A Null Mutation in Murine CD36 Reveals an Important Role in Fatty Acid and Lipoprotein Metabolism*

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A null mutation in the scavenger receptor gene CD36 was created in mice by targeted homologous recombination. These mice produced no detectable CD36 protein, were viable, and bred normally. A significant decrease in binding and uptake of oxidized low density lipoprotein was observed in peritoneal macrophages of null mice as compared with those from control mice. CD36 null animals had a significant increase in fasting levels of cholesterol, nonesterified free fatty acids, and triacylglycerol. The increase in cholesterol was mainly within the high density lipoprotein fraction, while the increase in triacylglycerol was within the very low density lipoprotein fraction. Null animals had lower fasting serum glucose levels when compared with wild type controls. Uptake of 3H-labeled oleate was significantly reduced in adipocytes from null mice. However, the decrease was limited to the low ratios of fatty acid/bovine serum albumin, suggesting that CD36 was necessary for the high affinity component of the uptake process. The data provide evidence for a functional role for CD36 in lipoprotein/fatty acid metabolism that was previously underappreciated.

Scavenger receptors are integral membrane glycoproteins, distinct from the classic low density lipoprotein (LDL) receptor, that mediate binding and uptake of native and modified lipoproteins by macrophages (1–8). There are at least two major classes of mammalian monocyte/macrophage scavenger receptors, SR-A and SR-B, based on molecular sequence and protein structural homology (1, 2, 9–11). Scavenger receptors have broad ligand specificity and may have evolved from the primitive immune system as pattern recognition molecules, which are able to recognize common structural motifs on microbial surfaces (1, 6, 12–17). They also function in the recognition and clearance of damaged, senescent, or apoptotic cells before lysis, tissue damage, and inflammation can ensue (11, 18–21) and in the modulation of cytokine release and host immune responses (14, 15, 22). Scavenger receptors may be important in the pathogenesis of atherosclerosis, since there is significant evidence in support of the hypothesis that uptake of oxidatively modified LDL by monocytes/macrophages is one of the key early events in lesion development (23–26).

The class A receptors, which are expressed on liver sinusoidal endothelial and Kupffer cells (27–29), and monocytes/macrophages (9, 10, 30) result from an alternative splice from a single gene (31, 32). SR-AI/II are trimeric, integral membrane glycoprotein receptors for oxidized LDL, acetylated LDL, and other anionic ligands including polyinosinic acid and maleylated albumin (5, 9, 10, 33–35). Monocytes/macrophages isolated from a null mouse carrying a mutation in the class A receptors showed partial loss in the ability to bind and internalize oxidized LDL (~30%) (36), and a lack of murine SR-AI/II receptors in the context of an atherogenic environment was partially protective against the formation of atherosclerotic lesions, decreasing lesion size by 40% (15).

Although functionally related to SR-AI/II, the class B receptors differ significantly in structure. CD36, the defining member of this class, is a highly glycosylated, single chain 88-kDa protein that binds oxidized LDL, fatty acids, anionic phospholipids (including phosphatidylinositol and phosphatidylserine) and the proteins collagen and thrombospondin (2, 37–40). As a result of the broad ligand specificity of CD36, multiple roles for this protein have been proposed. In vitro and in vivo studies indicate that a significant proportion of binding and internalization of oxidized LDL by tissue-differentiated macrophages occurs through CD36 (41–43). Antibodies to CD36 have been shown to block up to 50% of oxidized LDL binding and uptake in normal peripheral blood-derived macrophages (41, 42). Monocytes/macrophages from donors with a human polymorphism associated with lack of CD36 expression (Nak represents the bound and internalized 40% less oxidized LDL and accumulated 40% less cholesterol ester (43).

Recently, CD36 has been shown to be involved in the differentiation of monocytes and accumulation of lipid in macrophages as a result of exposure to oxidized LDL, and through the induction of the peroxisome proliferator-activated receptor γ (PPARγ) (44, 45), a member of the nuclear hormone receptor superfamily (46–52). This receptor also strongly regulates expression of genes involved in lipid metabolism; PPARγ heterodimerizes with the 9-cis-retinoic acid receptor and has been shown to be essential in the regulation of the differentiation of adipocytes and cellular lipid uptake by these cells (49, 53, 54). In addition to its role as a scavenger receptor, CD36 is also one of five structurally unrelated proteins that have been proposed to function as long chain fatty acid transporters and facilitate...
uptake of long chain fatty acids in adipocytes, mammary epithelia, small intestine, and cardiac and skeletal muscle (55–58). The physiologic role of these transporters remains controversial, however, because there is indirect evidence in support of an alternative theory that fatty acids may freely diffuse into cells (59–61). The evidence that CD36 plays a role in fatty acid transport includes its identification by reaction with a fatty acid derivative and subsequent binding of fatty acid in vitro (62–64). The expression of CD36 correlates strongly with tissues active in fatty acid metabolism, and it predominates in oxidative red muscle fibers (55, 56). CD36 is up-regulated, together with fatty acid uptake, by electrical stimulation and muscle contraction and is increased in the muscle of diabetic animals and those fed a high fat diet (65, 66). Association between CD36 deficiency or mutation with hypertrophic cardiomyopathy has been reported in humans and linked to impaired uptake of long chain fatty acid by the myocardium (67–69).

There is mounting evidence implying at least a correlation between expression of these putative long chain fatty acid transporters such as CD36, and adipocyte transcriptional regulators, such as PPARγ and others (70, 71), and thus a role for these proteins in adipocyte differentiation (55, 72, 73). These reports and those involving macrophage storage of oxidized LDL imply new parallels in two very different contexts of lipid storage and suggest a role for CD36 in normal lipid metabolism that has been thus far unappreciated.

To further characterize the role of CD36 in fatty acid and lipoprotein metabolism and oxidized LDL binding and internalization, we have generated a mouse null for CD36 by homologous recombination. Our data show that monocytic/macrophages from these mice had an impaired ability to bind and internalize oxidized LDL, a well characterized function of CD36. Adipocytes from null mice exhibited a significantly decreased ability to transport the long chain fatty acid, oleate, at low fatty acid:BSA ratios. Incorporation of oleate into triacylglycerol was reduced in adipocytes from null mice, while incorporation into diacylglycerol was increased. These animals had an increase in fasting serum/plasma cholesterol, an increase in nonesterified free fatty acid and triacylglycerol levels, and a decrease in fasting plasma glucose, revealing a more significant role for CD36 in lipoprotein and fatty acid metabolism than previously appreciated.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture reagents including leukemia-inhibitory factor (Esaro) were obtained from Life Technologies, Inc.; bovine serum was from Gemini Bio-Sciences, Inc.; total cholesterol, triacylglycerol, albumin, and glucose quantitative assay kits and calibrators and miscellaneous chemicals were from Sigma; standards and a quantitative assay kit for serum nonesterified fatty acids were from Wako; FITC-conjugated goat anti-rabbit IgG was from Kirkegaard and Perry Laboratories; rabbit anti-rat CD36 IgG was prepared as described by Ryeom et al. (74); FITC-conjugated rat anti-mouse SR-A antibody (2F8) was from Serotec, Ltd.; carrier-free 125I-NaCl was from Amersham Pharmacia Biotech; [α-32P]dCTP, 14C-labeled palmitate, and 3H-labeled oleate were from NEN Life Science Products; the targeting vector, pPNT, was a gift from H.-C. Liou (Weill Medical College of Cornell University); the murine CD36 cDNA was a gift from G. Endemann (Scios Nova Inc.); CJ7 embryonic stem (ES) cells, passage 7, and neo−/− embryonic fibroblasts as described (76). For electroporation, ES cells were resuspended at a concentration of 2.5 × 10^6/ml in phosphate-buffered saline (PBS) lacking calcium and magnesium. 0.9-mM aliquots were mixed with 20 μg of the linearized targeting vector and pulsed at 250 V, 500 microfarads (Bio-Rad Gene Pulser). The cells were then diluted in culture medium and plated at 5 × 10^5 cells/100-mm dish. After 24 h, G418 (Life Technologies) selection (250 μg/ml) was begun, and selection with ganciclovir (2 μM; Cytovene from Syntex Laboratories, Inc.) began on day 3. Resistant colonies were picked on days 8–10, dissociated with trypsin, and divided into two wells of a 96-well plate. Upon confluence, one well of ES cells was frozen and the other was expanded for DNA isolation and Southern blot analysis.

Genomic DNA was prepared from ES cells and tail biopsies by digestion at 55 °C with proteinase K. Approximately 10 μg of genomic DNA were digested with the appropriate restriction endonuclease, electrophoresed through a 1% agarose gel, transferred to Zeta Probe GT (Bio-Rad), and hybridized with the random primed 32P-radiolabeled probe indicated in Fig. 1. The probe/enzyme strategy was such that a different size restriction fragment would result from the endogenous locus, integration of the plasmid nonspecifically, and homologous recombination. A second probe/restriction enzyme strategy and probes to both the thymidine kinase and neo' genes were used to ensure that homologous recombination and not random integration of the targeting vector had occurred (data not shown). Primers specific to exon 3 were designed, and these failed to give rise to product from genomic DNA of null mice in polymerase chain reactions while yielding a fragment of the correct size in wild type animals (data not shown). Two separate ES cell clones were injected into C57BL/6 blastocysts as described (76) and resulted in chimeras with 75–99% agouti coats. Male chimeras were bred with C57BL/6 females, and agouti offspring were screened for the presence of the mutated CD36 gene by Southern blot hybridization. Offspring heterozygous for the mutation were interbred, and mice homozygous for the CD36 disrupted allele were identified by Southern blot hybridization.

**Immunoprecipitation and Western Blot Analysis**—Tissues were excised from euthanized mice after perfusion with PBS and resuspended in radiomunue precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 1 μg/ml aprotinin, and 2 μg/ml pepstatin). The tissue was once homogenized, insoluble material was removed by centrifugation, and the extracts were stored at −20 °C. Protein concentration was determined using the BCA reagent (Pierce). Immunoprecipitation was carried out using a polyclonal rabbit anti-rat CD36 antiserum (74), followed by incubation with protein A-Sepharose. After immunoprecipitation for 2 h at 4 °C, beads were thoroughly washed, and bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with the same antisem. Proteins were visualized with an ECL detection system (Bio-Rad). Human and mouse platelets served as positive controls. To demonstrate specificity, an identical blot was incubated with normal rabbit serum. Isolation, Labeling, and Oxidation of LDL—LDL (density 1.019–1.063 g/ml) was prepared from human plasma and isolated by sequential ultracentrifugation, extensively dialyzed against HEPES-buffered saline containing 0.3 mM EDTA, sterilized by filtration (0.22 μm), and stored under nitrogen gas at 4 °C. Protein concentration was determined using the BCA reagent. LDL was iodinated by the method of Goldstein et al. (77), using carrier-free 125I-NaCl. LDL and 125I-LDL were oxidized by dialyzing against 5 μmol/liter CuSO4 in PBS at 37 °C for 16 h. The purity and charge of the lipoproteins were evaluated by forming nitrocellulose electrophoretic gels in an agarose gel. The degree of oxidation was determined by measuring the amount of thiobarbituric acid-reactive substances. LDL had thiobarbituric acid-reactive substances of <1 nmol/mg. Oxidized LDL had thiobarbituric acid-reactive substances of >10 and <30 nmol/mg. The specific activity of the 125I-oxidized LDL was approximately 0.06–0.1 μCi/μg. Isolation and Cultivation of Monocytes/Macrophages—Resident
peritoneal macrophages and thioglycolate-elicited macrophages (4 days after intraperitoneal injection of 2–4 ml of 4% sterile thioglycolate solution) were collected by lavage into ice-cold PBS and cultured in Dulbecco’s modified Eagle’s medium containing 5% heat-inactivated fetal bovine serum, 1% glutamine, and 1% penicillin/streptomycin for 1–2 days.

Flow Cytometry Analysis—Blood was collected into heparinized syringes by heart puncture. Buffy coat cells were collected after centrifugation and incubated with rabbit anti-rat CD36 antiseraum (74) or PBS for 30 min at room temperature, followed by incubation with FITC-conjugated goat anti-rabbit IgG for 30 min. The cells were fixed with 1% fresh paraformaldehyde (PFA) to prevent loss of identity during the washing steps. The body-reactive population was accomplished on a Coulter Epics XL flow cytometer. The sizing parameters were set to analyze the mononuclear fraction of cells. Approximately 30,000 cells were analyzed in each group.

Thioglycolate-elicited macrophages were harvested as above and incubated with anti-SR-A/II (2F8) in PBS containing 0.5% BSA or with FITC-goat anti-rabbit secondary antibody alone for 30 min at 25 °C. After washing twice with PBS, cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry. Approximately 5000 cells were analyzed per group.

Cell Association of Lipoproteins by Peritoneal Macrophages—Cell association of 125I-oxidized LDL was carried out by the methods of Goldstein et al. (77). Cold ligand (100-fold excess) and fucoidan (50 μg/ml) were added 15 min prior to labeled ligand. Incubations for 5 h at 37 °C in serum-free medium, after which the cells were washed three times with PBS and solubilized in 0.1 N NaOH. Binding of 125I-oxidized LDL was carried out at 4 °C for 1 h, using ice-cold reagents, after which cells were washed and solubilized as above. To ensure that no internalization and degradation occurred during these experiments, non-trichloroacetic acid-precipitable radioactivity was measured, and no increase over input amounts was observed (data not shown). Specific binding accounted for 90% of total binding in these experiments. Radioactivity was quantified by γ spectroscopy, and aliquots were removed for protein concentration determination. Results are the average of triplicates, expressed as mean ± S.E.

Analysis of Cholesterol, Triacylglycerol, Fatty Acids, Albumin, and Glucose—Age- and sex-matched 6–16-week-old mice were fasted for 8–12 h, and tail vein blood was collected into heparinized or nonheparinized tubes. Serum or plasma was promptly separated from cells and stored at 4 °C or frozen at −20 °C. Assays were performed within 24 h of specimen collection. Cholesterol esters were converted to cholesterol by reaction with cholesterol esterase. Total cholesterol was then measured enzymatically by a modification of the method of Allain et al. (78). Triacylglycerol levels were determined by coupled enzymatic reactions that assayed glycerol release following hydrolysis of lipoproteins by lipoprotein lipase (79). Serum nonesterified fatty acid levels were measured by an enzymatic colorimetric method developed by Wako. Albumin levels were measured with an assay based on specific binding to bromocresol green (80). Plasma glucose levels were determined by a modification of the Trinder reaction (81), using tail vein blood from animals fasted overnight, drawn into sodium fluoride and promptly centrifuged to remove erythrocytes. The absence of glucose in the urine of fed and fasted age- and sex-matched animals was determined using Bili-Labstix (Bayer). Results were analyzed by unpaired Student’s t test.

Lipoprotein fractionation of pooled fasted plasma from three per group, age- and sex-matched animals, was accomplished by separation over two Superose 6 columns (Amersham Pharmacia Biotech) in series. The fractions were analyzed for protein, phosphatidylcholine, triacylglycerol, and cholesterol on a COBAS FARA automated analyzer, using appropriate standards and controls. The experiment was done twice.

Long Chain Fatty Acid Transport and Lipid Incorporation into Adipocytes—Adipocytes were isolated from epididymal fat pads of nonfasted (normal chow) age- and sex-matched 6–18-week-old mice by collagenase (type 1) digestion (1 mg/ml, Worthington) in Krebs-Ringer medium (pH 7.4) lacking phosphate, buffered with HEPES (KRH) and containing 2% BSA (Fraction V, fatty acid-free), 2 mM glucose (55, 82–84), and 200 mM adenosine to inhibit lipolysis (85). Dispersed cells were washed three times with KRH with 2% BSA and then twice with KRH with 0.1% BSA. Cells were suspended in the lower BSA buffer at a cell density of 25–30%. Cell density (volume of cells/volume of cell suspension), or lipocrit, was estimated from the packed cell volume obtained after centrifugation of a capillary tube containing 8-μl aliquot of mixed cell suspension. Transport of oleate was measured at 25 °C and was begun by adding to 30 μl of a mixed cell suspension 30 μl of isotopic solution (20,000 cpm/nm oleate), containing oleate complexed to BSA at ratios between 0.4 and 2. Uptake was stopped by the addition of 3 ml of cold buffer, and cells were separated from medium by low pressure vacuum filtration (about 50 mm Hg) using Gelman A/E glass fiber filters. Cell-associated radioactivity was obtained by counting the washed filters in 3 ml of aqueous scintillation fluid (Amersham Pharmacia Biotech) in a Beckman LS330 scintillation counter. Results are the average of triplicates, expressed as mean ± S.E.

RESULTS

Injection of the two different clones of ES cells into blastocysts resulted in 31 pups, eight of which were high level chimeras (75–90%) as judged by agouti coat color, which is carried by the ES cell line. Significantly, all were male. The male chimeras were backcrossed to C57BL/6 females, and all but one of the six survivors sired offspring. All were agouti, indicating germ line transmission of the ES cell genome. Southern blot analysis of genomic DNA isolated from tail tips of pups resulting from this cross confirmed the presence of the disrupted and endogenous alleles in F1 heterozygotes. These were interbred, and their offspring were genotyped as before. Fig. 1B shows the result of Southern blot screening of tail DNA from mice resulting from a cross of two heterozygotes, which was hybridized with the probe shown in Fig. 1A. The expected size
CD36 Null Mice Have Altered Lipid Metabolism

The absence of CD36 protein in null mice was further confirmed by immunofluorescence flow cytometric analysis ofuffy coat blood cells. Polyclonal anti-CD36 antibody, followed by a fluorescein-conjugated secondary antibody, was used to detect CD36 protein expression on mononuclear cells from wild type mouse blood. Incubation with secondary antibody alone was similar to that of the null animal.

CD36 null animals were born in the correct Mendelian ratio and were viable, and both males and females were fertile. The mice appeared grossly phenotypically normal, and thus far they appear to have a normal life span. The oldest null animal survived to about 2 years of age. The null mutation has been backcrossed four times to a C57Bl/6 background. All results in this report have been confirmed on this background.

Immunoprecipitation and Western blot analysis of tissues from the null mutant with a polyclonal rabbit anti-CD36 antisera (74) showed no expression of CD36. Fig. 2A demonstrates CD36 protein from fat tissue in a wild type animal (+/+), while the wild type animal had abundant expression of CD36, as inferred from the decrease in cell association observed in cells from CD36 null animals. Open symbols demonstrate cell association in the presence of 50 μg/ml fucoidan.

To determine the consequences of the absence of CD36 on lipoprotein metabolism, we studied binding and internalization of oxidized LDL by macrophages isolated from wild type and null animals. The amount of surface-bound oxidized LDL was significantly reduced on elicited peritoneal macrophages from null mice as compared with wild type controls (40–47% decrease at saturation; data not shown). Fig. 3 demonstrates that the decreased binding of oxidized LDL to CD36 null macrophages resulted in diminished lipoprotein transport into the cells. After a 5-h incubation with 30 μg/ml at 37 °C, cell-associated oxidized LDL was decreased by 63% in elicited macrophages, compared with cells from wild type control animals. Similarly, at this concentration of lipoprotein, cell association was reduced by 50% in resident macrophages from CD36 null mice (data not shown). Incubation with fucoidan (Fig. 3, open symbols), a polyanionic SR-AI/II ligand, decreased cell association to wild type and CD36 null elicited macrophages by 48 and 76%, respectively.

To examine the role of CD36 in transport of long chain fatty acids, we assayed and compared 3H-labeled oleate transport into adipocytes isolated from epididymal fat of CD36 null and wild type mice. Oleate transport was only slightly decreased in oleate-BSA ratio of 1.5, but the decrease was much more prominent when a low ratio (0.6) was used (Fig. 4). Transport in wild type cells saturated with a K_m of about 6 nM (Fig. 4, inset), which was in the range of K_m previously determined in other cell types (65, 83, 84, 86). In contrast, a K_m could not be estimated for CD36 null cells, since transport rates appeared to increase linearly with the concentration of unbound fatty acid in the medium. These data indicated that the CD36 null cells lacked the high affinity saturable component of oleate transport, which was present in cells from wild type mice. Blood albumin concentration in wild type and CD36 null animals was not different (average = 2.54 ± 0.16 and 2.59 ± 0.14 mg/dl, respectively; n = 5 per group). Basal levels of lipolysis in isolated adipocytes from wild type and CD36 null animals were similar (49 ± 2.7 μmol of glycerol/mg.

Fig. 2. A, immunoprecipitation/Western blot analysis of CD36. Protein from fat was prepared by solubilization in radioimmune precipitation buffer containing protease inhibitors. Fat was chosen because of the high expression of CD36 in this tissue. CD36 was immunoprecipitated with a polyclonal antibody against the rat homologue of CD36 and then identified by SDS-PAGE followed by Western blotting with the same antibody. CD36 protein was detected in wild type (+/+), mice but was absent in null mice (−/−), as indicated by the 88-kDa band designated with the arrowhead. B, flow cytometry analysis of Buffy coat blood cells. Polyclonal anti-CD36 antibody, followed by a fluorescein-conjugated second antibody, was used to detect CD36 protein expression on mononuclear cells from wild type mouse blood. Incubation with second antibody alone was similar to that of the null animal.
CD36 Null Mice Have Altered Lipid Metabolism

This report describes the generation by homologous recombination of a mouse null in the class B scavenger receptor, CD36. Our conclusions are based upon genetic (Southern blot hybridization, polymerase chain reaction), protein (immunoprecipitation/Western blot, flow cytometry), and functional analyses (oxidized LDL binding/cell association, oleate transport into adipocytes). These mice do not have a generalized defect in expression of all scavenger receptors, since we detected normal expression of SR-A (flow cytometry) and residual binding and cell association of oxidized LDL. These data are consistent with previous reports indicating the presence of multiple scavenger receptors, which would include SR-A and CD68 (86).

Peritoneal macrophages derived from CD36 null animals had a 40–47% decrease in binding of oxidized LDL at saturation and a 63% decrease in cell association at 37 °C at a ligand concentration of 30 μg/ml. These results are similar to those reported with antibody blockade of CD36 (41, 42) and strongly suggest that SR-AI/II and CD36 account for nearly all uptake of oxidized LDL by macrophages. Thus, targeted inhibition of these two scavenger receptors may be an effective therapeutic strategy to prevent early atherosclerotic lesion development. The SR-AI/II null mutation in mice was partially protective against experimental atherosclerosis (15), and studies of the effect of the CD36 null mutation in atherogenesis are currently in progress.

In addition to its role in accumulation of oxidized lipoproteins by monocytes/macrophages, CD36 has been proposed to function as a membrane receptor/transporter for long chain fatty acids. Strong evidence has accumulated to support the existence of a facilitated component for fatty acid transfer and a role for CD36 in this process (reviewed in Ref. 87). For example, a strong correlation was shown between expression of CD36 and cellular activity in fatty acid uptake and metabolism (65). Expression of CD36 in fibroblasts lacking the protein induced a high affinity saturable component of fatty acid uptake (72, 83). Increases in CD36 levels and fatty acid transport were reported in differentiating preadipocytes and following exposure to fatty acids (55, 70–73). In sarcolemmal vesicles from stimulated muscle, fatty acid transport and CD36 levels were both increased and by a similar magnitude (65). More recently, genetic linkage studies indicated that CD36 deficiency may underlie defects of fatty acid metabolism, hypertriglyceridemia, and the insulin resistance characteristic of the spontaneously hypertensive rat, an animal model for diabetes type II (88). Although the above data strongly supported an important physiological role for CD36 in lipid metabolism, the evidence remained indirect. The CD36 null mouse we describe in this report provides direct functional evidence for an in vivo role.

Oleate uptake by adipocytes from CD36 null mice was markedly reduced at low fatty acid:BSA ratios, indicating the absence of high affinity uptake in these cells. Uptake was less affected at the high fatty acid:BSA ratios. This supports the interpretation that CD36 allows cells to recruit fatty acids at normal physiological fatty acid:BSA ratios, when concentrations of unbound fatty acid are in the low nanomolar range (89). Data for CD36 null mice yields a line parallel to the x axis, so no Km could be obtained over the range of unbound fatty acid concentrations used; the Km is apparently very high, or the process is not saturable.●, wild type; ■, CD36 null.

of DNA/30 min for both groups). Interestingly, there was a trend toward increased incorporation of labeled palmitate into diacylglycerol in adipocytes from null animals (percentage of cell-associated radioactivity as diacylglycerol for CD36 null: 24.1 ± 0.4; for wild type: 18.5 ± 0.2; p < 0.001).

The changes in cellular fatty acid and lipoprotein binding and internalization were associated with abnormal levels of blood lipids. Fasted null animals had consistently higher levels of total cholesterol, free fatty acids, and triacylglycerol (Table I) and lower plasma glucose than wild type mice (Fig. 5). Plasma cholesterol levels were also increased in the unfasted state (data not shown). The increase in circulating cholesterol level was mainly due to an increase in the HDL fraction of the lipoprotein pool (~33%) (Fig. 6A). HDL particles were larger in null animals and contained increased phospholipid, as shown by phosphatidylcholine content (Fig. 6B). We also observed an increase in very low density lipoprotein (VLDL)-associated triacylglycerol, while intermediate density lipoprotein/LDL particles from null animals were relatively triacylglycerol-poor (Fig. 6C).

DISCUSSION

FIG. 4. Fatty acid transport into adipocytes of wild type and CD36 null animals. Data are from pooled adipocytes isolated from three animals per group per experiment and shown for fatty acid:BSA ratios of 1.5 (●, ○) and 0.5 (●, ■). Rates were obtained from five time measurements (0–120 s) and normalized to the DNA content as determined on aliquots of the cell suspension. ○ and ●, wild type; □ and ■, CD36 null. The mean ± S.E. of triplicates is plotted. A representative experiment is shown. Inset, Hanes plot of fatty acid uptake by adipocytes as a function of unbound fatty acid concentration. The Hanes plot of Slope versus S, where S represents unbound fatty acid concentration in nm and v represents velocity or rate in nmol/min/mg of DNA, has its x intercept as the −Km, and its slope is 1/Vmax. The data were derived from two experiments conducted at the different fatty acid:BSA ratios shown. In each experiment, complete time courses were performed for each ratio, and these were used to obtain the rates per minute. Unbound fatty acid concentrations were calculated using a computer routine as described previously (83) and were based on the fatty acid:BSA dissociation constants provided by Richieri and Klienfeld (89). Data were fitted by linear regression (wild type: r2 > 0.9). Data for CD36 null mice yields a line parallel to the x axis, so no Km could be obtained over the range of unbound fatty acid concentrations used; the Km is apparently very high, or the process is not saturable.●, wild type; ■, CD36 null.

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CD36 Null Mice Have Altered Lipid Metabolism

**TABLE I**

**Cholesterol, triacylglycerol, and free fatty acid levels**

| Plasma cholesterol (mg/dl) | Serum triacylglycerol (mg/dl) | Serum free fatty acids (mM) |
|---------------------------|-------------------------------|-----------------------------|
| **Wild type male**        | 100.4 ± 6.6 (8)               | 97.27 ± 5.89 (10)           | 1.73 ± 0.091 (10) |
| **CD36 null male**        | 130 ± 5.88 (10)              | 137.77 ± 10.04 (11)        | 3.28 ± 0.117 (11) |
| **Wild type female**      | 91.56 ± 3.72 (10)            | 68.81 ± 4.48 (10)          | 0.963 ± 0.062 (10) |
| **CD36 null female**      | 132.65 ± 9.81 (10)           | 86.25 ± 2.45 (20)          | 2.12 ± 0.075 (20) |

Age- and sex-matched wild type and CD36 null mice were starved, and blood was taken from the tail vein for analysis. Values are ±S.E., and values compared between same sex animals are significant at *p < 0.005. n values are shown in parentheses.

A recent report suggests that cardiac muscle utilizes a large proportion of very low density lipoprotein-derived fatty acids (90). In a lipoprotein lipase knockout transgenic, where lipoprotein lipase was reintroduced only in cardiac muscle but not in skeletal or adipose tissue, the hypertriglyceridemia and reduced HDL levels that were characteristic of the lipoprotein lipase null animal were corrected, despite a postheparin plasma lipase level of only 34% of control (90). Adipose and skeletal muscle showed no pathological changes and were able to synthesize fatty acids de novo. Interestingly, there was no rise in plasma free fatty acids in this model, indicating near total utilization by cardiac muscle of those released by VLDL catabolism. The correction of HDL cholesterol levels supports the hypothesis that VLDL exchanges cholesterol with HDL during catabolism by lipoprotein lipase. In the CD36 null animal, we hypothesize that the increase in fatty acid load reaching the liver as a result of inefficient fatty acid utilization by peripheral tissues is reflected in an increase in triacylglycerol-rich VLDL. Tissue catabolism of VLDL may be similar or increased, and HDL cholesterol increases as a consequence of increased levels/catabolism of VLDL.

Data obtained from the CD36 null animal are likely to have human relevance. CD36 deficiency in humans has been noted in a small fraction (1–3%) of the Asian population (40). Several recent reports correlate CD36 deficiency in humans and lack of fatty acid uptake by heart muscle, using the iodinated fatty acid derivative, 15-([p-iodophenyl]-3R,S-methyl pentadecanoic acid (67). A potential association between CD36 deficiency in humans with hypertrophic cardiomyopathy and a link to poor fatty acid utilization by heart tissue has been proposed (68). In a recent study, where a large number of patients were screened for lack of 15-([p-iodophenyl]-3R,S-methyl pentadecanoic acid uptake, those analyzed with this abnormality were found to be CD36-deficient (69). This study was flawed, however, because it did not note if any subjects with normal 15-([p-iodophenyl]-3R,S-methyl pentadecanoic acid uptake were CD36-deficient. Interestingly, three out of seven of those with CD36 deficiency additionally had type II diabetes.

CD36 null mice have a small increase in LDL, and this may be a consequence of increased VLDL. The decrease in triacylglycerol in this fraction supports our hypothesis that peripheral tissues are relatively starved for fatty acids and thus catabolize lipoproteins to release as much as possible. An alternative mechanism accounting for the increase in HDL cholesterol may be through direct binding of HDL by CD36 and subsequent clearance. Although recent reports have shown that CD36 can bind HDL (91–93), it should be noted that these studies used CD36-overexpressing transfected cell lines and did not measure HDL-CD36 association in a physiologically relevant cell. In one case, the CD36 protein that was expressed was of a different size than endogenous CD36 (91), and in another the transfected CD36 bound HDL but did not transfer cholesterol (92), which differentiates it from SR-BI and SR-BII (8, 94, 95). Most recently, Connelly et al. (93) reported that rat CD36 expressed in COS cells could mediate cholesterol ester uptake but at a much less efficient rate (15%) when compared with SR-BI. SR-BI heterozygous null animals, in which there is a 50% decrease in SR-BI expression, had a 25–40% rise in plasma cholesterol (94), similar to what we report for the CD36 null animal. The much lower capacity of CD36 to exchange HDL cholesterol strongly suggests different mechanisms for the rise of HDL in the two models; i.e., the rise in cholesterol in the CD36 null appears to be too large to be accounted for by the mechanism of cholesterol exchange mediated by CD36. Further studies are necessary to elucidate the exact mechanism, however. What has emerged from this null animal is that CD36 has a physiologic role in lipoprotein/fatty acid metabolism. This significantly differentiates it from SR-A receptors; there is no alteration in cholesterol, triacylglycerol, nonesterified fatty acid levels, and lipoprotein composition in the CD36 null animal (15). Furthermore, the lack of a fatty acid/lipoprotein-related phenotype in the SR-AII null animal gives independent, indirect support to the role of CD36 as a physiologic transporter of fatty acids.

CD36 is both a receptor for lipoproteins and a receptor for long chain fatty acids. Whether these seemingly different ligands have related structural properties that allow for this multifunctionality remains to be determined. Of significance are reports that it is the lipid component of the oxidized lipoprotein that is recognized by type B scavenger receptors (20, 41, 82, 96). Most of the surface lipid in lipoproteins is in the form of phosphatidylcholine, and it has been recently demonstrated that an oxidized derivative of this phospholipid specifically inhibited oxidized LDL binding and uptake by murine-resident peritoneal macrophages (97). These authors postulated that the oxidatively modified phospholipid may mimic the physiochemical properties of phosphatidylserine. The significance of this is that the...
receptors have a fundamental role in the removal of apoptotic cells (18–21). Indeed, CD36 has been shown to bind apoptotic cells (in conjunction with other receptors) and to bind phosphatidylserine liposomes (18, 19, 82, 96). Terpstra et al. (97) hypothesized that polar fatty acid degradation products may result from oxidation of cell membrane lipids and that these may form a surface configuration that in charge and polarity resembles phosphatidylinerine-rich liposomes. Thus, the recognition by CD36 of fatty acids, oxidized lipoproteins, and apoptotic membranes is entirely consistent, in that these ligands may share similar physiochemical properties.

The emerging evidence for similarity of regulation of lipid metabolism in monocytes/macrophages and adipocytes by identification of common regulator molecules such as PPARα and CD36 and the complex interrelationships among fatty acid, lipoprotein, and cholesterol trafficking, storage, and utilization provide new paradigms in which the study of null and transgenic animals becomes particularly useful. Only in the whole animal can a mutation in a gene product be appreciated for the interrelated effects it may have in normal and abnormal physiology and its contribution to the overall homeostasis of the animal be assessed. The ability to then further manipulate gene expression via transgenic technology, gene therapy, and bone marrow transplantation allows for selective examination of the null phenotype in a specific metabolic pathway or organ system. In this way, CD36 null animals will provide a powerful tool to fully elucidate the role of this protein in lipid metabolism.

FIG. 6. Fast protein liquid chromatography of lipoproteins from wild type and null animals. Plasma was collected by heart puncture from 4–6 age- and sex-matched animals of each genotype. Total cholesterol (TC; panel A), phosphatidylcholine (PC; panel B), and triacylglycerol concentration (TG; panel C) were measured in each fraction and are shown. An increase in cholesterol was apparent in all lipoprotein subclasses in the CD36 null animal, with the greatest increase present in the HDL fractions. HDL particles were notably larger and had an increase in phosphatidylcholine. There was a large increase in VLDL-associated triacylglycerol in CD36 null animals and an increase in intermediate density lipoprotein (IDL) particles.

appearance of phosphatidylserine in the outer leaflet of cell membranes is an early sign that a cell is undergoing apoptosis and a signal to professional and nonprofessional phagocytes to remove that cell before further damage ensues from lysis (98). Scavenger

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