Increased lipolysis in transgenic animals overexpressing the epithelial fatty acid binding protein in adipose cells

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Abstract **Fatty acid binding proteins (FABPs) are low-molecular-mass, soluble, intracellular lipid carriers. Previous studies on adipocytes from adipocyte fatty acid binding protein (A-FABP)-deficient mice have revealed that both basal and isoproterenol-stimulated lipolysis were markedly reduced (Coe et al. 1999. *J. Lipid Res.* 40: 967–972). Herein, we report the construction of transgenic mice overexpressing the FABP5 gene encoding the epithelial fatty acid binding protein (E-FABP) in adipocytes, thereby allowing evaluation of the effects on lipolysis of increased FABP levels and of type specificity. In adipocytes from FABP5 transgenic mice, the total FABP protein level in the adipocyte was increased to 150% as compared to the wild type due to a 10-fold increase in the level of E-FABP and an unanticipated 2-fold down-regulation of the A-FABP. There were no significant differences in body weight, serum FFA, or fat pad mass between wild-type and FABP5 transgenic mice. Importantly, both basal and hormone-stimulated lipolysis increased in adipocytes from the FABP5 transgenic animals. The molecular composition of the fatty acid pool from either the intracellular compartment or that effluxed from the adipocyte was unaltered. These results demonstrate that there is a positive relationship between lipolysis and the total level of FABP but not between lipolysis and a specific FABP type.**—Hertzel, A. V., A. Bennaars-Eiden, and D. A. Bernlohr. **Increased lipolysis in transgenic animals overexpressing the epithelial fatty acid binding protein in adipose cells. *J. Lipid Res.* 2002.** 43: **2105–2111.**

Supplementary key words adipocytes • hormone-sensitive lipase • free fatty acids

Mammalian fatty acid binding proteins (FABPs) are expressed from a nine-member multi-gene family in tissues involved with active lipid metabolism (1–3). Some cells contain a single member of the family, whereas others express multiple family members. Structural analyses have documented, despite considerable divergence of the primary sequence, that the FABPs fold as a conserved β-barrel forming an interior water-filled cavity into which the ligand noncovalently resides (4). Typically, a single long-chain fatty acid or other hydrophobic molecule is bound within the cavity, although the liver FABP binds two ligands (5). Even though specific in vivo functions have remained elusive, FABPs have been thought to provide solubility and/or intracellular trafficking of long-chain fatty acids and other hydrophobic ligands between metabolic enzymes and/or membranes (1, 3, 6, 7).

Adipocytes play a dynamic role in lipid metabolism and homeostasis, including the traditional function of storing triacylglycerol during conditions of energy excess and mobilizing fatty acids for utilization by other tissues in times of energy depletion (8). Additionally, adipocytes secrete cytokines in response to various metabolic and hormonal signals indicating an alteration in the energy status of the organism. These secreted proteins can function in a paracrine, autocrine, or endocrine manner, thereby regulating overall body energy metabolism (9–12). Two genes from the FABP family are expressed in adipocytes: FABP4 [encoding the major form termed adipocyte fatty acid binding protein (A-FABP), also called aP2 or ALBP (A-form)], as well as FABP5 [encoding a minor form termed epithelial fatty acid binding protein (E-FABP), also called mal-1 or KLBP (E-form)]. In the wild-type mouse, E-FABP is approximately 1% to 5% that of A-FABP in the adipocyte (13) making the A/E protein ratio approximately 20:1. Both proteins bind fatty acids with comparable affinities as well as similar specificities (14–16). In addition to expression in adipocytes, A-FABP is expressed in macrophages, whereas E-FABP expression is more widespread, including lens, macrophage, retina, tongue, lung, brain, and skin, as well as a variety of epithelial and endothelial cells (6, 17–20).

Knockout mouse models of several members of this family have been developed with the goal of identifying metabolic phenotypes linked to functional differences in

Abbreviations: A-FABP, adipocyte fatty acid binding protein; E-FABP, epithelial fatty acid binding protein; FABP, fatty acid binding protein; HSL, hormone-sensitive lipase; Tg, trangenic.

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FABP biology (6, 21–23). Utilizing such a strategy, FABP4-disrupted mice exhibit reduced basal as well as isoproterenol-stimulated lipolysis in situ and in vivo, suggesting a role for the protein in facilitating fatty acid efflux from the adipocyte (13, 21). Consistent with this view, A-FABP has been shown to physically interact with hormone-sensitive lipase (HSL) and stimulate its activity (24, 25). Interestingly, FABP4 null animals up-regulate the expression of the FABP5 gene in adipocytes, resulting in an animal with reduced total FABP level as well as a switch in FABP types relative to wild-type animals (13, 26). As such, the reduced lipolysis in FABP4 null mice could be linked to the loss of the A-FABP, the up-regulation of the E-FABP, or a generalized reduction in the total FABP content.

To further characterize the relationship between levels of FABPs as well as of the specificity the type of FABP to lipolysis, a transgenic mouse was generated that overexpresses the FABP5 gene in the adipocyte driven by the FABP4 promoter. Herein, we report that such animals exhibit an increased total FABP level (150% of wild type) due to an increased level of E-FABP and a surprising decrease in the level of A-FABP resulting in an A/E ratio of 0.4. Adipocytes from such FABP5 transgenic mice exhibited increased basal and isoproterenol-stimulated lipolysis, indicating that lipolysis is linked to total FABP content and not to a specific FABP type.

MATERIALS AND METHODS

Chemicals

Adenosine deaminase, isoproterenol, BSA (fatty acid free), N6-(*R*-1-methyl-2-phenethyl) phenylisopropyl adenosine (PIA), dinonyl phthalate, and boron trifluoride were obtained from Sigma Chemicals, St. Louis, MO. Fatty acid standards for gas chromatography were obtained from Nucheck Prep, MN. Bond Elut-aminopropyl silica gel columns were obtained from Varian Sample Preparations, Harbor City, CA. ECF Western blotting kit was obtained from Amersham Biosciences, Inc., Piscataway, NJ. The colorimetric nonesterified fatty acid (NEFA) assay kit was obtained from Wako, Richmond, VA.

Animals

C57Bl/6J wild-type, FABP4 null mice, and FABP5 transgenic mice were fed a standard low-fat diet ad libitum. All mice were weaned at 3 weeks of age and experiments were performed on 12 to 20 week old animals. To avoid anesthetic-induced artifacts in lipolysis, the mice were killed by cervical dislocation and blood samples were obtained through a cardiac puncture prior to dissection of the epididymal fat pads. Serum samples were frozen at −20°C prior to further analysis. All procedures were reviewed and approved by the University of Minnesota Animal Care and Use Committee.

FABP5 transgenic mice

The complete murine FABP5 structural gene (27), from the transcriptional start site through the poly A site, was cloned downstream of the FABP4 5.4 kb promoter/enhancer (28) using the following strategy. Site-directed mutagenesis (29) was used to introduce a *Sac*II site upstream of the FABP4 promoter/enhancer as well as two *Not*I sites flanking the FABP5 structural gene. The FABP5 gene was cloned into the *Not*I site at the 3′ side of FABP4 in pSKIIaP2 (28). Subsequently, the vector sequences were removed by digestion with *Sac*II, followed by gel purification and dialysis of the fragment corresponding to the promoter/enhancer and structural gene. The DNA was microinjected into fertilized murine C57Bl/6J oocytes, and embryos were reimplanted in the uterus of a surrogate mother. Potential founders were identified by isolation of total genomic DNA and digestion with *Xba*I, followed by Southern blot analysis with an FABP5 intron 1 probe. The probe hybridized to a 2.5 kb band from an FABP5 transgenic mouse, whereas the corresponding wild-type band would be >20 kb. Three FABP5 transgenic lines were identified and backcrossed a minimum of three times to separate out independent integration sites. Initial analysis indicated that all lines behaved equivalently, and one was chosen for further study.

Protein levels of FABPs

Epididymal fat pads were dissected, homogenized with a Brinkman Polytron in PBS [10 mM sodium phosphate buffer (pH 6.8) containing 167 mM NaCl, 1 ml/g tissue] containing protease inhibitors, and centrifuged at 100,000 *g* for one h to produce a soluble extract. Varying amounts of protein were separated by SDS-PAGE along with known amounts of purified A-FABP and E-FABP standards (prepared in this laboratory). The proteins were transferred to a polyvinylidene difluoride membrane and blocked with TBS [10 mM Tris-HCl, 150 mM NaCl (pH 8.0)] containing 0.1% Tween-20 and 0.1% BSA. Rabbit affinity-purified polyclonal antibodies [directed toward either A-FABP (1:10,000 dilution) or E-FABP (1:1,000 dilution) prepared in this laboratory] were incubated overnight at room temperature and washed three times, and a secondary fluorescein-conjugated antibody was incubated for 1 h. Following additional washes, the fluorescent signal was obtained on a Storm Trooper Storm 840 Densitometer and analyzed using NIH Image software. The FABP concentration was determined from the standard curve plotted for each protein within the linear range of the signal.

Isolation of primary adipocytes

Murine epididymal fat pads from three to seven mice were pooled, minced, and suspended in a Krebs Ringer HEPES (KRH) buffered solution [118 mM NaCl, 4.75 mM KCl, 1.2 mM KH_2PO_4, 2.44 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.52 mM $CaCl_2$, 2.0 mM glucose, 200 nM adenosine, and 25 mM HEPES (pH 7.4)] containing 1 mg/ml collagenase (CLS1, Worthington Biochemicals) and 20 mg/ml BSA (Type V) prewarmed to 37°C. Adipocytes were isolated by collagenase digestion at 37°C for 60 min with vigorous shaking. The adipocytes were recovered by centrifugation at 2,000 rpm for 10 min at room temperature and washed twice in BSA-free KRH buffer supplemented with 200 nM adenosine, and subsequently resuspended in KRH buffer containing 2.5% BSA. The viability of the cells was verified by trypan blue exclusion analysis, and cell number was quantitated using an improved hemacytometer (Neubauer, American Scientific Products) prior to analyses.

Lipid extraction and separation

Intracellular fatty acids were extracted by homogenization of individual fat pads in a chloroform-methanol-phosphate-buffered saline solution (2:1:1.5; v/v/v) using a Brinkman Polytron tissue homogenizer. The homogenates were centrifuged at 4,000 rpm for 10 min at 25°C to generate a phase separation. The organic layer was removed and the extraction was repeated. The organic layers were pooled and concentrated under nitrogen to 1 ml. To separate the lipid classes, a Bond Elut-aminopropyl silica gel column (500 mg) was used (30). Briefly, the lipids were loaded onto the column and washed sequentially with chloroform-2-propanol (2:1; v/v) to elute the neutral lipids and diethyl

ether containing 2% acetic acid (98:2; v/v) to elute the FFAs. Test extractions using radioactive samples indicated that >98% of the input FFAs were recovered in the diethyl ether elution. For serum FFA analysis, total lipids were extracted from the serum in chloroform-methanol-water (2:1:1.5; v/v/v) using a volume five times the sample volume. The samples were vortexed for 1 min and centrifuged at 2,500 rpm for 10 min. The organic layer was removed and the extraction repeated. The organic phases were pooled and lipid classes were separated as described above.

Fatty acid analysis

The extracted FFAs were converted into methyl esters using 14% boron trifluoride in methanol. Fatty acid derivatives were separated by gas chromatography using an HP 5890 gas chromatograph (Agilent Technologies) equipped with a flame ionization detector and integrator. Chromatography was performed using an Omegawax 320 fused silica capillary column (30 m× 0.32 mm internal diameter, 0.25 μm film thickness; Supelco, Bellefonte, PA). Individual fatty acid derivatives were identified by comparison of their retention times to those of authentic purified fatty acid standards. The colorimetric NEFA assay was used to quantify the total nonesterified FFAs as per the manufacturer's instructions.

Analysis of adipocyte lipolysis

Approximately 25,000 cells were incubated in a final volume of 500 μl in KRH buffer supplemented with 200 nM adenosine, adenosine deaminase (1 unit/ml) and 10 μM PIA. Lipolysis was stimulated by the addition of 100 μM isoproterenol for 30 min at 37°C with vigorous shaking. The reaction was stopped by the addition of dinonyl phthalate oil followed by a low-speed centrifugation to separate the adipocytes from the incubation media. The total as well as individual fatty acids effluxed was analyzed as previously described. The data represent the mean value from pools of at least three mice each (n = 5) analyzed in triplicate for each genotype.

Analysis of primary adipocyte fatty acid uptake

Approximately 50,000 cells were incubated in 550 μl KRH buffer supplemented with 2 mM glucose. BSA and oleic acid were preincubated for 30 min at 37°C. Uptake was initiated by addition of BSA/oleic acid (final concentration 50 μM each; [^{3}H]oleic acid:1 Ci/mol) to the cells, resulting in a free oleic acid concentration of 6.85 nM. Reactions were maintained at 37°C for 30 and 300 s in quadruplicate. The reactions were stopped by the addition of 5 ml ice-cold 0.2 mM phloretin with 0.1% BSA, followed by vacuum filtration on glass microfiber filters to separate the unincorporated fatty acids from the cells. The filters were washed three times with 5 ml cold phloretin/BSA solution and dried, and the amount of [^{3}H]oleic acid in the cells was determined by direct scintillation counting.

Statistical analyses

All values are expressed as mean ± SEM unless otherwise noted. Statistical significance was determined by the two-tailed Student's *t*-test. *P* values < 0.05 are considered significant.

RESULTS

To test the hypothesis that FABPs facilitate fatty acid efflux during lipolysis, a transgenic mouse was developed that expresses high levels of FABP5 in adipocytes. Because the complete FABP5 gene is relatively small, a transgene containing the 5.4 kb FABP4 promoter/enhancer driving adipocyte expression of the complete FABP5 gene (**Fig. 1A**) was constructed and injected into fertilized murine C57Bl/6J oocytes. Successful insertion of the transgene was confirmed by Southern blot analysis (Fig. 1B). Mice with the strongest band on the Southern blot were mated to wild-type C57Bl/6J mice, and their offspring were analyzed for the transgene. Backcrossing to wild type allowed maintenance of the transgene in the heterozygous state. Three lines were continued and backcrossed a minimum of three times to allow segregation of multiple insertion sites. Copy number was also determined by Southern blot

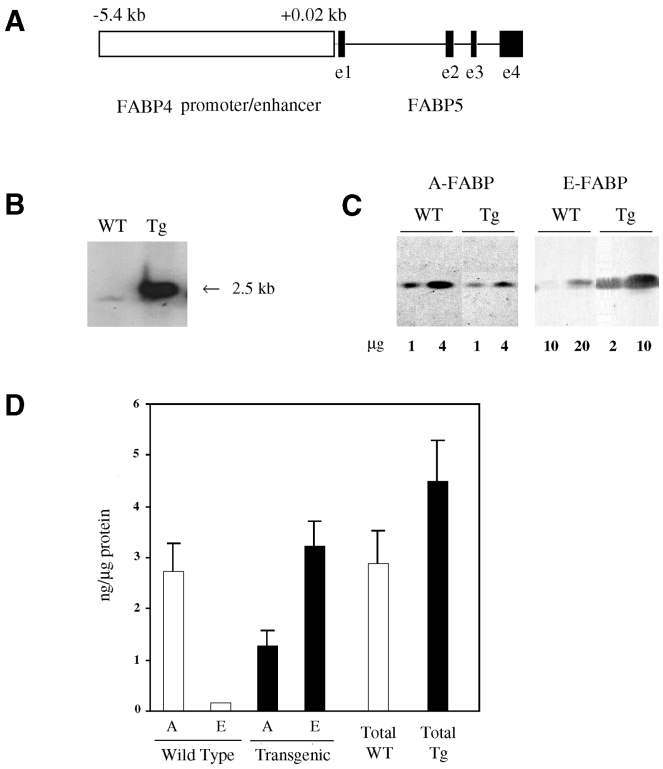

Fig. 1. Generation of transgenic mice overexpressing FABP5 in adipocytes. A: Schematic of the transgene. The open box indicates the FABP4 promoter/enhancer region including −5.4 kb to +0.02 kb downstream of the start of transcription. The closed boxes indicate the coding exons of FABP5, and the lines represent the intervening introns. B: Southern blot analysis of FABP5 transgenic mice. Total genomic DNA was isolated, digested, and used for blotting. The blot was hybridized with a FABP5 intron 1 probe. The transgene will generate a band of 2.5 kb. The endogenous wild-type copy could not be detected because its very large fragment size (>20 kb) was not efficiently transferred. WT indicates DNA from wild-type mice; Tg indicates DNA from FABP5 transgenic mice. C: Western blot analysis of fatty acid binding proteins (FABPs) in adipocytes from wild-type and FABP5 transgenic mice. Various amounts of adipocyte protein extracts (indicated below each lane) were separated by SDS-PAGE, blotted, and incubated with affinity-purified polyclonal antibodies against adipocyte fatty acid binding protein (A-FABP) or epithelial fatty acid binding protein (E-FABP). Individual mouse samples were assayed with a minimum of 10 independent samples. D: Quantitation of adipocyte FABPs from Western blot analysis. Graph depicting individual A-FABP (A) or E-FABP (E) and total FABP levels in wild-type and FABP5 transgenic adipocytes, plotted as mean ± SEM.

TABLE 1. Physical and metabolic parameters of wild-type and FABP5 transgenic mice

	Wild-Type	Transgenic
Transgene copy number	—	10 ± 1
Body weight (g)	28.17 ± 2.41	27.92 ± 1.65
Epididymal fat pad wt (g)	0.39 ± 0.08	0.43 ± 0.04
A-FABP (ng/μg)	2.73 ± 0.54	1.26 ± 0.31[a]
E-FABP (ng/μg)	0.15 ± 0.02	3.22 ± 0.49[b]
Total FABP (ng/μg)	2.88 ± 0.66	4.48 ± 0.80
% Adipocytes with diameter:		
0–2,000 (microns)	83	68
2,001–4,000	14	24
4,001–6,000	2	3
>6,000	1	5

A-FABP, adipocyte fatty acid binding protein; E-FABP, epithelial fatty acid binding protein. The transgene copy number was determined by Southern blot analysis and compared to the intensity of a single-copy gene. Fatty acid binding protein (FABP) levels were determined by Western blot analysis using chemifluorescence, and the signals were compared to known concentrations of purified protein. Adipocyte diameter was determined by light phase microscopy. Analyses were done on individual mice/samples from a minimum of 10 mice. Statistical significance comparing wild-type to FABP5 transgenic mice according to Student's *t*-test.

[a] $P < 0.05$.

[b] $P < 0.001$.

analysis comparing a fragment from the wild-type FABP5 gene (single copy) to the transgene that indicated approximately 10 copies of the transgene. The FABP5 transgenic mice had no apparent defect in fertility or growth, with no significant differences in body weight, offspring sex ratio, or epididymal fat pad mass (**Table 1**). Interestingly, approximately 10% of the adipocytes from the FABP5 transgenic mice were larger than the wild-type cells. Analyses were performed on all three lines with similar results; data from one line is reported herein.

Western blot analyses were performed on adipocyte protein extracts to examine whether the transgene was expressing FABP5 in adipocytes (Fig. 1C). Total levels of A-FABP and E-FABP were calculated by comparison to standard curves of each protein (Fig. 1D) using homogenous protein and monospecific polyclonal antibodies, both prepared in this laboratory. In wild-type adipocytes, E-FABP levels are 0.15 ng/μg protein while A-FABP levels are 2.73 ng/μg protein, leading to a total FABP level of 2.88 ng/μg protein and an A/E ratio of approximately 20:1. In the adipocytes from FABP5 transgenic animals, E-FABP levels were up-regulated to 3.22 ng/μg protein while A-FABP levels were down-regulated to 1.26 ng/μg protein, leading to an overall 150% increase in FABP content and an A/E ratio of 0.4:1. This is in contrast to the FABP4 null mice, which express only 10% adipocyte FABP levels (13).

Although the fatty acid binding specificity and affinity of E-FABP and A-FABP are similar, they are not identical (2, 14, 16). To determine if the overexpression of E-FABP in the FABP5 transgenic mice alters the composition of intracellular FFAs (the sum of bound and unbound), gas chromatography analysis was performed on lipids extracted from the fat pads of wild-type and FABP5 transgenic mice. From the two genotypes, 14 different fatty acids were identified and analyzed, with relative mol% ranging from 0.1% to 30.1% (**Table 2**). The predominant fatty acids identified were long-chain 16 and 18 carbon atoms with zero to two double bonds (mainly C16:0, C18:1, and C18:2) that accounted for 94% to 97% of the total fatty acids identified. No major significant differences in the composition of intracellular fatty acids were noted between the two genotypes. These results suggest that despite a change in A/E ratio from 20 to 0.4, the composition of the total intracellular FFA pool remained unaltered.

To determine if the serum concentration of FFAs or the serum composition was altered in FABP5 transgenic animals relative to wild-type, samples were collected and the fatty acids extracted and analyzed by gas chromatography. Levels of plasma FFA were indistinguishable among the three genotypes (**Table 3**). Likewise, no significant differences were observed in the serum composition of the FFAs (data not shown).

Lipolysis is an adipocyte function critical to maintaining energy homeostasis. Previous results on primary adipocytes from the FABP4 null mice demonstrated a 50% reduction in basal and isoproterenol-stimulated lipolysis. To investigate the relationship between total FABP levels, the A/E ratio, and the process of lipolysis, primary adipocytes from wild-type and FABP5 transgenic mice were incubated for 30 min in the absence or presence of isoproterenol, and the FFAs released in the media were quantified. As shown in **Fig. 2**, basal efflux of FFAs was elevated (2.3-fold) in the FABP5 transgenic mice as compared to the wild-type animals (34.8 ± 13.7 vs. 14.8 ± 8.9 FFA nmol/10,000 cells/30 min; $P < 0.003$). As expected, a robust stimulation in lipolysis was observed in response to the addition of isoproterenol for both genotypes (3.5- to 3.8-fold). Similar to the basal conditions, stimulation resulted in a 2.1-fold increase in lipolysis in the FABP5 transgenic compared to the wild-type adipocytes (121.2 ± 14.6 vs. 56.6 ± 21.6; $P < 0.0001$ FFA nmol/10,000 cells/30 min). Thus, both basal and stimulated lipolysis are elevated in

TABLE 2. Intracellular fatty acid composition in wild-type and FABP5 transgenic mice

Fatty Acid	Wild-Type	FABP5 Transgenic
	mol%	
C14:0	1.3 ± 0.2	1.67 ± 0.3
C14:1	1.7 ± 0.4	1.97 ± 0.3
C16:0	23.97 ± 1.0	25.17 ± 0.4
C16:1	5.87 ± 0.7	5.57 ± 0.3
C18:0	5.77 ±1.7	6.27 ± 1.0
C18:1	27.47 ± 0.9	27.37 ± 1.4
C18:2	30.07 ± 1.8	28.37 ± 0.5
C18:3	2.57 ± 0.1	2.47 ± 0.1
C20:1	0.37 ± 0.0	0.37 ± 0.0
C20:2	0.77 ± 0.3	0.87 ± 0.2
C20:3	0.07 ± 0.0	0.17 ± 0.1
C20:4	0.67 ± 0.2	0.57 ± 0.0
C22:0	0.17 ± 0.1	0.27 ± 0.1
C22:1	0.47 ± 0.2	0.57 ± 0.0

FFAs were esterified to methyl esters and analyzed by gas chromatography. The analysis was repeated three times for each genotype, with a pool of at least three mice per experiment. Values are expressed as mean ± SEM. Statistical significance was not identified, because all *P* values were > 0.05.

TABLE 3. Serum FFA levels in male mice

	FFA
	mM
Wild-type	0.76 ± 0.11
FABP4 null	0.55 ± 0.06
FABP5 transgenic	0.81 ± 0.11

Quantitation of serum FFAs in wild-type, FABP4 null, and FABP5 transgenic mice. Serum was collected and FFAs were measured using a nonesterified fatty acid (NEFA) Wako kit. Analysis was done on at least seven mice from each genotype. Data are expressed as mean ± SEM.

the FABP5 transgenic mice. Although the levels of lipolysis are enhanced in the FABP5 transgenic adipocytes, it appears that the degree of stimulation is maintained, suggesting no alteration in the signaling pathway. The change in lipolysis shows a positive relationship to the total levels of FABP present within the adipocyte.

Analysis by gas chromatography of the types of fatty acids released during lipolysis of primary fat cells revealed 9 major different fatty acids, the major subtypes of which were long-chain fatty acids (palmitic, oleic, and linoleic). Few differences were found among the three genotypes (**Table 4**). Among the minor subtypes of fatty acids, the profile of fatty acids effluxed during isoproterenol-stimulated lipolysis was very similar to the profile of the composition of intracellular FFAs within the adipocyte.

To further explore the role of FABPs in adipocyte lipid

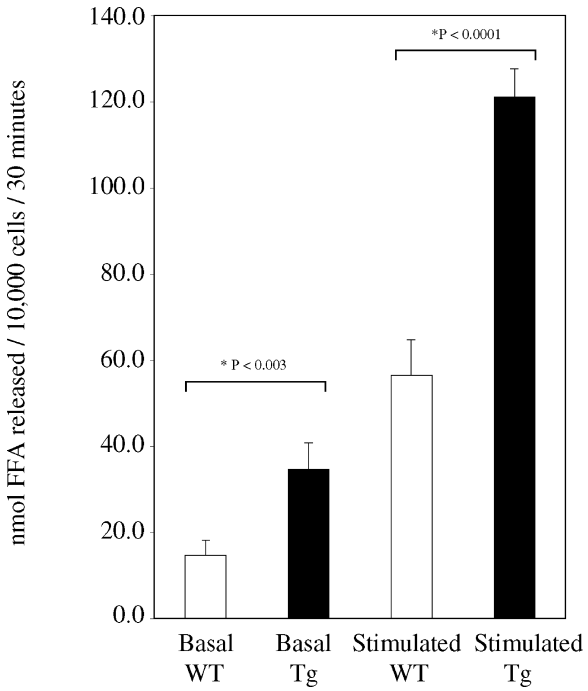

Fig. 2. Fatty acid efflux of primary cultured adipocytes from wild-type and FABP5 transgenic mice. Fatty acid release was determined from adipocytes in either basal or 30 min of isoproterenol stimulation. Experiments were performed in triplicate with adipocytes isolated from a pool of at least three mice from each genotype and were repeated at least five times. Values are expressed as mean ± SEM. Statistical significance determined by two-tailed Student's *t*-test. **P* values < 0.05.

TABLE 4. Composition of fatty acids released during isoproterenol-stimulated lipolysis in male mice in vitro

Fatty Acid	Wild-Type	FABP4 Null	FABP5 Transgenic
C14:0	1.96 ± 0.30	1.81 ± 0.18	1.03 ± 0.15
C16:0	24.94 ± 1.31	24.34 ± 0.87	23.57 ± 2.27
C16:1	8.18 ± 0.35	10.61 ± 0.80	7.60 ± 0.52
C18:0	2.17 ± 0.50	2.49 ± 0.24	3.29 ± 0.50
C18:1	26.57 ± 0.67	29.42 ± 4.23	33.17 ± 4.22
C18:2	32.89 ± 1.10	28.62 ± 3.23	24.34 ± 7.83
C18:3	2.84 ± 0.20	2.35 ± 0.61	3.83 ± 0.60
C20:3	0.13 ± 0.13	ND	ND
C20:4	0.31 ± 0.19	0.36 ± 0.15	0.70 ± 0.27

Composition of FFAs released from isoproterenol-stimulated cultured primary adipocytes from wild-type, FABP4 null, and FABP5 transgenic mice. FFAs were esterified to methyl esters and analyzed by gas chromatography. Numbers indicate mol% of the total FFA pools. The analysis was repeated at least four times for each genotype, with a pool of at least three mice per experiment. Values are expressed as mean ± SEM. Statistical significance was not identified, because all *P* values were > 0.05. ND, not detected.

homeostasis, the capacity to internalize exogenous fatty acids was determined. Primary adipocytes were isolated and incubated with a BSA-[^{3}H]oleic acid complex at 37°C for 30 s. Results showed that there was no difference in the ability to internalize fatty acids in the FABP5 transgenic adipocytes compared to the wild type (**Fig. 3**). Furthermore, incubating the adipocytes with the oleic acid for 5 min did not reveal a major difference in the ability to metabolize the internalized oleic acid. There may be a trend indicating a change in the metabolism of oleic acid

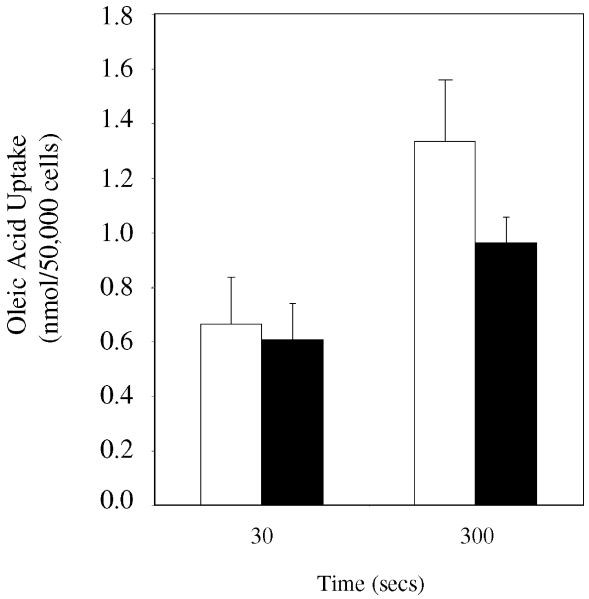

Fig. 3. Fatty acid uptake in primary cultured adipocytes from wild-type and FABP5 transgenic mice. Transport was carried out using 50 μM [^{3}H]oleic acid complexed with 50 μM BSA for 30 s and 5 min. Open box indicates wild type; closed box indicates FABP5 transgenic. Experiments were performed in quadruplicate with adipocytes isolated from a pool of a least four mice from each genotype. The experiment was repeated twice. A representative experiment is plotted. Values are expressed as mean ± SEM. Student's *t*-test for each time point indicated no significant differences with $P > 0.05$.

(as seen by lower levels of uptake in the FABP5 transgenics at 5 min); however, future studies are necessary to be able to fully interpret these results. Therefore, increasing (150%) or decreasing (10%) FABP levels in adipocytes does not result in a change in the level of fatty acid uptake in primary adipocytes.

DISCUSSION

We have successfully developed a system for increasing FABP content in adipocytes and for examining the specificity of the FABP type. The insertion into mice of a transgene expressing the FABP5 gene from the FABP4 promoter/enhancer resulted in dramatic overexpression of E-FABP in the adipocyte. An unexpected consequence of the overexpression of E-FABP was an approximate 50% reduction in the expression of A-FABP. This may be the result of "squelching" due to the dilution of a transcription factor(s) or coactivators by the 10 copies of the FABP4 promoter/enhancer from the transgene compared to that used by the normal single-copy native promoter. However, since a similar promoter/enhancer DNA binding site is used to drive the expression of many adipocyte genes whose functions are linked to lipid metabolism, the total number of such protein-DNA interaction(s) in the genome seems to be only marginally increased. As such, the molecular mechanism leading to the down-regulation of the FABP4 gene is not known. Despite the down-regulation of FABP4, the expression of FABP5 resulted in a significant increase of 150% in total adipocyte FABPs. This is in contrast to the FABP4 null mice, whose adipocytes contain approximately 10% of the total adipocyte FABPs compared to the wild type (13).

Comparing data from the FABP4 null animals and the FABP5 transgenic animals to those from the wild type provides some insight into the function of the FABPs. In this study, the FABP5 transgenic mice exhibited increased primary adipocyte lipolysis in both the basal and the stimulated states without any alteration in fatty acid transport or metabolism. This was demonstrated in a system that has a 2-fold reduction in A-FABP but a 20-fold increase in E-FABP, and therefore a 150% increase in total FABP. Previously, two reports showed that adipocytes from the FABP4 null mice have reduced lipolysis despite the 7- to 8-fold up-regulation of E-FABP(13, 21), although one report did not see this change (31). It is not clear why, in this latter report, the decrease in lipolysis was not seen, although this may have been due to differences in how the animals were maintained or in how the experiments were carried out. Examination of the change in lipolysis as a function of A-FABP level, E-FABP level, or total FABP level indicates that a positive relationship exists with only the total FABP abundance and not with any singular FABP type. That is, although the abundance of each FABP and the A/E ratio were experimentally manipulated in either the FABP4 null or FABP5 transgenic mice and compared to those of the wild-type animals, the changes in lipolysis correlate only with the total level of FABP.

Recently, A-FABP has been shown to directly interact with HSL, resulting in an increase in hydrolysis activity (24, 25) and providing a mechanistic basis for reduced lipolysis in the FABP4 null adipocytes. Current studies are evaluating the association of E-FABP with HSL, but our results predict that E-FABP, similar to A-FABP, would also interact with HSL and stimulate triacylglycerol hydrolysis. Although a specific additional function of A-FABP or E-FABP unlinked to lipolysis cannot be ruled out at this time, these results are consistent with a model in which both FABPs have overlapping functions in the adipocyte linked to facilitation of fatty acid efflux.

This study was conducted using animals fed a normal chow diet, containing a low amount (5%) of calories as fat. Previous studies have indicated that FABP4 null animals maintained on a high-fat diet (~40% of calories from fat) exhibit lower fasting serum glucose as well as improved insulin and glucose tolerance tests (26). While not part of this study, when fed a high-fat diet, the FABP5 transgenic mice exhibited increased fasting glucose levels as well as impaired glucose tolerance tests (unpublished observations). Interestingly, high-fat-fed FABP5 transgenic mice up-regulate both FABP4 and FABP5, resulting in an increase in the total FABP level relative to the wild type. As such, FABP4 null mice are relatively insulin sensitive while FABP5 transgenic mice are relatively insulin resistant. Although it is tempting to speculate that lipolysis may control insulin sensitivity, it is important to point out that serum FFA levels are not appreciably altered between the three genotypes of mice, even on a high-fat diet (21, 32). Therefore, the mechanistic linkage between FABP level, lipolysis, and the metabolic syndrome is more complex than FFA availability.

In summary, we have constructed a FABP5 transgenic mouse with elevated levels of E-FABP in adipocytes. Primary adipocytes contain 150% the total FABP content as compared to the wild type and have increased basal and stimulated lipolysis. The up-regulation of E-FABP in adipocytes did not alter the composition of FFAs within the adipocyte, in serum, or in the products of lipolysis. This indicates that A-FABP and E-FABP appear to function in an analogous manner and have a common function in facilitating fatty acid efflux from adipocytes.

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REFERENCES

1. Coe, N. R., and D. A. Bernlohr. 1998. Physiological properties and functions of intracellular fatty acid-binding proteins. *Biochim. Biophys. Acta.* **1391:** 287–306.
2. Bernlohr, D. A., M. A. Simpson, A. V. Hertzel, and L. J. Banaszak. 1997. Intracellular lipid-binding proteins and their genes. *Annu. Rev. Nutr.* **17:** 277–303.

3. Veerkamp, J. H., and R. G. Maatman. 1995. Cytoplasmic fatty acid-binding proteins: their structure and genes. *Prog. Lipid Res.* **34:** 17–52.
4. LaLonde, J. M., D. A. Bernlohr, and L. J. Banaszak. 1994. The up-and-down beta-barrel proteins. *FASEB J.* **8:** 1240–1247.
5. Haunerland, N., G. Jagschies, H. Schulenberg, and F. Spener. 1984. Fatty-acid-binding proteins. Occurrence of two fatty-acid-binding proteins in bovine liver cytosol and their binding of fatty acids, cholesterol, and other lipophilic ligands. *Hoppe Seylers Z. Physiol. Chem.* **365:** 365–376.
6. Hertzel, A. V., and D. A. Bernlohr. 2000. The mammalian fatty acid-binding protein multigene family: molecular and genetic insights into function. *Trends Endocrinol. Metab.* **11:** 175–180.
7. Bernlohr, D. A., N. R. Coe, and V. J. LiCata. 1999. Fatty acid trafficking in the adipocyte. *Semin. Cell Dev. Biol.* **10:** 43–49.
8. Bernlohr, D. A., and M. A. Simpson. 1996. Adipose tissue and lipid metabolism. *In* Biochemistry of Lipids, Lipoproteins and Membranes. Elsevier, Amsterdam. 257–281.
9. Shuldiner, A. R., R. Yang, and D. W. Gong. 2001. Resistin, obesity and insulin resistance-the emerging role of the adipocyte as an endocrine organ. *N. Engl. J. Med.* **345:** 1345–1346.
10. Kim, S., and N. Moustaid-Moussa. 2000. Secretory, endocrine and autocrine/paracrine function of the adipocyte. *J. Nutr.* **130 (Suppl.):** 3110–3115.
11. Trayhurn, P., and J. H. Beattie. 2001. Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proc. Nutr. Soc.* **60:** 329–339.
12. Wauters, M., R. V. Considine, and L. F. Van Gaal. 2000. Human leptin: from an adipocyte hormone to an endocrine mediator. *Eur. J. Endocrinol.* **143:** 293–311.
13. Coe, N. R., M. A. Simpson, and D. A. Bernlohr. 1999. Targeted disruption of the adipocyte lipid-binding protein (aP2 protein) gene impairs fat cell lipolysis and increases cellular fatty acid levels. *J. Lipid Res.* **40:** 967–972.
14. Kane, C. D., N. R. Coe, B. Vanlandingham, P. Krieg, and D. A. Bernlohr. 1996. Expression, purification, and ligand-binding analysis of recombinant keratinocyte lipid-binding protein (MAL-1), an intracellular lipid-binding found overexpressed in neoplastic skin cells. *Biochemistry.* **35:** 2894–2900.
15. Bernlohr, D. A., N. R. Coe, M. A. Simpson, and A. V. Hertzel. 1997. Regulation of gene expression in adipose cells by polyunsaturated fatty acids. *Adv. Exp. Med. Biol.* **422:** 145–156.
16. Simpson, M. A., V. J. LiCata, N. Ribarik-Coe, and D. A. Bernlohr. 1999. Biochemical and biophysical analysis of the intracellular lipid binding proteins of adipocytes. *Mol. Cell. Biochem.* **192:** 33–40.
17. De Leon, M., A. A. Welcher, R. H. Nahin, Y. Liu, M. A. Ruda, E. M. Shooter, and C. A. Molina. 1996. Fatty acid binding protein is induced in neurons of the dorsal root ganglia after peripheral nerve injury. *J. Neurosci. Res.* **44:** 283–292.
18. Jaworski, C., and G. Wistow. 1996. LP2, a differentiation-associated lipid-binding protein expressed in bovine lens. *Biochem. J.* **320:** 49–54.
19. Krieg, P., S. Feil, G. Furstenberger, and G. T. Bowden. 1993. Tumor-specific overexpression of a novel keratinocyte lipid-binding protein. Identification and characterization of a cloned sequence activated during multistage carcinogenesis in mouse skin. *J. Biol. Chem.* **268:** 17362–17369.
20. Owada, Y., T. Yoshimoto, and H. Kondo. 1996. Increased expression of the mRNA for brain- and skin-type but not heart-type fatty acid binding proteins following kainic acid systemic administration in the hippocampal glia of adult rats. *Brain Res. Mol. Brain Res.* **42:** 156–160.
21. Scheja, L., L. Makowski, K. T. Uysal, S. M. Wiesbrock, D. R. Shimshek, D. S. Meyers, M. Morgan, R. A. Parker, and G. S. Hotamisligil. 1999. Altered insulin secretion associated with reduced lipolytic efficiency in aP2-/- mice. *Diabetes.* **48:** 1987–1994.
22. Binas, B., H. Danneberg, J. McWhir, L. Mullins, and A. J. Clark. 1999. Requirement for the heart-type fatty acid binding protein in cardiac fatty acid utilization. *FASEB J.* **13:** 805–812.
23. Schaap, F. G., B. Binas, H. Danneberg, G. J. van der Vusse, and J. F. Glatz. 1999. Impaired long-chain fatty acid utilization by cardiac myocytes isolated from mice lacking the heart-type fatty acid binding protein gene. *Circ. Res.* **85:** 329–337.
24. Shen, W. J., Y. Liang, R. Hong, S. Patel, V. Natu, K. Sridhar, A. Jenkins, D. A. Bernlohr, and F. B. Kraemer. 2001. Characterization of the functional interaction of adipocyte lipid-binding protein with hormone-sensitive lipase. *J. Biol. Chem.* **276:** 49443–49448.
25. Shen, W. J., K. Sridhar, D. A. Bernlohr, and F. B. Kraemer. 1999. Interaction of rat hormone-sensitive lipase with adipocyte lipid-binding protein. *Proc. Natl. Acad. Sci. USA.* **96:** 5528–5532.
26. Hotamisligil, G. S., R. S. Johnson, R. J. Distel, R. Ellis, V. E. Papaioannou, and B. M. Spiegelman. 1996. Uncoupling of obesity from insulin resistance through a targeted mutation in aP2, the adipocyte fatty acid binding protein. *Science.* **274:** 1377–1379.
27. Hertzel, A. V., and D. A. Bernlohr. 1998. Cloning and chromosomal location of the murine keratinocyte lipid-binding protein gene. *Gene.* **221:** 235–243.
28. Graves, R. A., P. Tontonoz, K. A. Platt, S. R. Ross, and B. M. Spiegelman. 1992. Identification of a fat cell enhancer: analysis of requirements for adipose tissue-specific gene expression. *J. Cell. Biochem.* **49:** 219–224.
29. Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA.* **82:** 488–492.
30. Kaluzny, M. A., L. A. Duncan, M. V. Merritt, and D. E. Epps. 1985. Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J. Lipid Res.* **26:** 135–140.
31. Shaughnessy, S., E. R. Smith, S. Kodukula, J. Storch, and S. K. Fried. 2000. Adipocyte metabolism in adipocyte fatty acid binding protein knockout (aP2-/-) mice after short-term high-fat feeding: functional compensation by the keritinocyte fatty acid binding protein. *Diabetes.* **49:** 904–911.
32. Uysal, K. T., L. Scheja, S. M. Wiesbrock, S. Bonner-Weir, and G. S. Hotamisligil. 2000. Improved glucose and lipid metabolism in genetically obese mice lacking aP2. *Endocrinology.* **141:** 3388–3396.