

## Defective Uptake and Utilization of Long Chain Fatty Acids in Muscle and Adipose Tissues of CD36 Knockout Mice\*

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The transmembrane protein CD36 has been identified in isolated cell studies as a putative transporter of long chain fatty acids. In humans, an association between CD36 deficiency and defective myocardial uptake of the fatty acid analog 15-(*p*-iodophenyl)-3-(*R,S*)-methyl pentadecanoic acid (BMIPP) has been reported. To determine whether this association represents a causal link and to assess the physiological role of CD36, we compared tissue uptake and metabolism of two iodinated fatty acid analogs BMIPP and 15-(*p*-iodophenyl) pentadecanoic acid (IPPA) in CD36 null and wild type mice. We also investigated the uptake and lipid incorporation of palmitate by adipocytes isolated from both groups. Compared with wild type, uptake of BMIPP and IPPA was reduced in heart (50–80%), skeletal muscle (40–75%), and adipose tissues (60–70%) of null mice. The reduction was associated with a 50–68% decrease in label incorporation into triglycerides and in 2–3-fold accumulation of label in diglycerides. Identical results were obtained from studies of [<sup>3</sup>H]palmitate uptake in isolated adipocytes. The block in diglyceride to triglyceride conversion could not be explained by changes in specific activities of the key enzymes long chain acyl-CoA synthetase and diacylglycerol acyltransferase, which were similar in tissues from wild type and null mice. It is concluded that CD36 facilitates a large fraction of fatty acid uptake by heart, skeletal muscle, and adipose tissues and that CD36 deficiency in humans is the cause of the reported defect in myocardial BMIPP uptake. In CD36-expressing tissues, uptake regulates fatty acid esterification at the level of diacylglycerol acyltransferase by determining fatty acyl-CoA supply. The membrane transport step may represent an important control site for fatty acid metabolism *in vivo*.

Studies with isolated and cultured cells have provided evidence for the existence of a protein-facilitated component in the membrane transport of long chain fatty acids (FA)<sup>1</sup> in adipose

(1, 2), liver (3, 4), and muscle tissues (3, 5). Among the proteins proposed to enhance FA uptake is the transmembrane protein CD36. Expression of this protein in fibroblasts, which do not endogenously express CD36, was associated with an increase in FA uptake and incorporation into phospholipids (6). This increase reflected the appearance of a saturable, phloretin-sensitive component exhibiting a transport  $K_m$  within the physiologic range of unbound FA concentrations (7–10 nM) (7).

The distribution of CD36 favors tissues with a high metabolic capacity for FA such as adipose, heart, and skeletal muscle (8). In muscle tissues, CD36 expression is most abundant in red oxidative fibers and is up-regulated with muscle stimulation concomitant with an increase in the  $V_{max}$  of FA transport (9). Expression is also high in tissues experiencing large fluxes of FA such as capillary endothelia, mammary epithelia (10), or epithelia of the small intestine (11).

Mice null for CD36 were recently developed and found to exhibit increased serum FA, triglyceride, and cholesterol (12). Their adipocytes lack the high affinity component of FA transport observed in cells isolated from wild type mice. In contrast, transgenic mice with muscle-targeted CD36 overexpression exhibit a decrease in serum FA, triglyceride, and cholesterol (13). Soleus muscle from these mice displays an enhanced ability to oxidize FA in response to stimulation/contraction.

In humans, CD36 deficiency has a prevalence of 0.3–11% with the higher incidences in Asian and African populations (14). Deficient individuals are apparently healthy. However, a strong association between CD36 deficiency and defective myocardial uptake of the slowly oxidized FA analog 15-(*p*-iodophenyl)-3-(*R,S*)-methyl pentadecanoic acid (BMIPP) has been reported (15). To determine whether these two defects are indeed causally related and to directly explore the contribution of CD36 to FA uptake by various tissues, we examined uptake and metabolism of [<sup>3</sup>H]palmitate and of iodinated fatty acid analogs, BMIPP and 15-(*p*-iodophenyl)pentadecanoic acid (IPPA), in CD36 null mice.

### MATERIALS AND METHODS

**Animals**—CD36 null mice were generated by targeted homologous recombination and back-crossed 4 times to C57Bl/6. Wild type control littermates were bred from the same cross as the nulls and were therefore of identical genetic background (*i.e.* 93.75% C57Bl/6 and 6.25% 129SvJ) (12). Mice used in the experiments were 16–20 weeks in age and weighed 20–30 g. They were housed in a facility equipped with a 12-h light cycle, were given unlimited access to water, and were fed a standard pelleted diet *ad libitum*.

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<sup>1</sup> The abbreviations used are: FA, fatty acid; BMIPP, 15-(*p*-iodophenyl)-3-(*R,S*)-methyl pentadecanoic acid; IPPA, 15-(*p*-iodophenyl)pen-

ta-decanoic acid; BSA, bovine serum albumin; DGAT, diacylglycerol acyltransferase; FABP, fatty acid-binding protein; FABPpm, plasma membrane fatty acid-binding protein; and FATP, fatty acid transport protein.

**Analysis of Albumin and Fatty Acids**—Mice were either in the postprandial state or fasted for 8 h. Tail vein blood was collected into heparinized (for albumin determination) or EDTA-containing tubes (for FA determination). Serum was promptly separated from cells and stored at 4 °C. Nonesterified FA was measured by an enzymatic colorimetric assay (NEFA C, Wako Chemicals, New York) and albumin by specific binding to bromocresol green (16).

**BMIPP and IPPA Preparation**—BMIPP and IPPA were radioiodinated by the thallium-iodide exchange method as described previously (17). Purification was done over a Sep-Pak RP-18 Light cartridge (Waters Corp.). The specific activity of [<sup>125</sup>I]BMIPP was typically in the range of 2–4 Ci/mmol. The specific activity of [<sup>131</sup>I]IPPA was typically about 0.5 Ci/mmol. The radiochemical purity of each preparation as determined by TLC was greater than 99%. The purified compounds were dissolved in a minimal amount of warm absolute ethanol and added dropwise to a stirred solution of 6% FA-free bovine serum albumin (BSA) at 40 °C. The solution was sterile-filtered (0.22 μm, Millipore) before injection.

**Tissue Distribution of BMIPP and IPPA**—In each experiment, animals were sex-matched, but similar results were obtained with either sex. Each mouse was injected in a lateral tail vein with 200 μl of the isotope solution (14–75 μCi). The animals were sacrificed by cervical dislocation after 2 h, unless noted otherwise. The tissues were removed, rinsed with saline, and blotted dry. Tissues were weighed and counted in a NaI auto-gamma counter. A sample of the injected solution was also counted to determine the total injected dose. The thyroid was included as an indicator of the level of free iodine in the injected solution. Blood samples to be used for lipid extraction were added directly to vials containing 0.5 ml of a 4 mg/ml EDTA solution.

**Analysis of BMIPP/IPPA Lipid Incorporation**—Lipids were extracted from frozen tissue by the method of Folch *et al.* (18). Aliquots were chromatographed next to known standards on aluminum-backed silica gel plates (Merck, from Analtech, Inc.). A petroleum ether:diethyl ether:glacial acetic acid (70:30:1, v/v/v) solvent system was used to resolve polar lipids, diglycerides, fatty acids, and triglycerides. The distribution of BMIPP in each lipid class was determined as a percentage of total counts on the plate.

**Determination of BMIPP-labeled Acyl-CoA**—For BMIPP-labeled acyl-CoA determination, tissues were excised, rinsed with saline, and promptly frozen in liquid nitrogen. Frozen tissues were ground to a fine powder under liquid nitrogen, and the powdered tissue (20–90 mg) was quickly weighed and homogenized in 100 mM KH<sub>2</sub>PO<sub>4</sub>, pH 4.9 (2 ml). Total long chain acyl-CoA was isolated by solid phase extraction on an oligonucleotide purification cartridge (Applied Biosystems) according to Deutsch *et al.* (19). BMIPP-labeled acyl-CoA was determined by counting total long chain acyl-CoA in a NaI auto-gamma counter.

**Isolation of Adipocytes**—Adipocytes were isolated from epididymal fat pads of age-matched mice by collagenase (type 1, Worthington) digestion (1 mg/ml) in Krebs-Ringer medium lacking phosphate, buffered to pH 7.4 with 10 mM HEPES (KRH), and containing 2% BSA (fraction V, fatty acid-free), 2 mM glucose, and 200 nM adenosine to inhibit lipolysis. Isolated cells were washed three times with KRH with 2% BSA and then twice with KRH with 0.1% BSA and were suspended (30% v/v) in the 0.1% BSA KRH buffer. Cell density, or lipocrit, was estimated from centrifugation of 8-μl of mixed cell suspension in a hematocrit tube.

**Assay of Palmitate Uptake and Lipid Incorporation into Isolated Adipocytes**—Uptake of [<sup>3</sup>H]palmitate was evaluated at 25 °C as described previously (2). Briefly, 30 μl of the mixed cell suspension was added to 30 μl of transport solution containing palmitate (4000 cpm/μl) complexed to BSA at an FA to BSA molar ratio of 0.25. Uptake was stopped after 10–60 s by addition of 3 ml of cold buffer, and cells were separated from the medium by low pressure vacuum (about 50 mm Hg) filtration (Gelman A/E filters). Cell-associated radioactivity was obtained by counting the washed filters in 4 ml of aqueous scintillation fluid (Amersham Pharmacia Biotech) in a Beckman LS330 scintillation counter. Zero time radioactivity was determined from samples where cold buffer was added before cells.

To determine palmitate incorporation into lipids, cells were incubated with [<sup>3</sup>H]palmitate/BSA as described above, except that incubations were for 15–30 min. Cells on the filters were Folch-extracted and the lipids analyzed by TLC on glass-backed silica gel 60 plates (Whatman). Lipid classes were resolved using a petroleum ether:diethyl ether:glacial acetic acid (70:30:1, v/v/v) solvent system. The plates were marked in 55 equal fractions, which were scraped and counted in 4 ml of aqueous scintillation fluid. Peaks were identified by standards run on each plate stained separately with iodine. This procedure gave clear separation between the major lipid fractions of interest as shown in Fig.

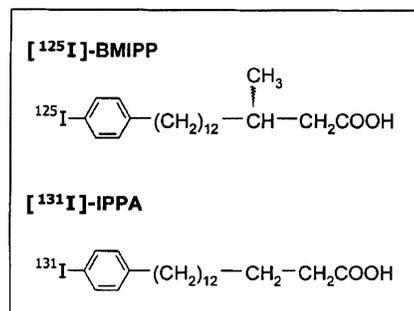


FIG. 1. Chemical structures of the FA analogs [<sup>125</sup>I]BMIPP and [<sup>131</sup>I]IPPA. The 3-methyl group of BMIPP inhibits  $\beta$ -oxidation resulting in prolonged tissue retention without affecting its incorporation into complex lipids. The straight chain analog IPPA is rapidly oxidized in tissues, and its incorporation into cardiac lipids is similar to native long chain fatty acids.

4. The distribution of [<sup>3</sup>H]palmitate in each lipid class was determined as a percentage of total counts on the plate.

**Preparation of Microsomes for Assays of Enzymatic Activity**—Microsomes were prepared as described by Coleman (20) and stored in aliquots at –70 °C until use. Protein was determined by the Peterson-modified Lowry assay (21) following lipid removal and protein precipitation according to Wessel *et al.* (22). BSA was used as a standard.

**Assay of Diacylglycerol Acyltransferase Activity**—Microsomal diacylglycerol acyltransferase activity was determined as described by Coleman (20). Control reactions containing no microsomal protein were performed in parallel to provide a background for each point. Under the assay conditions given the reaction followed zero-order kinetics with respect to substrate concentrations. Tissue-specific activities are expressed as nanomoles of triglyceride produced per min per mg of protein.

**Assay of Acyl-CoA Synthetase Activity**—Microsomal long chain acyl-CoA synthetase activity was determined according to the procedure of Tanaka *et al.* (23) except that [<sup>3</sup>H]palmitate was solubilized with FA-free BSA in a 3:1 FA to BSA molar ratio rather than with Triton X-100 as described. Control reactions containing no microsomal protein were performed in parallel to provide a background for each point. Under the assay conditions given the reaction followed zero-order kinetics with respect to substrate concentrations. Tissue-specific activities are expressed as nanomoles of acyl-CoA produced per min per mg of protein.

**Statistical Analyses**—Results are presented as means  $\pm$  S.E. The significance of differences between means was analyzed by the unpaired Student's *t* test (two-tailed).

**Materials**—All reagents and standards were of the highest purity available. Iodine-125, iodine-131, [<sup>14</sup>C]palmitoyl-CoA, and [<sup>3</sup>H]palmitate were from NEN Life Science Products. All other reagents were obtained from Sigma unless otherwise noted.

## RESULTS

**FA Uptake Is Reduced in Muscle and Adipose Tissues of CD36 Null Mice**—To evaluate directly the contribution of CD36 to FA utilization by various tissues *in vivo*, we compared the biodistribution of the slowly oxidized FA analog [<sup>125</sup>I]BMIPP (shown in Fig. 1) between wild type and CD36 null mice. The usefulness of BMIPP as a metabolic tracer for FA utilization has been demonstrated extensively in studies on both humans and laboratory animals (24). Like native FA, tissue extraction of BMIPP from the blood equilibrates within 2–3 min (25, 26). The inhibitory effect of the 3-methyl group on  $\beta$ -oxidation results in prolonged tissue retention of the FA without affecting its incorporation into phospholipids, diglycerides, or triglycerides (27). The stable iodination of BMIPP coupled with its prolonged tissue retention make it an ideal tracer for sensitive comparisons of tissue capacities for FA uptake *in vivo*.

Mice were injected with BMIPP in the postprandial state since under these conditions serum FA concentrations were not significantly different between null and wild type animals (Table I). The biodistribution data for BMIPP are shown in Fig. 2.

TABLE I  
Serum albumin and fatty acid concentrations for CD36 null and wild type mice

Values are means  $\pm$  S.E. The number of animals sampled is given in parentheses.

	Serum albumin <i>mg/dl</i>	Fasted (8-h)		Fed	
		Serum FA	FA:albumin	Serum FA	FA:albumin
		<i>mM</i>		<i>mM</i>	
Wild type					
Male	2.7 $\pm$ 0.3 (5)	1.73 $\pm$ 0.09 (10)	4.3 $\pm$ 0.5	0.44 $\pm$ 0.06 (6)	1.1 $\pm$ 0.2
Female	2.5 $\pm$ 0.2 (4)	0.96 $\pm$ 0.06 (10)	2.6 $\pm$ 0.3	0.24 $\pm$ 0.04 (7)	0.6 $\pm$ 0.1
CD36 Null					
Male	2.3 $\pm$ 0.1 (5)	3.3 $\pm$ 0.1 (11)	9.6 $\pm$ 0.5	0.47 $\pm$ 0.04 (6)	1.4 $\pm$ 0.1
Female	2.9 $\pm$ 0.1 (5)	2.12 $\pm$ 0.08 (20)	4.9 $\pm$ 0.2	0.28 $\pm$ 0.03 (8)	0.65 $\pm$ 0.07

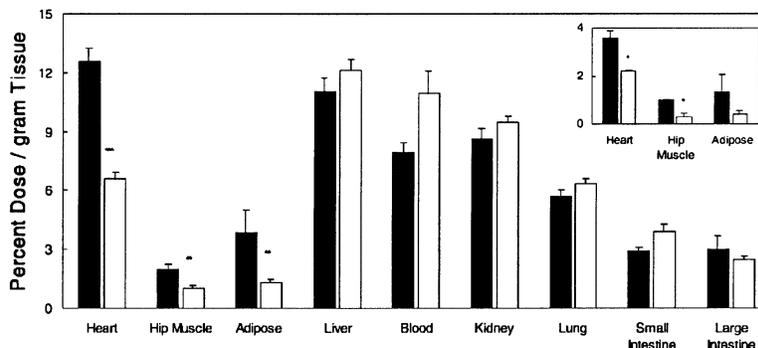


FIG. 2. The biodistributions of BMIPP and IPPA show significantly decreased uptake in CD36 null heart, muscle, and fat tissues. Equivalent results were obtained with BMIPP and IPPA (*inset*) although the recovery of IPPA is greatly reduced by its rapid oxidation. No significant differences in uptake were observed with either FA in tissues that do not express CD36 in the wild type mouse. For the experiments shown, mice were injected in the postprandial state with 42  $\mu$ Ci of [ $^{125}$ I]BMIPP or 75  $\mu$ Ci of [ $^{131}$ I]IPPA (*inset*) suspended in 200  $\mu$ l of a 6% FA-free BSA saline solution. Tissues were removed 2 h after injection. Uptake is expressed as percent of injected dose per g of tissue. Means are shown  $\pm$  S.E. Results are from one experiment typical of three others. ■, wild type (BMIPP,  $n = 5$ ; IPPA,  $n = 2$ ); □, CD36 null (BMIPP,  $n = 10$ ; IPPA,  $n = 2$ ). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; and \*\*\*,  $p < 0.0001$ .

Of the nine tissues examined, significant impairment of BMIPP uptake was observed only in the heart, skeletal muscle, and fat of CD36 null mice. In these tissues, uptake was reduced by 50–80% in comparison to wild type controls. In muscle tissues, the magnitude of the defect increased with increasing oxidative capacity of the muscle (Fig. 3), consistent with the pattern of CD36 expression (28). For example, diaphragm muscle, which in the mouse is almost exclusively (95.8%) oxidative (29), exhibited a defect in BMIPP uptake nearly 3 times that of hip muscle, which is predominantly glycolytic (30).

**Triglyceride Synthesis Is Inhibited in Adipose and Muscle from CD36 Null Mice**—Previous results with mice overexpressing CD36 in muscle tissues suggested that uptake may play a rate-limiting role in determining FA oxidation in muscle (13). To determine whether FA esterification in muscle and adipose tissues is similarly limited by FA uptake, we examined whether these tissues from CD36 null mice exhibit significant reductions in FA incorporation into complex lipids. Uptake and metabolism of the native FA palmitic acid were compared in adipocytes isolated from wild type and CD36 null mice. In agreement with the BMIPP data, adipocytes from CD36 null mice exhibited a 60% decrease in the uptake of [ $^3$ H]palmitate (data not shown). Evaluation of lipid extracts by TLC (Fig. 4) showed that the percentage of [ $^3$ H]palmitate present as free, unesterified FA was 75% lower in adipocytes isolated from CD36-deficient mice as compared with the wild type controls. The incorporation of [ $^3$ H]palmitate into triglycerides was decreased by 24% in CD36 null adipocytes, whereas incorporation into diglycerides was increased by more than 3-fold.

To determine whether the altered lipid incorporation observed in isolated adipocytes was representative of lipid incorporation in intact tissues *in vivo*, we analyzed the lipid pool distribution of BMIPP from several tissues. No differences

were observed between wild type and CD36 null mice in blood, liver (Table II), or lung (not shown). In contrast, CD36-deficient heart, skeletal muscle, and adipose tissues (Table II) exhibited dramatic decreases in labeled triglycerides (63, 31, and 50%, respectively) with equally dramatic increases in labeled diglycerides (3.1-, 1.5-, and 1.8-fold, respectively). In CD36-deficient heart muscle the diglyceride/triglyceride ratio was increased 8-fold in comparison to wild type. No statistically significant differences were observed in BMIPP recovered as free FA, although the general trend in all experiments was toward lower levels in adipose and muscle tissues of CD36 null mice. BMIPP incorporation into polar lipids, which do not migrate in the solvent system used and would include phospholipids, monoglyceride 3-phosphate, and phosphatidic acid, was not significantly altered except in skeletal muscle where a decrease was observed.

To confirm these results, mice were co-injected with [ $^{125}$ I]BMIPP and the straight chain analog [ $^{131}$ I]IPPA (Fig. 1). IPPA has been used extensively for evaluation of cardiac metabolism. In contrast to BMIPP, it is rapidly oxidized in tissues. Its fractional distribution in cardiac lipids has been shown to closely agree with values reported for [ $^3$ H]oleic acid and [ $^{14}$ C]palmitic acid (31). The biodistribution of IPPA (Fig. 2, *inset*) and its lipid incorporation (not shown) were identical to results obtained with BMIPP.

**Decreased Triglyceride Synthesis in CD36 Null Adipose and Muscle Is a Result of Limiting Acyl-CoA**—The biosynthetic pathway for triglyceride, shown in Fig. 5, highlights the steps that are altered in tissues of CD36-null mice. The observation that diglyceride accumulation was coupled to a decrease in triglycerides indicated that triglyceride synthesis was inhibited at the level of diacylglycerol acyltransferase (DGAT). To determine if this reflected a tissue-specific down-regulation of

the enzyme in CD36 null mice, we assayed this enzyme in microsomal fractions derived from several tissues. As seen in Fig. 6A, no significant difference in specific activity was observed between null and wild type mice in any of the tissues tested.

This suggested that the inhibition of DGAT activity was at the substrate level. Because diglycerides accumulated in these tissues, inhibition was a likely result of decreased acyl-CoA. As expected, CD36 null tissues exhibiting a defect in BMIPP uptake showed a decrease of the same magnitude in BMIPP-labeled acyl-CoA (data not shown). To determine if the reduction in labeled acyl-CoA could be directly attributed to the defect in FA uptake, we assayed the activity of microsomal long chain acyl-CoA synthetase from several tissues. As with DGAT, no significant differences were observed between null and wild type tissues (Fig. 6B).

#### DISCUSSION

The present study was undertaken to evaluate the role of CD36 in FA uptake and metabolism *in vivo* and to examine directly the association between CD36 deficiency and depressed myocardial FA uptake in humans (15). CD36-mediated

uptake was determined *in vivo* by comparing the biodistribution of BMIPP and IPPA in tissues from CD36 null and wild type mice and *in vitro* by examining [ $^3$ H]palmitate utilization by adipocytes isolated from both groups. Defects in uptake were confined to tissues where CD36 expression is normally high (fat, heart, and skeletal muscle), whereas no defect was observed in tissues where CD36 expression is low or undetectable (liver, kidney, lung, and large intestine). Of notable exception was the small intestine, which in the wild type mouse exhibits a high expression of CD36 (8, 11). As shown in Fig. 2, the small intestine from null mice showed no reduction in BMIPP uptake. Although this may indicate a limited contribution of CD36 to FA uptake in this tissue, a more likely explanation is that it reflects the distribution of CD36 in the intestine, where it is localized to the luminal membrane of brush border cells (11). Contribution of CD36 to FA uptake in the small intestine is therefore most likely confined to the absorption of dietary FA and would not have been apparent with intravenously administered BMIPP.

Although a physical chemistry analysis of the process of FA diffusion through the bilayer suggests that proteins are not in principle essential for the transport of FA across membranes (32, 33), an abundance of biochemical data supports the existence of a protein component in the membrane transfer of long chain FA (reviewed in Ref. 34). The data presented here indicate that a membrane protein facilitating uptake (*i.e.* CD36) is essential for normal rates of FA uptake by fat and muscle tissues *in vivo*. At FA to albumin ratios less than unity, the concentration of unbound FA in the plasma and interstitium is exceedingly low (1–10 nM) (7). Membrane proteins such as CD36 with high affinity for FA (35) may be required to compete effectively with albumin for uptake of FA. An additional function may be to aid in channeling the FA to metabolic sites thereby preventing efflux back into the albumin-containing medium. For example, interactions between CD36 and cytosolic FA-binding proteins (FABPs) may account for the similarly dramatic defects in BMIPP uptake observed in heart and skeletal muscle of mice lacking heart-type FABP (36). Indeed,

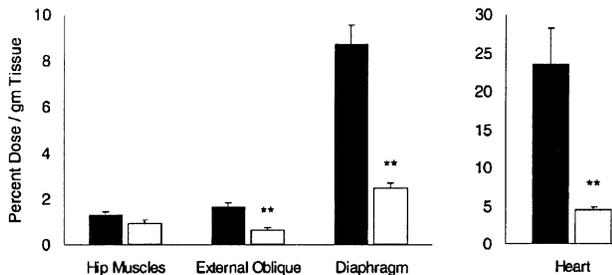


FIG. 3. The defect in BMIPP uptake in CD36 null muscle increases with increasing oxidative capacity of the muscle. Muscles are shown in order of increasing oxidative capacity (29, 30). Mice were injected with 14  $\mu$ Ci of [ $^{125}$ I]BMIPP. Tissues were removed 30 min after injection. Uptake is expressed as percent of injected dose per g of tissue. Values are means  $\pm$  S.E. ■, wild type ( $n = 8$ ); □, CD36 null ( $n = 6$ ). \*\*,  $p < 0.01$ .

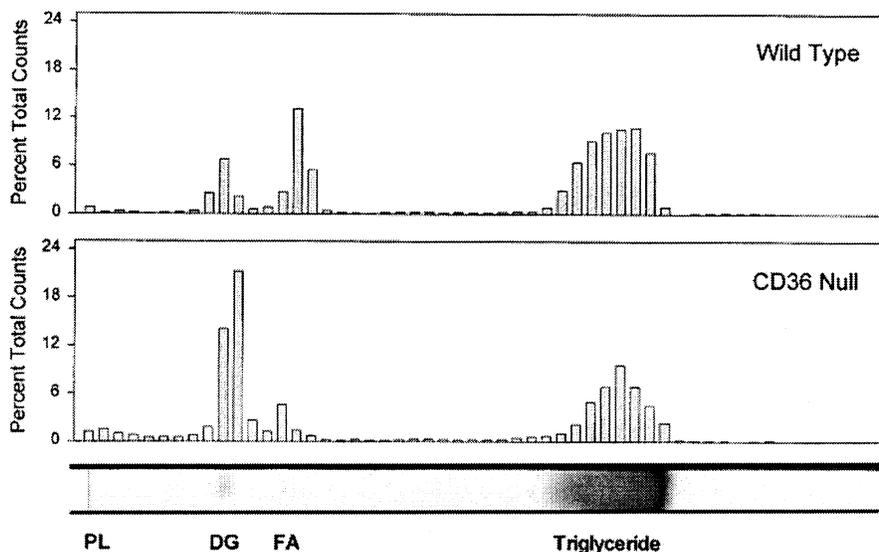


FIG. 4. TLC analysis of FA incorporation into complex lipids in isolated adipocytes. Isolated adipocytes were incubated for 30 min with a [ $^3$ H]palmitate solution complexed with BSA in an FA to BSA molar ratio of 0.25. The cells were filtered and washed, and lipids were Folch-extracted. An aliquot of extracted lipid (20,000 cpm) was analyzed by TLC for [ $^3$ H]palmitate incorporation. Lanes were divided into 3-mm fractions, scraped, and counted for activity. Peak fractions were identified by standards run on each plate. A representative lane stained with iodine is shown aligned with the activity profiles. The data are representative of three separate determinations using fat pads pooled from three mice per group. They are typical of three other experiments. PL, polar lipids; DG, diglyceride.

TABLE II  
The lipid pool distribution of BMIPP

Tissue samples from the experiment shown in Fig. 2 were Folch-extracted and the lipids evaluated by TLC for BMIPP incorporation. Values reflect the percent of total counts on the TLC plate recovered in each lipid fraction. Polar lipids include phospholipids, phosphatidic acid, and monoacylglycerol 3-phosphate. Means, shown  $\pm$  S.E., are from one experiment typical of two others.

	Fatty acid	Diglyceride	Triglyceride	Polar lipids
Wild type ( $n = 3$ )				
Blood	11 $\pm$ 4	47 $\pm$ 3	20 $\pm$ 2	15.5 $\pm$ 0.5
Liver	1.8 $\pm$ 0.2	34 $\pm$ 2	27 $\pm$ 3	35 $\pm$ 4
Adipose	2.6 $\pm$ 0.2	34 $\pm$ 6	60 $\pm$ 7	3.3 $\pm$ 0.6
Skeletal muscle	2.65 $\pm$ 0.07	50 $\pm$ 5	27 $\pm$ 5	18.6 $\pm$ 0.5
Heart	3.1 $\pm$ 0.5	17 $\pm$ 5	65 $\pm$ 9	14 $\pm$ 3
CD36 Null ( $n = 5$ )				
Blood	8 $\pm$ 2	47 $\pm$ 3	24 $\pm$ 2	15.8 $\pm$ 0.5
Liver	1.5 $\pm$ 0.5	38 $\pm$ 1	26 $\pm$ 2	33 $\pm$ 1
Adipose	1.8 $\pm$ 0.4	60 $\pm$ 3 <sup>a</sup>	31 $\pm$ 3 <sup>a</sup>	5.5 $\pm$ 0.7
Skeletal muscle	2.6 $\pm$ 0.1	76 $\pm$ 1 <sup>a</sup>	8.5 $\pm$ 0.6 <sup>a</sup>	9.4 $\pm$ 0.9 <sup>a</sup>
Heart	1.9 $\pm$ 0.3	52 $\pm$ 4 <sup>a</sup>	24 $\pm$ 3 <sup>a</sup>	11 $\pm$ 1

<sup>a</sup>  $p < 0.005$ .

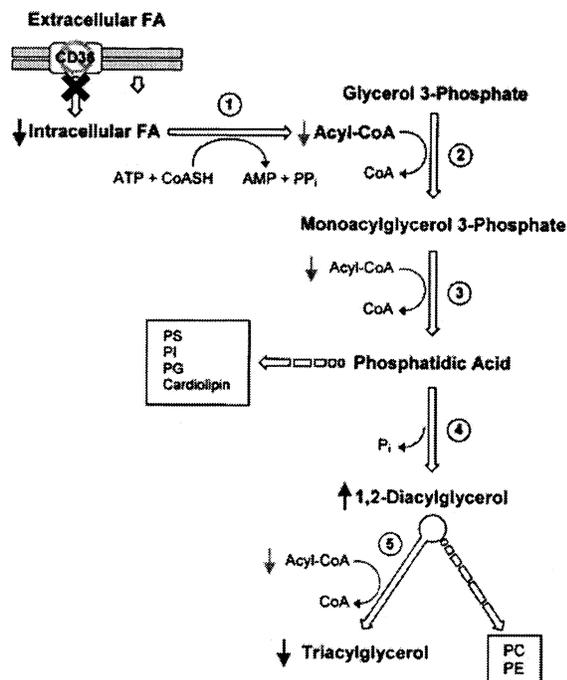


FIG. 5. The triacylglycerol biosynthetic pathway. Diagram of the FA esterification pathway showing key steps and highlighting those altered in muscle and adipose tissues of CD36 null mice. The enzyme diacylglycerol acyltransferase catalyzes step 5 at the main bifurcation between triglyceride and phospholipid formation. Step 1, acyl-CoA synthetase; step 2, glycerol-3-phosphate acyltransferase; step 3, monoacylglycerol-3-phosphate acyltransferase; step 4, phosphatidic acid phosphatase; step 5, diacylglycerol acyltransferase. Broken arrows represent steps not shown. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol.

CD36 has been shown by co-immunoprecipitation to associate with cytosolic FABP in mammary epithelial cells (37).

Clearly some portion of the FA uptake observed in muscle and adipose tissues does represent the simple diffusion of FA across the membrane. However, given the FA to serum albumin ratios (0.6–1.4) measured in the mice in the postprandial state (Table I), the contribution of passive diffusion is expected to be low. Studies with isolated cells have shown that passive diffusion contributes less than 15% to uptake at FA to albumin ratios below unity (1, 3). The contribution of other known FA transporters expressed in these tissues, plasma membrane

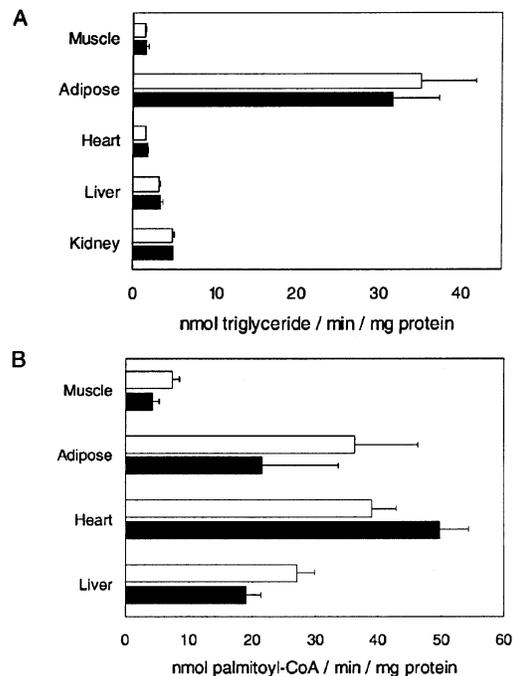


FIG. 6. Activities of the microsomal enzymes (A) DGAT and (B) long chain acyl-CoA synthetase. Activities of both enzymes are not significantly different between wild type and CD36 null tissues. Assays were performed under conditions of zero-order kinetics with respect to substrate concentrations. Values are the means of measurements done in triplicate on tissues from 4 wild type (■) and 4 CD36 null mice (□) and are shown  $\pm$  S.E.

FABP (FABPpm) (4) and fatty acid transport protein (FATP) (38), may account for much of the residual uptake observed in the absence of CD36. It is apparent, however, that the contributions of CD36, FABPpm, and FATP to FA uptake in muscle and adipose tissues are not entirely redundant. Otherwise, these tissues would be able to compensate more effectively for the lack of CD36 expression. It is also possible that the three proteins may work synergistically to enhance FA uptake and that deletion of either FABPpm or FATP alone would have consequences similar to those shown here.

A major goal of this study was to utilize the CD36 knockout mouse to evaluate directly the apparent association between CD36 deficiency and defective myocardial uptake of FA reported in humans (15). Co-existence of the two defects could have been coincidental or secondary to a third and primary

defect. Our results definitively show that the reported association between CD36 deficiency and defective myocardial BMIPP uptake in humans does indeed reflect a direct causal relation. Interestingly, the depression in BMIPP uptake observed in hearts of null mice is of identical magnitude to that reported in CD36-deficient humans (39). The CD36 null mouse may therefore represent an appropriate model for examining the role of CD36 in myocardial FA utilization in humans as well as for studying cardiomyopathies thought to result from depressed myocardial FA utilization (40).

Our results further suggest that defects in FA utilization in humans with CD36 deficiency would also extend to skeletal muscle and adipose tissues. Decreased FA metabolism by non-hepatic peripheral tissues on the large scale observed in CD36 null mice would undoubtedly impact glucose and amino acid homeostasis and might predispose deficient individuals to the development of metabolic disorders such as obesity and insulin resistance. CD36 deficiency has recently been linked to defective FA metabolism in spontaneously hypertensive rats and may contribute to the insulin resistance observed in this rodent model of type II diabetes (41). In line with this, CD36 null mice<sup>2</sup> and transgenic mice overexpressing CD36 (13) exhibit altered levels of glucose and insulin. The possible role of CD36 in the development of insulin resistance is currently under investigation.

The atypical lipid incorporation of tritiated palmitate and of the FA analogs BMIPP and IPPA observed in adipose and muscle tissues from null mice nicely illustrates the mechanisms controlling cellular FA esterification. In these tissues, a block in diglyceride to triglyceride conversion occurred despite normal specific activities of the key enzymes long chain acyl-CoA synthetase and DGAT. Since DGAT was presumably saturated with diglyceride, the data strongly suggest that the rate of triglyceride synthesis is determined by FA supply and a relatively low affinity of DGAT for long chain acyl-CoA. The lower affinity of DGAT for acyl-CoA as compared with other enzymes in the FA esterification pathway (Fig. 5) has been suggested from results of enzymatic assays in intact and permeabilized hepatocytes (42, 43). However, the findings presented here mark the first demonstration of a key regulatory role for DGAT *in vivo* and point to the pivotal role of membrane FA transport in maintaining acyl-CoA at the levels required for optimal DGAT activity.

Regulation of FA esterification at the branch point between phospholipid and triglyceride synthesis makes sense physiologically since it would serve to first secure FA for pathways essential to the cell, namely  $\beta$ -oxidation and phospholipid synthesis. Only when the FA needs of these pathways are met, as reflected by a rise in long chain acyl-CoA, would triglyceride synthesis proceed optimally through DGAT. This would ensure that triglyceride deposition would not compete with  $\beta$ -oxidation when the FA supply is low and might explain why CD36-deficient animals appear healthy under normal and non-metabolically challenged conditions. However, it is likely that CD36 null animals may be unable to adapt as efficiently as wild type animals to fasting, exercise, or high fat diets. Furthermore, although CD36 deficiency by itself may be largely asymptomatic, if present with other metabolic defects it could conceivably give rise to an overtly abnormal phenotype such as insulin resistance.

In summary, the present study demonstrates *in vivo* that CD36 mediates a major fraction of the FA uptake by myocardial, skeletal muscle, and adipose tissues. The data firmly

establish a causal link between defective myocardial FA uptake and CD36 deficiency in humans (15). It is further shown that FA esterification to triglycerides in these tissues is regulated at the level of DGAT and that facilitated uptake of FA is necessary to maintain acyl-CoA at the levels required for optimal activity of this enzyme. These findings together with the previously documented rate-limiting role of membrane transport in muscle FA oxidation (13) would indicate that the transport step is a potentially important regulatory site for FA metabolism. Changes in CD36 expression associated with different nutritional and metabolic factors as well as resulting from pharmacological agents (44–49) may be responsible for much of their observed physiologic effects.

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