Both supernatant fractions were pooled, and Percoll and aprotinin were added to final concentrations of 16% (vol/vol) and 10 mg/ml, respectively. The resulting suspension was placed at the bottom of a density gradient consisting of a 3-ml middle layer of 4% Nycodenz (wt/vol) and a 1-ml KCl-MOPS upper layer. This sample was centrifuged at 60 *g* for 45 min at room temperature. Subsequently, the vesicles were harvested from the interface of the upper and middle layers, diluted in KCl-MOPS, and recentrifuged at 900 *g* for 10 min. The resulting pellet was resuspended in KCl-MOPS to a final protein concentration of 2–3 mg/ml.

Palmitate uptake studies were performed as previously described (3, 4, 22, 24). Briefly, 40 μl of 0.1% BSA in KCl-MOPS containing unlabeled (15 mM) and radiolabeled 0.3 mCi [^{3}H]palmitate and 0.06 mCi [^{14}C]mannitol were added to 40 μl of vesicle suspension. The incubation was carried out for 15 s. Palmitate uptake was terminated by the addition of 1.4 ml of ice-cold KCl-MOPS (2.5 mM $HgCl_2$ and 0.1% BSA). The sample was then quickly centrifuged at 12,000 rpm for 1 min. The supernatant was discarded, and the radioactivity was determined in the tip of the tube. Nonspecific uptake was measured by adding the stop solution and then the radiolabeled palmitate solution.

Western Blotting

The protein content of LCFA transporters fatty acid translocase (FAT/CD36) and plasma membrane-associated bind-

ing protein (FABPpm) was determined in both homogenates and plasma membranes of the giant vesicles by use of Western blotting, as we have previously described (3, 4, 22, 24). For detection of FAT/CD36 and FABPpm, we used an antibody directed against CD36 in mice (25) (gift from Dr. N. N. Tandon, Otsuka America Pharmaceutical, Rockville, MD) and a rabbit polyclonal anti-FABPpm antiserum (6) (gift from Dr. Calles-Escandon, Wake Forest University, Winston-Salem, NC). The content of H-FABP was determined in soleus muscles by using Western blotting with a monoclonal anti-human H-FABP antibody, as previously reported (28).

LCFA Metabolism in Isolated Soleus Muscles

LCFA metabolism was determined in intact isolated soleus muscles (7.5 ± 0.2 mg) obtained from mice, with procedures previously described (9, 11, 12, 20, 32). Briefly, soleus muscles were removed from mice anesthetized with pentobarbital sodium (100 mg/100 g body mass). Immediately after the muscle tissue was obtained, the mice were euthanized with a second intraperitoneal injection of pentobarbital sodium (100 mg/100 g body mass). Isolated soleus muscles were tied at the tendons and placed in a 2-ml glass incubation reservoir (Radnoti Glass Technology, Monrovia, CA). The lower tendon was secured by passing it through a two-way stopcock and closing the valve. The stopcock also permitted drainage of the incubation medium. The upper tendon was attached to a force transducer. The muscles were preincubated for 20 min, followed by a 30-min incubation period. For the resting conditions, muscle remained suspended at resting length, while for the contraction experiments muscles were stimulated with 150-ms trains comprised of 0.1-ms impulses (20–40 V; 60 Hz) at 20 tetani/min. The incubation medium contained 0.5 mM palmitate and 5 mM glucose and 2 μCi of [1-^{14}C]palmitate or 0.5 μCi of D-[U-^{14}C]glucose (Amersham Life Science, Oakville, Ontario, Canada) to monitor LCFA or glucose oxidation as well as LCFA incorporation into the intramuscular lipids. Oxidation of palmitate and glucose was determined by acidifying 0.5 ml of incubation buffer with 1.0 ml of 1 M H_2SO_4 in a sealed glass vial holding a suspended center well containing benzethonium hydroxide. Center wells were placed in scintillation vials, and radioactivity was counted using standard liquid scintillation techniques.

To determine radiolabeled palmitate incorporation into intramuscular lipid pools, previously published procedures were employed (9-12). For these purposes, soleus muscles were blotted, weighed, and ultrathuraxed (Polytron homogenizer) in 1.5 ml of ice-cold 1:2 chloroform-methanol solution. Connective tissue was blotted, weighed, and subtracted from the muscle wet weight. Samples were then spotted on silica gel plates (Silica Gel GF 250 mm, Analtech, Newark, DE) and resolved in solvent (60:40:4, heptane-isopropyl ether-acetic acid) for 45 min. Plates were air-dried, sprayed with dichlorofluorescein dye (0.02% wt/vol in ethanol), and visualized under long-wave ultraviolet light. Individual lipid bands were marked on the plate with a scalpel and scraped into vials for liquid scintillation counting.

Radiochromatography of Soleus Muscles Incubated with Fatty Acid

All procedures were done on ice or at 4°C. Both solei from two freshly killed mice of the same genotype were washed with PBS (20 mM K-phosphate pH 7.3, 150 mM NaCl, and 0.02% sodium azide) and then homogenized in 0.3 ml of PBS and 1 mM phenylmethanesulfonyl fluoride by 30 strokes with a Dounce homogenizer. A second homogenate from mice of the alternative genotype was prepared immediately thereafter. The homogenates were cleared by centrifugation in an Eppendorf microfuge (first for 10 min at 2,000 rpm, and second for 20 min at 14,000 rpm) and then in a Sorvall RC M120 microultracentrifuge (90 min at 105,000 *g*). Protein concentrations of the final supernatant fractions were determined using a bicinchoninic acid kit (BCA; Pierce) and equalized by adding PBS to the more concentrated sample. Portions of the supernatant (0.25 ml, containing 0.6 and 0.7 mg protein) were mixed with 2.5 μCi (30–60 Ci/mmol) of [^{3}H]palmitic acid in ethanol (final ethanol concentration was 0.8%, vol/vol). After incubation for 15 min at room temperature, each mix was passed over a Superdex 75 HR10/30 column (Amersham-Pharmacia) run with PBS at 1 ml/min, and 1-ml fractions were collected. In each fraction, absorbance (280 nm) and radioactivity content (cpm/100 μl) were determined.

Data Analyses

The data were analyzed using analysis of variance (ANOVA) procedures, and a Fisher protected least significant difference post hoc test was used to test significant differences revealed by the ANOVA. Significance was accepted at $P < 0.05$. All data are reported as means ± SE.

RESULTS

We confirmed that H-FABP was expressed in the wild-type mice but not in the skeletal muscle of the H-FABP-null mice (Fig. 1). Lower concentrations of CP (−41%), ATP (−22%), glycogen (−34%), and lactate (−31%) ($P < 0.05$, Table 1) were observed in the soleus muscle of the H-FABP-null mice. No differences were observed in CS and β-HAD activities ($P > 0.05$, Table 1) nor in the intramuscular TAG depots ($P < 0.05$, Table 1) in wild-type and H-FABP-null muscles.

Fatty Acid Transport and Metabolism

Fatty acid transport. The total content of fatty acid transporters FAT/CD36 and FABPpm and their location at the plasma membrane did not differ in muscles of wild-type and H-FABP-null mice ($P > 0.05$, Fig. 2*A*). On the other hand, palmitate transport into giant vesicles was reduced 30% in the H-FABP-null mice ($P < 0.05$, Fig. 2*B*).

TAG hydrolysis. Soleus muscle TAG concentrations were reduced during the incubations in resting mus-

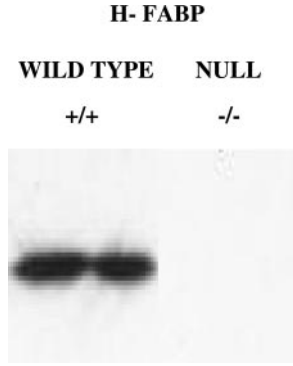

Fig. 1. Western blots of skeletal muscle demonstrating heart-type fatty acid-binding protein (H-FABP) in wild-type mice and its absence in H-FABP-null mice.

Table 1. *Concentrations of ATP, CP, TAG, and glycogen, and activities of CS and β-HAD in soleus muscles of wild-type and H-FABP-null mice*

Group	Concentration, μmol/g dry wt					Enzyme Activity, $\mu mol \cdot g^{-1} \cdot h^{-1}$	
	CP	ATP	TAG	Glycogen	Lactate	CS	β-HAD
H-FABP$^{+/+}$	30.5 ± 5.7	15.4 ± 1.1	9.6 ± 0.9	61.8 ± 5.7	20.7 ± 1.4	3.6 ± 0.1	4.2 ± 0.2
H-FABP$^{-/-}$	18.2* ± 2.7	12.0* ± 0.9	11.0 ± 1.6	41.0* ± 7.2	14.2* ± 2.5	3.6 ± 0.1	4.2 ± 0.3

Values are means ± SE; n = 6–9 muscles in each group. CP, creatine phosphate; TAG, triacylglycerol; CS, citrate synthase; β-HAD, β-3-hydroxyacyl-CoA dehydrogenase; H-FABP, heart-type fatty acid-binding protein. *$P < 0.05$, H-FABP$^{-/-}$ vs. H-FABP$^{+/+}$.

cles of wild-type and H-FABP-null mice ($P < 0.05$, Fig. 3). With muscle contraction there was a further decrease in intramuscular TAG content in the wild-type (−50%) and H-FABP-null mice (−27%) ($P < 0.05$, Fig. 3). Despite similar TAG concentrations in freshly obtained soleus muscles of wild-type and H-FABP-null mice, the H-FABP-null mice hydrolyzed less TAG in the incubated resting and contracting soleus muscles than in the soleus muscles of wild-type mice ($P < 0.05$, Fig. 3).

Palmitate metabolism. At rest, the total palmitate uptake (sum of CO_2 production + TAG esterification + phospholipid synthesis) by isolated soleus muscle was reduced (−56%) in H-FABP-null mice ($P < 0.05$, Fig. 4*A*). This was attributable to lowered rates of TAG esterification (−49%, $P < 0.05$, Fig. 4*B*), CO_2 production (−71%, $P < 0.05$, Fig. 4*C*), and fatty acid incorporation into phospholipids (−30%, $P < 0.05$, Fig. 4*D*).

Muscle contraction was used to increase the metabolic rate in soleus muscle. In wild-type muscle, total palmitate uptake was increased (+45%, $P < 0.05$, Fig. 4*A*) due to increases in TAG esterification (+20%, $P < 0.05$, Fig. 4*B*), CO_2 production (+77%, $P < 0.05$, Fig. 4*C*), and fatty acid incorporation into phospholipids (+19%, $P < 0.05$, Fig. 4*D*).

Despite the absence of H-FABP, the soleus muscles retained a substantial capacity to increase palmitate metabolism during the contraction experiments. Compared with the resting solei of H-FABP-null mice, large increases in total palmitate uptake (+85%), TAG esterification (+72%), CO_2 production (+120%), and incorporation into phospholipids (+67%) ($P < 0.05$) occurred in the contracting H-FABP-null solei (Fig. 4). However, the rates of total palmitate uptake (−44%), TAG esterification (−26%), and CO_2 production (−64%) were lower in contracting H-FABP-null solei compared with the rates observed in contracting wild-type solei. No differences in palmitate incorporation into phospholipids were observed in the contracting soleus muscles from the two groups of mice ($P > 0.05$). Interestingly, TAG esterification rates and palmitate oxidation rates were only 12 and 34% lower, respectively, in contracting muscle of H-FABP-null mice compared with the resting muscles of the wild-type mice.

Compensatory Responses in H-FABP-Null Muscles

To examine what, if any, compensation occurred in the H-FABP-null mice, we examined whether palmitate binding in the cytosol was altered and whether the mitochondrial protein content in mouse hindlimb muscles was altered.

Palmitate binding in the cytosol. Soleus muscle homogenates from wild-type and H-FABP-null mice were incubated with [^{3}H]palmitate and size-fractionated. The absorbance patterns were identical in extracts of

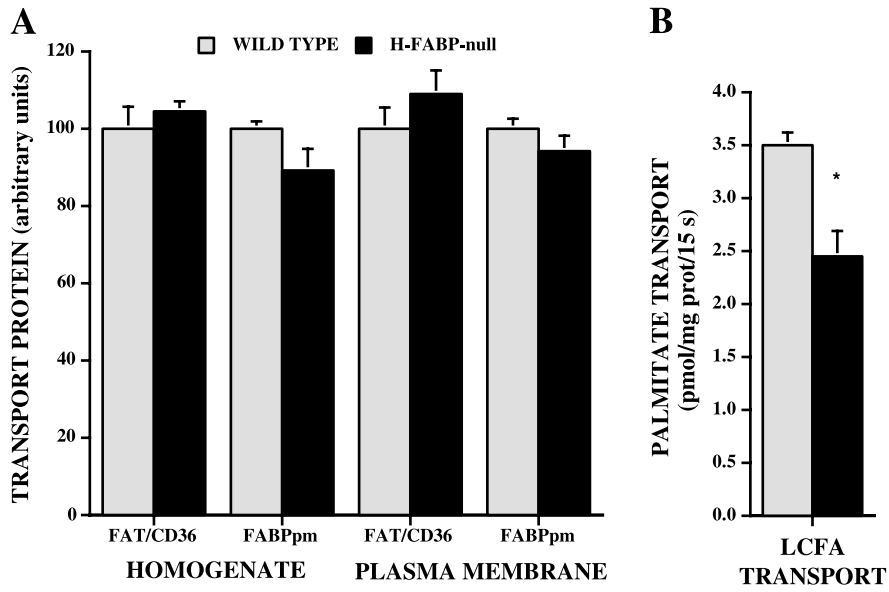

Fig. 2. Fatty acid transport proteins in muscle homogenates and plasma membranes (*A*) and palmitate transport into giant sarcolemmal vesicles in hindlimb muscles of wild-type and H-FABP-null mice (*B*). Fatty acid transporter values (means ± SE, *A*) are shown in 5–10 sets of hindlimb muscle preparations. Fatty acid transport values (means ± SE, *B*) are shown in 14 wild-type and 20 H-FABP-null mice. *$P < 0.05$, H-FABP-null vs. wild-type mice.

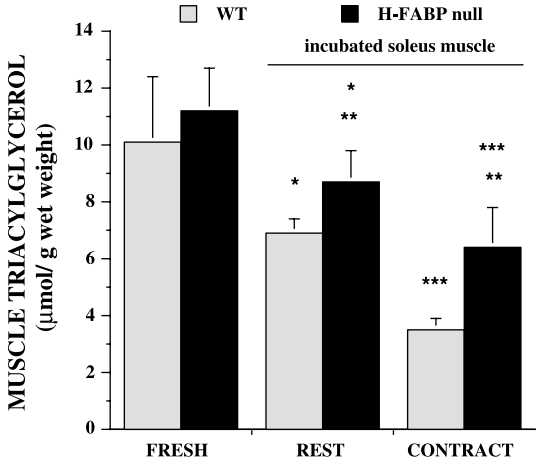

Fig. 3. Soleus muscle triacylglycerol concentrations in freshly excised muscle (fresh) and at the end of the incubation period in resting (rest) and contracting (contract) muscles in wild-type (WT) and H-FABP-null mice. Values are means ± SE; n = 6–11 soleus muscles. *$P < 0.05$, incubated resting muscle vs. fresh muscle; **$P < 0.05$, H-FABP-null vs. wild-type mice; ***$P < 0.05$, contraction vs. rest.

the two genotypes (Fig. 5*A*), but the the radioactivity profiles were different (Fig. 5*B*). Specifically, the peak (P2) corresponding to the known size of H-FABP was missing in the H-FABP knockout muscle extract (Fig. 5*B*), whereas no change was observed in the two flanking peaks, P1 and P3. Of these, only the larger peak (P1) corresponds to protein, whereas the smaller peak (P3) is also seen after chromatography of tracer alone (data not shown), and thus P3 corresponds to unbound [^{3}H]palmitate. The size of P3 relative to the other peaks increased when higher amounts of radioactivity were added (data not shown), indicating that [^{3}H]palmitic acid was still present in excess under the conditions used.

Mitochondrial protein. IMF and SS mitochondria were isolated from mouse hindlimb muscles. Mitochondrial IMF protein content did not differ between the two groups of mice (Fig. 6). In contrast, the SS mitochondrial protein was increased 43% in the H-FABP-null mice ($P < 0.05$, Fig. 6).

Carbohydrate Metabolism: Glucose Oxidation and Glycogen Depletion

We also examined whether changes in fatty acid metabolism in H-FABP-null muscles resulted in changes in carbohydrate metabolism. Glucose oxidation was examined in resting and contracting soleus muscles. In H-FABP-null mice, glucose oxidation was lower at rest and during contraction than in the wild-type mice (Fig. 7*A*). Although glycogen concentrations at rest were lower in the H-FABP-null mice, the glyco-

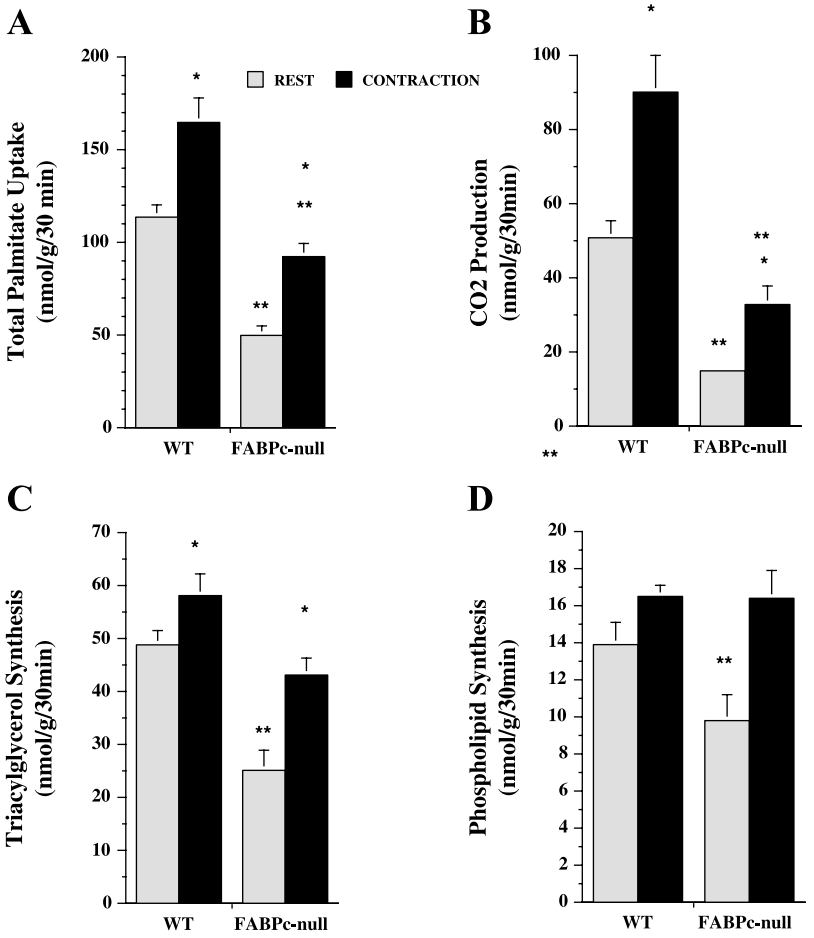

Fig. 4. Palmitate metabolism at rest and during contraction in isolated soleus muscles from wild-type and H-FABP (FABPc)-null mice. Values are means ± SE; n = 5–6 soleus muscles per treatment. *$P < 0.05$, contraction vs. rest; **$P < 0.05$, H-FABP-null vs. wild-type mice.

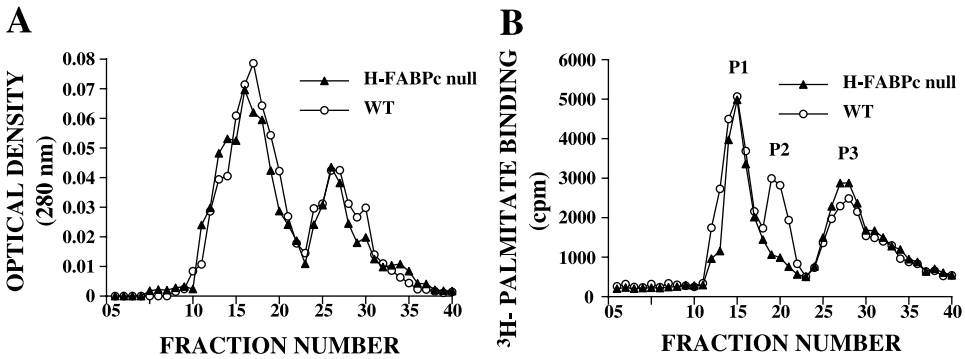

Fig. 5. Soleus homogenates from wild-type (○) and H-FABP-null (▲) mice were incubated with [^{3}H]palmitic acid and size-fractionated. *A*: optical density shown by UV light absorbance; *B*: radioactivity profile. From *left* to *right*: positions of the 3 radioactivity peaks of wild-type cytosol (c) mice are identical with those of the following standards: P1, bovine serum albumin (67 kDa); P2, bovine pancreatic ribonuclease A (13.7 kDa), the range where H-FABP (14–15 kDa) is normally located; P3, free radiolabeled palmitate. This figure is representative of 3 independent experiments.

gen utilization during 30-min contraction did not differ between wild-type and H-FABP-null mice ($P > 0.05$; Fig. 7*B*).

DISCUSSION

These studies have shown that deletion of H-FABP can have a profound impact on *1*) reducing fatty acid transport and metabolism, *2*) the proportions of mitochondrial subfractions, and *3*) reducing the concentrations of glycogen, ATP, and CP but not intramuscular TAGs. However, *4*) it is also important to note that the ability to respond to a metabolic challenge (muscle contraction) by increasing fatty acid oxidation was not fully impaired in H-FABP-null muscles. Nevertheless, our results demonstrate clearly that, in skeletal muscle, H-FABP has a key role in cytosolic LCFA transport and metabolism.

Alterations in Fatty Acid Metabolism

In the absence of H-FABP, palmitate transport across the plasma membrane was impaired. The reduction in LCFA transport would seem to be directly due to the absence of H-FABP in the null mice, since neither the expression level of FAT/CD36 and FABPpm nor their plasmalemmal content was altered in the H-FABP-null mice. This concurs with observations in hearts of H-FABP-null mice, in which initial rates of LCFA uptake by cardiac myocytes were reduced (−45%) but the expression of fatty acid transporter mRNAs was not altered (27). We have previously shown that LCFA transport is regulated by these transport proteins when H-FABP concentrations are not perturbed (4, 22, 23, 24). We (4, 24) have also argued previously that it is essential to have adequate quantities of H-FABP in giant vesicles to act as a metabolic sink, since LCFAs are not metabolized in this vesicle preparation (4, 24). The present study confirms this requirement for H-FABP to maintain an optimal rate of LCFA transport. However, the reduction in LCFA transport, given the complete absence of H-FABP, is more modest than might be expected if H-FABP was the only available cytosolic FABP.

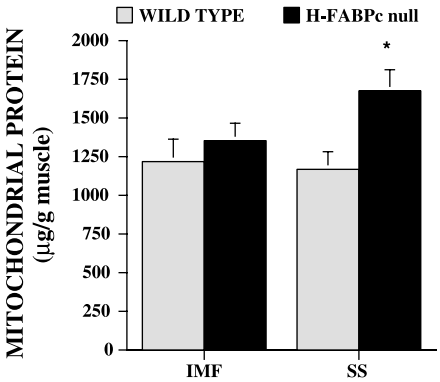

Fig. 6. Intermyofibrillar (IMF) and subsarcolemmal (SS) mitochondrial protein in wild-type and H-FABP-null mouse muscles. Values are means ± SE; $n = 4$–5 animals. *$P < 0.05$, H-FABP-null vs. wild-type mice.

The marked reduction in LCFA metabolism (TAG esterification −49%; palmitate oxidation −71%) seems also to be directly attributable to the lack of H-FABP, since activities of selected enzymes (CS and β-HAD) were not altered in muscles of H-FABP-null mice. Similarly, in hearts of H-FABP-null mice, CS and β-HAD activities were also not altered, whereas the oleate oxidation rate was reduced by 45% (27). Thus, in quiescent skeletal muscle (present study) and in heart (27), a null mutation in H-FABP markedly reduced, but did not fully inhibit, LCFA oxidation and esterification.

When presented with a metabolic challenge (muscle contraction), there was a substantial increase in LCFA metabolism, not only in the wild-type soleus muscles (TAG esterification +20% and palmitate oxidation +77%) but also in the H-FABP-null soleus muscles (TAG esterification +72% and palmitate oxidation +120%). Contraction-induced increases in both palmitate oxidation and TAG esterification in isolated rat soleus muscles have previously been shown by us (9, 20). In contrast to the present observations, it was recently reported that electrical stimulation increased the rate of oleate oxidation in cardiac myocytes from wild-type mice but not H-FABP-null mice (27). Clearly, the ability of H-FABP-null soleus muscles to increase LCFA metabolism during contraction differs markedly from the inability to do so in the heart. It is not immediately obvious why the responses in heart and skeletal muscle differ.

The concentration of intramuscular TAGs did not differ in the soleus muscles of H-FABP-null and wild-type mice. Similar TAG concentrations were also observed in hearts of H-FABP-null and wild-type mice

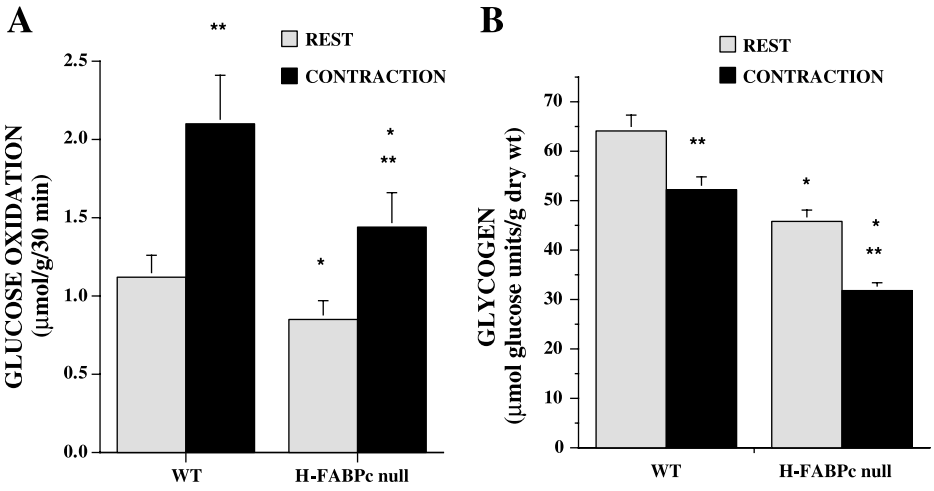

Fig. 7. Glucose oxidation (*A*) and glycogen utilization (*B*) in isolated soleus muscles of wild-type and H-FABP-null mice. Values are means ± SE; $n = 5–8$ muscles in each treatment. $^{**}P < 0.05$, contraction vs. rest; $^{*}P < 0.05$, H-FABP-null vs. wild-type mice.

(27). The present studies indicate that, although LCFA uptake is reduced and the rate of TAG esterification is lower in H-FABP-null mice, the rate of TAG hydrolysis is also lower in H-FABP-null mice. Thus it appears that TAG can accumulate to normal levels in the H-FABP-null mice because of a reduced rate of TAG utilization at rest and during contraction.

Adaptive Response to H-FABP-Null Mutation

Although the IMF mitochondrial protein was not altered in H-FABP-null mice, there was a 43% increase in SS mitochondrial protein in these mice compared with wild-type mice. This suggests strongly that there was an increase in SS mitochondria. Previously, it has been observed that the deletion of the soluble Ca^{2+}-binding protein parvalbumin (7) or creatine kinase (34) also increases muscle mitochondrial content to compensate for the disturbances created in muscle metabolism by these alterations. The selective increase in SS mitochondria, in H-FABP-null mice, is an interesting adaptive strategy. Presumably this increase is designed to reduce, within muscle cells, the diffusional distance for LCFAs to reach a greater number of mitochondria where LCFAs can be oxidized. In contrast, in hearts from H-FABP-mice, there did not appear to be an altered subcellular localization of mitochondria, as determined from electron microscopy (27).

H-FABP and Cytosolic Trafficking of Fatty Acids

From the present studies, it seems that H-FABP is perhaps somewhat more important for the trafficking of LCFAs to the mitochondria than to the TAG depots, because in quiescent soleus muscle, palmitate oxidation (−71%) was reduced more than TAG esterification (−49%). Increasing the metabolic rate with 30 min of contraction increased palmitate oxidation by +49 $nmol \cdot mg^{-1} \cdot 30\ min^{-1}$ in wild-type muscle but by only +18 $nmol \cdot mg^{-1} \cdot 30\ min^{-1}$ in H-FABP muscle. In contrast, TAG esterification was increased by only +9 $nmol \cdot mg^{-1} \cdot 30\ min^{-1}$ in wild-type muscle, whereas it was increased by +18 $nmol \cdot mg^{-1} \cdot 30\ min^{-1}$ in H-FABP muscle. These latter results may also be attributable to the reduced rate of TAG hydrolysis in the present studies, which would reduce the rate of TAG turnover. However, on balance it does appear that a null mutation of H-FABP in muscle has a greater quantitative effect on palmitate oxidation than on TAG esterification.

Partially Impaired Fatty Acid Metabolism May Indicate Presence of Other Cytosolic Binding Proteins

Despite the complete absence of H-FABP, it was quite striking that neither LCFA transport nor LCFA metabolism was completely impaired in either skeletal muscle (present study) or cardiac myocytes of H-FABP-null mice (27). The increase in SS mitochondria may partly account for this in the intact muscle. In addition, with muscle contraction there was still a considerable increase in LCFA metabolism in muscles of H-FABP-null mice, such that rates of TAG esterification in contracting H-FABP-null muscles (43 ± 3.2 $nmol \cdot g^{-1} \cdot 30\ min^{-1}$) were similar to those observed in resting wild-type muscle (49 ± 2.7 $nmol \cdot g^{-1} \cdot 30\ min^{-1}$). Moreover, the rates of palmitate oxidation in contracting H-FABP-null muscles doubled from 15 ± 1.1 $nmol \cdot g^{-1} \cdot 30\ min^{-1}$ at rest to 33 ± 5.0 $nmol \cdot g^{-1} \cdot 30\ min^{-1}$ during contraction. This oxidation rate during contraction in H-FABP-null muscle was therefore only 35% lower than in the resting soleus muscle from wild-type mice. These observations may indicate that *1*) there are other FABPs also involved in shuttling LCFA within the cytoplasm, *2*) a certain amount of fatty acid oxidation can occur via a cytosolic FABP-independent mechanism, or *3*) alternatively, other FABPs are expressed in response to the H-FABP-null mutation. There is evidence for this latter phenomenon. The null mutation of the adipose tissue FABP aP2 resulted in the increased expression of keratinocyte FABPs (18). These binding proteins substituted for the function normally performed by aP2 (30). Whether such an adaptive response occurs in muscle tissue of H-FABP-null mice is not known. Previously, in hearts of H-FABP-null mice, mRNAs encoding other FABPs were not found (2).

The fatty acid-binding profile of size-fractionated cytosol that we obtained with wild-type mouse soleus muscles is similar to that previously described for rat soleus and other skeletal muscles (26). Miller et al. (26) correlated the area of the smaller (~12 kDa) binding peak (i.e., see P2 in Fig. 6*B* in the present study) with the immunoreactivity to anti-H-FABP antibodies. Here, we found that this peak (P2) is completely absent in H-FABP knockout muscle, providing definitive evidence that fatty acid binding in the lower-molecular-weight region is caused mainly by H-FABP. The position of the other, larger fatty acid-binding peak (P1) corresponds to that of serum albumin, although Miller et al. could not prevent its presence by hindlimb perfusion before homogenization. However, even small amounts of albumin trapped within the muscle interstitium might bind significant amounts of label because of the presence of multiple fatty acid-binding sites on serum albumin, as opposed to only one site on H-FABP. The nature of this larger peak is of significant interest: if it is albumin, then H-FABP is the only principal LCFA-binding protein in soleus cytosol. However, if it is something other than albumin, then this might explain the substantial residual capacity to alter LCFA metabolism in H-FABP-deficient soleus muscle. Thus this peak (P1) requires further investigation, including complete removal of contaminating albumin with suitable antibodies.

H-FABP Mutation Also Exerts Effects on Carbohydrate Metabolism

The absence of H-FABP also resulted in other alterations in muscle metabolism besides those noted for fatty acids. Reductions in the concentrations of PC, ATP, and muscle glycogen were observed in H-FABP-null muscles. The latter suggests that there may be a greater reliance on muscle glycogen to act as a substitute for fatty acid metabolism, because it is well known that skeletal muscle can shift rather easily the proportions of carbohydrate and fatty acids that are metabolized. However, muscle glycogen utilization in the contracting muscle did not differ. Moreover, we also observed that rates of glucose oxidation at rest and during contraction were lower in H-FABP-null mice. This downregulation of both glucose and fatty acid oxidation, and similar rates of glycogen utilization in wild-type and H-FABP-null muscles, are unexpected. This would imply that, in the H-FABP-null soleus muscle, more of the glycogen is metabolized aerobically to provide a higher yield of ATP.

Comparison of the Effects of H-FABP Deletion on Metabolism in Soleus Muscle and the Heart

The metabolic milieu in H-FABP-null skeletal muscles does not parallel completely that observed in H-FABP-null hearts. For example, in H-FABP-null mice, cardiac glycogen content was 60% greater and glucose oxidation in quiescent cardiac myocytes was 80% greater than in wild-type mice (27). In contrast, in skeletal muscle of H-FABP-null mice, glycogen concentrations were 34% lower and glucose oxidation rates were 24% lower than in wild-type mice. Electrical stimulation failed to increase glucose oxidation further in cardiac myocytes from H-FABP-null mice (27), whereas the same treatment in skeletal muscle increased glucose oxidation in both wild-type (+87.5%) and H-FABP-null mice (+70%), although the absolute rates of glucose oxidation remained 33% lower during muscle contraction in the H-FABP-null mice. Although there is no obvious explanation for these differences in hearts and skeletal muscles of H-FABP-null mice, the data do indicate that there are tissue-specific responses to the null mutation of H-FABP. The greater reliance on glucose by the H-FABP-null heart may reflect the greater energetic demands of this tissue on an ongoing basis, and therefore it is more critical to metabolize carbohydrates as a substitute for fatty acid metabolism in this tissue. On the other hand, when fatty acid metabolism is impaired in skeletal muscle, reducing the contractile activity may be an effective strategy to cope with such a reduced LCFA availability. This would then obviate the need to metabolize glucose at a higher rate. It is quite possible that the activity patterns of H-FABP-null mice are reduced, since they are known to have a reduced exercise capacity and may not adapt well to exercise training (2).

Summary

Our studies have shown that fatty acid metabolism is reduced, both at rest and during muscle contraction, in skeletal muscles of H-FABP-null mice. This indicates that H-FABP is a key FABP involved in the trafficking of LCFAs in the myocyte. In contrast to the heart, glucose metabolism in H-FABP-null skeletal muscle does not appear to compensate for these reductions. Instead, the considerable increase in SS mitochondria may aid the maintenance of LCFA oxidation by reducing the diffusional distance to the mitochondria within the myocyte. It is, however, striking that the reductions in LCFA metabolism are far less than one would surmise if H-FABP were the only cytoplasmic FABP in muscle. Indeed, H-FABP-null muscles respond reasonably well to increased muscle contraction.

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