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The Fatty Liver Dystrophy (fld) Mutation:
Developmentally Related Alterations in Hepatic Triglyceride Metabolism
and Protein Expression

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SUMMARY

Fatty liver dystrophy (fld) is an autosomal recessive mutation in mice characterized by hypertriglyceridemia and development of a fatty liver in the early neonatal period. Also associated with the fld phenotype is a tissue-specific deficiency in the expression of lipoprotein lipase and hepatic lipase, as well as elevations in hepatic apolipoprotein A-IV and apolipoprotein C-II mRNA levels. Although these lipid abnormalities resolve at the age of weaning, adult mutant mice exhibit a peripheral neuropathy associated with abnormal myelin formation. The fatty liver in fld/fld neonates is characterized by the accumulation of large triglyceride droplets within the parenchymal cells, and these droplets persist within isolated hepatocytes maintained in culture for several days. To identify the metabolic defect that leads to lipid accumulation, we investigated several aspects of cellular triglyceride metabolism. The mutant mice exhibited normal activity of acid triacylglycerol lipase, an enzyme thought to be responsible for hydrolysis of dietary triglycerides in the liver. Metabolic labeling studies performed with oleic acid revealed that free fatty acids accumulate in the liver of 3 day old fld/fld mice, but not in adults. This accumulation in liver was mirrored by elevated free fatty acid levels in plasma of fld/fld neonates, with levels highest in very young mice and returning to normal by the age of one month. Quantitation of fatty acid oxidation in cells isolated from fld/fld neonates revealed that oxidation rate is reduced 60% in hepatocytes and 40% in fibroblasts; hepatocytes from adult fld/fld mice exhibited an oxidation rate similar to those from wild-type mice. Previously, a single mutation affecting fatty acid oxidation in the mouse has been reported. This mutation, in the butyryl CoA dehydrogenase (bcd) gene, arose spontaneously in the BALB/cByJ mouse strain, which coincidentally is the same strain in which the fld mutation occurred. Using a PCR assay to distinguish between normal and mutant alleles of the bcd gene, we determined that both fld/fld and unaffected littermates of the BALB/cByJ-fld strain are homozygous for the normal bcd gene. These results indicate that the fld mutation must have occurred in the BALB/cByJ strain sometime after the isolation of the fld mutants in 1981, and that the fld defect represents a

distinct mutation affecting fatty acid oxidation. Finally, using quantitative two-dimensional gel electrophoresis, we have detected 25 proteins with significantly altered expression levels ($p < 0.001$) in the fld/fld fatty liver. Further characterization of these proteins may lead to a better understanding of normal cellular triglyceride metabolism and provide specific candidates for the fld gene product.

The fatty liver dystrophy (fld) mutation is an autosomal recessive mutation that occurred spontaneously in the BALB/cByJ mouse strain in 1981 (1). The mutant phenotype is characterized by fatty liver and hypertriglyceridemia during neonatal development, and by a peripheral neuropathy which progresses throughout the lifetime of the animal (2, 3). Also associated with the fld phenotype during the neonatal period are tissue-specific alterations in expression of several proteins involved in lipoprotein metabolism. For example, lipoprotein lipase (LPL)¹ in white adipose tissue is reduced to 6% of normal activity, although LPL activity is reduced by only 50% in brown adipose and heart. In addition, hepatic lipase activity in the circulation is reduced to 20% of normal levels, whereas mRNA levels for apolipoproteins A-IV and CII are significantly elevated in liver (100-fold and 6-fold, respectively), yet appear normal in intestine (2).

Interestingly, the fatty liver and the abnormalities in triglyceride metabolism, lipase and apolipoprotein expression in fld/fld pups spontaneously resolve during the suckling/weaning transition (postnatal days 13-15) (2). Although this reversion coincides with the change from a diet that is rich in triglycerides (mother's milk) to a diet that is low in fat and high in carbohydrates (laboratory chow), the resolution appears to be effected by a genetically programmed event and not the result of a change in diet. Thus, in fld/fld mice in which weaning has been delayed, the triglyceride levels, as well as apo A-IV mRNA and apo C-II mRNA levels, return to normal on the same schedule as in weaned mice (2). In addition, the lipid abnormalities evident in neonatal fld/fld mice cannot be elicited by feeding adult animals a high fat diet rich in triglycerides (2). These results suggest that the mutation at the fld locus disrupts the normal expression or activity of proteins required for lipid homeostasis during the neonatal period.

The peripheral neuropathy, which is manifested in an unsteady gait shortly before weaning and progresses throughout the lifetime of fld/fld mice, may also be associated with a defect in lipid metabolism. Sciatic nerve fibers from fld/fld adult mice exhibit deficiencies in phospholipids, glycosphingolipids, and neutral lipids, and elevated cholesterol esters (3). In contrast, no differences are evident in the composition of brain lipids between fld/fld mice and their unaffected littermates ✓

suggesting that the mutation affects peripheral but not central nerve tissue. The morphology of sciatic nerve in mutant mice revealed a variety of gross abnormalities in myelin structure including thin, poorly compacted myelin sheaths, ongoing myelin breakdown, and enlarged Schwann cell mitochondria and nuclei (3). Coincident with these morphologic changes, altered levels of peripheral nerve myelin- and axon-specific proteins also exist. Taken together, the varied aspects of the fld mutant phenotype indicate that the fld mouse is an excellent model in which to investigate potentially related mechanisms controlling lipid metabolism in newborn mice and those contributing to the development and maintenance of peripheral nerve.

In this report we further characterize the metabolic deficiencies contributing to the fatty liver in fld/fld mice during the neonatal period. During the fatty liver stage, triglyceride droplets accumulate within hepatocytes of fld/fld mice. This phenotype, together with elevated apo A-IV mRNA and reduced hepatic lipase activity, persists in hepatocytes isolated from neonatal mice and maintained in culture. We have examined several aspects of hepatic triglyceride metabolism in fld/fld mice and determined that neonatal fld/fld hepatocytes do not exhibit a constitutive increase in triglyceride synthesis, but rather accumulate fatty acids. Furthermore, the rate of fatty acid oxidation in cultured neonatal fld/fld hepatocytes is significantly reduced compared to wild-type cells. Only a single other mouse mutation has been described which affects fatty acid oxidation; co-incidentally, that mutation occurred in the same mouse strain as the fld mutation. Using a definitive PCR assay, we determined that the fld mutation is distinct from the previously described mutation and represents a second mouse model with a defect in fatty acid oxidation. Finally, using quantitative two-dimensional electrophoresis, we demonstrate that, in addition to lipases and apolipoproteins, expression levels of at least 25 proteins are significantly altered in the fld/fld neonatal liver. The identification of these proteins may lead to a better understanding of normal cellular triglyceride metabolism in neonatal liver and reveal candidates for the fld gene product.

METHODS

Animals—Non-tested breeding pairs of mouse strain BALB/cByJ-fld were obtained from the Mouse Mutant Resource colony at the Jackson Laboratory (Bar Harbor, ME). Pairs were bred continually for up to 6 months to produce fld/fld and wild-type (+/?) offspring used in studies. All mice were maintained in a 12 hour light/dark cycle and fed Purina Mouse Chow 5001 *ad libitum*.

Microscopy of liver sections—Mice were lightly anaesthetized with isoflurane (Forane, Ohmeda PPD, Inc.) and livers fixed by whole body intracardiac perfusion of a solution consisting of 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer. Portions of the right lobes of liver were excised, diced into 1-2 mm blocks, and fixed for 30 min. in the paraformaldehyde/glutaraldehyde fixative. Tissue blocks were rinsed in phosphate buffer, dehydrated in ethanol and embedded in Polybed 812 epoxy resin. Deplasticized semi-thin sections were cut and stained with toluidine blue and photographed with an Olympus AH-2 light microscope.

Primary hepatocyte isolation—Hepatocytes were isolated by recirculating perfusion of livers from 10-12 day old and adult mice as described (4). Perfusion of the livers from young mice was made possible by the use of extra fine tubing (0.5 mm diameter) for cannulation of the portal vein (BioTime, Inc., Berkeley, CA). Primary hepatocytes were cultured in arginine-free DMEM containing 10% fetal calf serum at 37°C in 5% CO₂. The use of arginine-free medium prevents the growth of fibroblasts which may be present in hepatocyte preparations from neonatal mice.

RNA Analysis—Total RNA was prepared from hepatocytes using Trizol™ (Gibco-BRL). RNA (10 µg) was electrophoresed in formaldehyde gels, blotted, and hybridized with cDNA probes for mouse apo A-IV and apo A-I as described (5).

In vivo metabolism of [³H]oleic acid—[9,10(n)-³H]oleic acid (10.0 Ci/mmol, Amersham) was evaporated in a glass tube and resuspended in NaOH at a molar ratio of 1:4 (oleate-NaOH). 50 µCi of saponified oleate was dissolved in 30 µl (for 3 day old mice) or 100 µl (for adult mice) of PBS and injected I.P. Initial trials were also performed by direct perfusion of liver which had been cannulated through the portal vein and excised. After a 15 min pulse, mice were killed (or perfusion stopped) and

livers collected. Livers were homogenized in buffer containing 0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 1 mM DTT.

Lipid analyses—Lipids were prepared from liver homogenates by extraction with chloroform and methanol (6). Phospholipid concentration was estimated by quantitation of phosphorus (7). Neutral lipids were separated by thin-layer chromatography on high performance silica gel plates (Aldrich Chemical Co.) in hexane-diethyl ether-acetic acid (80:20:1), and visualized by charring in 3% cupric acetate/8 % phosphoric acid or by incorporation of iodine vapor (7). Radioactivity of neutral lipid species from oleate labeling experiments were determined by scintillation counting of silica gel spots in EcoLite (ICN). For quantitation of radiolabeled phospholipid, silica gel spots were incubated for 5 min. in 0.1 ml of 0.1 M KOH in ethanol at 37°C and neutralized with 10 µl acetic acid before addition of scintillation cocktail.

Plasma free fatty acid levels were determined enzymatically using reagents from Wako Chemical (Austin, TX) by the method of Shimizu et al. (8).

Primary skin fibroblast culture—Primary fibroblast cultures were established from skin explants of 10 day old fld/fld and wild-type mice. Skin samples 1 cm square were placed into 30 mm petri dishes in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and incubated at 37°C in 5% CO₂. After a few days, fibroblasts migrating out from the explants were removed from the dish by treatment with trypsin and passaged to fresh dishes. Fibroblasts were used in oxidation experiments between passages 3-5.

Triacylglycerol lipase assays—Hepatic lipase activity in plasma and hepatocyte culture medium was determined utilizing a tri[³H]oleate substrate (9). Assays were performed in duplicate with 2 µl plasma from 3-4 mice of each type, or with 50 µl culture medium from 2-4 culture plates after incubation with 10 U/ml heparin for 24 hours. Liver samples used for acid lipase activity determinations were prepared as 20% (w/v) homogenates in 0.15 M NaCl, 0.005 M barbital buffer, pH 7.4, and cleared by centrifugation at 48,200 x g for 30 min at 4°C. Lipase activity was determined as above, except with samples buffered to pH 5.0. Assays were run in duplicate using 1 µl (100 µg protein) of homogenate

from 3 animals of each age and phenotype. In measurements presented, one mU of enzyme activity represents the release of 1 nmol of fatty acid per min.

Palmitate oxidation assay—Freshly isolated hepatocytes or skin fibroblasts (passage 3-5) were plated at a density of 10^5 cells/well in 24-well tissue culture plates (one well = 2 cm^2). Plates were incubated 20 h at 37°C in 5% CO_2 . Palmitate oxidation was assayed using a $[9,10(n)^3\text{H}]$ palmitate substrate (54 Ci/mmol, Amersham) by the method of Moon and Rhead (10). Briefly, cell monolayers were rinsed twice with phosphate buffered saline and incubated in 0.2 ml of substrate mixture containing 22 μM unlabeled palmitate + 5 μCi $[^3\text{H}]$ palmitate in Hank's basic salt solution (Gibco-BRL) containing 0.5 mg/ml BSA. Negative controls were prepared by treating the monolayer with methanol for 30 s to abolish cellular metabolism. In initial studies, cells were incubated in the palmitate reaction mixture for times ranging from 15 min to 3 h to ascertain linearity of the assay. For data presented, 2 hr were employed, at which time the reaction medium was collected from the cell monolayers and treated with 0.2 ml 10% trichloroacetic acid. Cell monolayers were washed with an additional 0.1 ml PBS and the wash pooled with the initial reaction medium. The protein precipitate was removed by centrifugation at 8500 g for 5 min. Supernatants were treated with 70 μl of 6 N NaOH, and applied to a 1 ml Dowex-1 (Sigma Chemical) column. The $^3\text{H}_2\text{O}$, which is an end product of oxidation, passes through the column. This sample, together with a 1 ml water wash, was directly quantitated by scintillation counting in 10 ml EcoLite (ICN, Irvine, CA).

To determine total cellular protein of cultures used for oxidation assays, 1 ml of water was added to cell monolayers after removal of reaction medium and cells were subjected to 3 cycles of freezing (-70°C) and thawing (37°C). Proteins were determined using a modified Bradford protein assay reagent (BioRad, Richmond, CA).

Polymerase chain reaction—The polymerase chain reaction (PCR) was used to distinguish wild-type and mutant alleles for the butyryl-CoA dehydrogenase (bcd) gene in BALB/cByJ-fld mice and mice of additional BALB/c sublines. Genomic DNA was isolated from the tail of both 10 day old and adult fld/fld and unaffected mice using proteinase K digestion followed by phenol/chloroform extractions and ethanol precipitation (11). Genomic DNA from control strains BALB/cJ, BALB/cBy,

and BALB/cByJ was purchased from the Jackson Laboratory (Bar Harbor, ME). The PCR was performed using primers that flank a deletion occurring in the *bcd* mutant allele (5' primer, 5'-TGGCGTGCTGCCATGTTGA-3'; 3' primer, 5'-CCTTGTGTTCTTGCCTCCGA-3') (adapted from reference 12). Each reaction contained 1 µg genomic DNA, 15 pmol each primer, 50 mM KCl, 10 mM Tris, pH 8.8, 2.5 mM MgCl₂, 200 µM each deoxyribonucleoside triphosphate, 0.1% Triton X-100, and 2 U Taq polymerase (Perkin-Elmer/Cetus). The amplification was performed for 30 cycles of 1 min 95°C, 1 min 57°C, and 1 min 72°C. Products were analyzed on a 2% NuSieve agarose gels (FMC Bioproducts, Rockland, ME).

Quantitative 2-dimensional gel electrophoresis—Livers were harvested from 5-7 day old *fld/fld* and wild-type mice and immediately frozen in liquid nitrogen until processing. In preparation for electrophoresis, livers were homogenized in 1 ml of a solution containing 9M urea, 4% Nonidet P-40, 5% β-mercaptoethanol and 2% pH 9-11 ampholytes per 250 mg (frozen tissue weight) (13). The homogenate was centrifuged for 5 min at 100,000 rpm (435,000 x g) in a Beckman TL 100 ultracentrifuge, and the supernatant decanted.

2-dimensional gel electrophoresis (2DE) was carried out from 10 individual livers of each phenotype (ie. 10 *fld/fld* and 10 wild-type) as described (13). For electrophoresis in the first dimension, 10 µl of liver homogenate (^{0.25}~~2.5~~ mg protein) was loaded onto polyacrylamide rods containing an equal mixture of Biolyte pH 3-10 and pH 5-7 ampholytes. Second dimension electrophoresis was performed on 10-17% polyacrylamide gradient gels. Gels were fixed and stained in 0.125% Serva blue R (Coomassie blue R250)/2.5% (vol/vol) H₃PO₄/50% ethanol, and were destained in 20% ethanol. 2DE patterns were digitized using an Eikonix 1412 scanner and spot matches made by an automatic matching algorithm. Spot densities were integrated to detect proteins showing statistically significant differences between *fld/fld* and wild-type samples ($P < 0.001$). (Reference: Giometti and Taylor)

Statistical Analyses—Values for fatty acid levels, lipase activities, fatty acid oxidation rates, and 2DE protein levels are given as mean ± S.D. The Student's t test was used to test for differences between *fld/fld* and wild-type mice. Differences were considered statistically significant at $p < 0.05$, $p < 0.01$, and $p < 0.001$ as specified in text and figures.

RESULTS

Accumulation of triglyceride in hepatocytes from neonatal fld/fld mice—As originally described by Sweet et al. (1), fld/fld mice can be distinguished from their unaffected littermates in the first few postnatal days by the appearance of their liver, which is increased in size and white in color. To examine the morphology of the fatty liver, liver tissue from 9 day old fld/fld and unaffected mice was examined by light microscopy. As shown in Figure 1a, liver from 9 day old fld/fld mice is characterized by a massive accumulation of large lipid droplets within the parenchymal cells, in contrast to the small, sparse lipid droplets in liver parenchyma of unaffected, age-matched mice (Fig. 1b). In addition, hypertrophy is evident in the fld/fld hepatocytes due to the large mass of intracellular triglyceride, resulting in the hepatomegaly characteristic of the fld defect (2). At 3 months of age, however, the large lipid droplets from fld/fld liver have disappeared (Fig. 1c) and the liver becomes indistinguishable from wild-type (Fig. 1d). Isolated neonatal fld/fld hepatocytes appear identical to those examined *in situ* (see Fig. 1a), and could be maintained in culture for 4 days without loss of lipid from the cells (data not shown). Analysis by thin layer chromatography of lipids from the fatty liver of 7 and 14 day old fld/fld mice revealed that the excess lipid is virtually all triglyceride, with a slight elevation in diglycerides, but no apparent changes in cholesterol, cholesteryl esters, or phospholipids (Fig. 2). An elevation in free fatty acid is also evident in the fatty liver of 7 day old mice. Lipids from the livers of 30 and 90 day old fld/fld mice appear completely normal (Fig. 2). In quantitative triglyceride assays of liver homogenates prepared from 10 day old fld/fld mice, triglyceride levels were elevated approximately 6-fold compared to wild-type littermates (43.7 vs. 7.6 μg triglyceride/mg liver protein for fld/fld and wild-type mice, respectively).

In addition to triglyceride accumulation, hepatocytes isolated from neonatal fld/fld mice and cultured *in vitro* exhibit dramatically elevated levels of apo A-IV mRNA. As shown in Fig. 3, apo A-IV mRNA is nearly undetectable in hepatocytes isolated from 10 day old wild-type mice, but is greatly elevated in cells from fld/fld littermates; mRNA for a related protein, apolipoprotein A-I, is present at similar levels in wild-type and fld/fld hepatocytes. In addition, hepatic lipase activity measured in the media from hepatocytes maintained in culture for 4 days shows a 2-fold reduction in cells from fld/fld

compared to wild-type mice (0.85 ± 0.17 mU/ml culture medium for fld/fld vs. 1.48 ± 0.10 mU/ml for unaffected), and mirrors the relative levels of hepatic lipase activity in plasma from 12 day old mice (47.2 ± 3.2 mU/ml plasma for fld/fld vs. 112.6 ± 6.3 mU/ml for wild-type mice). Thus, several hallmarks of the fatty liver phenotype *in vivo* (triglyceride accumulation, elevated apo A-IV mRNA, and reduced hepatic lipase activity) are present in hepatocytes maintained in culture for several days, suggesting that a cellular defect within the hepatocyte contributes to the fld mutant phenotype.

Altered triglyceride catabolism in fld/fld fatty liver—The diet of suckling rodent pups contains about 10 times the amount of fat in an adult rodent diet (14). During the suckling period, virtually all of the triglyceride in the bloodstream is derived from chylomicrons secreted by the intestine (15). Furthermore, the liver does not attain the capacity to secrete triglyceride in the form of VLDL until the age of weaning (15). Thus, dietary triglyceride and fatty acids taken up by the neonatal liver in the form of chylomicron remnants must be efficiently catabolized within the liver in order to maintain homeostasis. We hypothesized that the fatty liver in suckling fld/fld mice may result from one of the following defects in triglyceride catabolism: an inability of the liver to hydrolyze incoming triglyceride, increased rates of triglyceride synthesis, or impaired fatty acid catabolism.

The hydrolysis of triglyceride present in chylomicron remnants taken up by the liver occurs in the lysosomes by lysosomal acid lipase (16). To determine whether accumulation of triglycerides in fld/fld hepatocytes may result from an inability to hydrolyze incoming triglycerides, we measured lipase activity in liver homogenates prepared from fld/fld and wild-type littermates. Liver homogenates were prepared from mice 7, 12, 14, 30, and 90 days of age, incubated with a radiolabeled-triolein emulsion at pH 5.0, and hydrolysis quantitated by the release of free fatty acids. Homogenates from mutant and wild-type mice showed similar rates of hydrolysis indicating that fld/fld mice do not have a defect in lysosomal triglyceride hydrolysis (Fig. 4).

We next investigated whether the accumulation of triglyceride in the fatty liver is due to increased constitutive triglyceride synthesis. The livers of fld/fld mice appear normal at birth, but become completely fatty within the first 24 hours of suckling. Since acid lipase hydrolytic activity appears normal in the fatty liver, it is likely that increased triglyceride synthesis occurs during the first

24 hours post-partum. If increased triglyceride synthesis represents the primary defect in the fld mutation, it should occur throughout the fatty liver stage. To address this possibility we examined the rate of fatty acid incorporation into triglyceride and other lipids in 3 day old mice in which the fatty liver has been fully established. Due to the small size of neonatal mice, we found it necessary to administer the label by intraperitoneal injection rather than by intravenous injection or direct perfusion of the liver. To confirm that I.P. injection gives representative results, we performed pilot studies in adult mice to compare the incorporation of radiolabeled-oleic acid into hepatic lipids when administered via direct perfusion of the hepatic portal vein or by I.P. injection. After a 15 minute pulse, the incorporation of label into specific hepatic lipid species was determined by separating neutral lipids using thin layer chromatography followed by scintillation counting of specific lipid species. The proportion of label incorporated into various lipid species (triacylglycerol, diacylglycerol, cholesteryl ester, phospholipid, and free fatty acid) was identical for perfusion and I.P. injection, although the labeling efficiency was several-fold higher for perfusion (data not shown). These results indicate that I.P. injection gives an accurate representation of the relative incorporation into lipid species and is a suitable method to use with neonatal mice which are too small for perfusion.

Results of oleic acid labeling studies performed with 3 day old fld/fld and wild-type mice are shown in Fig. 5a. The proportion of label incorporated into triacylglycerol, diacylglycerol, and cholesteryl ester fractions was similar between fld/fld and wild-type mice. However, the fld/fld mice exhibited 2.5 fold higher levels of free oleic acid (significant at $p > 0.01$), and a 25% lower incorporation of labelled oleate into phospholipids (not significant). Similar studies performed in adult mice in which the fatty liver has resolved revealed that the proportion of label occurring as free fatty acid in the fld/fld liver is reduced, and phospholipid incorporation increased, to wild-type levels (Fig. 5b). These results indicate that the maintenance of the fatty liver in fld/fld neonates is associated with increased levels of free fatty acid and not with increased triglyceride synthesis from fatty acid precursor. We also detected an age-dependent elevation of free fatty acid levels in the blood of fld/fld mice, with approximately 40% higher levels in 5 day old fld/fld mice, diminishing to normal levels by 1 month of age (Fig 5c).

The elevated levels of free fatty acid in fld/fld liver and blood are consistent with a defect in fatty acid metabolism. A characteristic finding in human subjects with genetic defects in fatty acid oxidation is the occurrence of hypoglycemia during fasting and acute episodes (17). Plasma glucose levels in pooled samples of 8 day old fld/fld and wild-type mice (4 mice/pool) were 67 mg/dl and 151 mg/dl, respectively. The occurrence of hypoglycemia together with elevated fatty acid levels in fld/fld neonates lead us to investigate whether there is a fatty acid oxidation deficiency in the fld/fld liver. For these studies we employed a radiolabeled fatty acid substrate ([9,10(n)- ^3H -palmitate]) that allows detection of medium and long chain fatty acid β -oxidation disorders (18). Primary hepatocytes, cultured overnight, were incubated with this substrate for 2 hours, and the release of $^3\text{H}_2\text{O}$ quantitated as a measure of the rate of β -oxidation (10).

Results of the palmitate oxidation assay are shown in Table 1. In assays performed with neonatal hepatocytes, fld/fld cells consistently exhibited a 60% reduction in the rate of palmitate oxidation compared to cells from wild-type mice. To ascertain whether reduced fatty acid oxidation is evident in other neonatal cell types which do not accumulate lipid, the fatty acid oxidation assay was performed in skin fibroblasts isolated from 10 day old fld/fld and unaffected mice. Impaired oxidation was evident in neonatal fibroblasts, but was less severe than in neonatal hepatocytes, with a 39% reduction in fld/fld compared to unaffected mouse cells (Table 1). We also used the palmitate oxidation assay to determine oxidation efficiency in hepatocytes from young adult mice (60 days old) that no longer accumulate intracellular triglyceride. In contrast to the phenotype seen with neonatal hepatocytes, cells from adult fld/fld and wild-type mice showed similar rates of oxidation (Table 1). Thus, fatty acid oxidation is impaired in neonatal but not adult fld/fld hepatocytes. These results parallel those described above indicating that free fatty acid levels are elevated in both liver and plasma of neonatal fld/fld mice, but return to normal in the adult animals. Taken together, the findings suggest that impaired fatty acid oxidation during neonatal development contributes to the formation and/or maintenance of the fatty liver.

BALB/cByJ-fld/fld mice do not carry null allele for bcd gene present in BALB/cByJ strain—Although several genetic disorders affecting fatty acid oxidation have been documented in

humans, a single spontaneously occurring mutation affecting fatty acid oxidation in the mouse has been reported (19, 20). This mutation occurred in the gene for mouse butyryl CoA dehydrogenase (also known as short chain acyl CoA dehydrogenase), an enzyme required for the complete oxidation of short chain fatty acids. Mice deficient for butyryl CoA dehydrogenase accumulate fatty acid-glycine conjugates and organic acids in the urine, and develop a fatty liver after 18 hours of fasting. The enzyme deficiency is the result of a deletion in the butyryl CoA dehydrogenase gene (*bcd-1*) which occurred spontaneously in the BALB/cByJ mouse strain (21). Significantly, this is the same BALB/c subline that carries the *fld* mutation. The *bcd-1* gene has been mapped to mouse chromosome 5 (22), a locus that is known to be distinct from the *fld* locus (Dr. E. Birkenmeier, personal communication). However, since the *fld* mutation occurred in the same BALB/c substrain that carries the *bcd* mutation, it is possible that the *fld* phenotype could result from a combination of mutations at the *bcd* locus and at a separate *fld* locus. Due to the position of the label in the substrate employed here for fatty acid oxidation assays (9,10[³H]palmitate), products resulting from the activity of butyryl CoA dehydrogenase would not have been detected.

To determine whether the *fld* mice carry the mutant *bcd* allele, we used a PCR assay to distinguish between the native and mutant *bcd-1* genes (12). This assay employs oligonucleotide primers that flank the deletion occurring in the mutant *bcd* gene so that PCR products of different sizes are produced for the native compared to the mutant allele. As shown in Fig. 6, using DNA from BALB/cJ and BALB/cBy, only the native PCR product of 870 bp is produced; in contrast, DNA from the BALB/cByJ strain produces a 592 bp product due to a 278 bp deletion in the mutant allele. Interestingly, DNA from BALB/cByJ-*fld* mice exhibiting both the wild-type and the *fld* phenotypes produce amplification products characteristic of the native *bcd* allele. This indicates that the BALB/cByJ-*fld* strain does not carry an underlying butyryl CoA dehydrogenase deficiency, and that the *fld* defect represents a distinct mutation associated with impaired fatty acid oxidation. Furthermore, these data demonstrate that the *bcd* mutation must have occurred in the BALB/cByJ parental strain recently, sometime after the BALB/cByJ-*fld* breeding stock was isolated in about 1981 (1).

Altered expression levels of several proteins in fld/fld fatty liver detected by quantitative 2-dimensional gel electrophoresis. The previous characterization of the fld fatty liver by Langner et al. (2) revealed altered expression levels of four proteins involved in lipid metabolism—hepatic lipase, lipoprotein lipase, apo A-IV, and apo C-II. In addition, in the current study we have presented biochemical evidence for altered activity of one or more proteins involved in fatty acid oxidation. To produce an overview of protein expression alterations in the fld fatty liver, we performed quantitative 2-dimensional gel electrophoresis (2DE) of total proteins in livers from 5 day old fld/fld and wild-type mice. Equivalent amounts of protein from liver homogenates were subjected to isoelectric focusing followed by electrophoresis in 9-17% SDS polyacrylamide slab gels, and protein patterns were analyzed ~~by digital scanning and integration of spot densities.~~ Liver samples from each of ten wild-type and ten fld/fld mice were examined; a typical 2DE pattern from an fld/fld liver is shown in Fig. 7. Homogenates from fld/fld and wild-type livers produced qualitatively similar 2DE patterns; however, quantitation of more than 400 distinct proteins detected by Coomassie Blue staining revealed significantly altered levels ($p < 0.001$) of 25 proteins in mutant compared to wild-type mice (arrows in Fig. 7 and Table 2). Of these, 23 proteins were expressed at significantly higher levels in fld/fld liver, whereas only 2 proteins (spots 36 and 112) were diminished in the mutant mice (Table 2). Interestingly, protein spot 112 was undetectable in 5 of the 10 fld/fld liver samples analyzed, and in the remaining fld/fld samples its level was reduced by 50%.

A comparison of the 2DE patterns with the mouse protein database maintained at Argonne National Laboratory (13) allowed identification of some of the 25 differentially expressed proteins. Proteins with elevated expression levels in fld/fld liver include keratin (spot 23), β -actin (spot 29), and γ -actin (spot 185). Additionally, spot 245 and 206 were identified as apo A-IV and apo E, respectively, by immunoblot analysis using specific antibodies (data not shown). Thus, in agreement with the previously detected elevations in mRNA for apo A-IV and apo E (2), apo A-IV protein levels are elevated 7-fold and apo E levels elevated 2.6-fold in the fatty liver of fld/fld mice. Expression levels of a related protein, apo A-I (spot 294 in the mouse protein database), are not altered in fld/fld liver, in agreement with our mRNA measurements (see Fig. 3). The database also provides information about

the relative abundance of proteins in three subcellular compartments (cytosol, mitochondria, and microsomes). The two proteins with diminished levels in fld/fld liver (36 and 112), as well as several of the proteins elevated in fld/fld, are most abundant in the cytosolic fraction (Table 2). The mouse protein database also contains information about the quantitative response of proteins to four different chemicals known to produce peroxisome proliferation in the liver (clofibrate, ciprofibrate, di-(2-ethylhexyl)-phthalate, and WY-14,463). Of the 17 proteins which show altered expression in fld/fld liver and for which data are available, 88% (15/17) respond to at least one of the four peroxisome proliferating agents, the majority of which (65%; 11/17) respond to one specific proliferating chemical, WY-14,463. Since only 61% (176/287) of all proteins identified in the mouse liver database respond to these proliferating agents, and of these only 33% (94/287) are affected specifically by WY-14,463, this suggests that many of the proteins whose levels are affected by the fld mutation may share common regulatory pathways that respond to agents such as WY-14,463. Peroxisome proliferators are known to induce changes in the expression of a specific subset of hepatic proteins, including a number of mitochondrial and peroxisomal enzymes. Thus, the altered expression levels in fld/fld liver of several proteins affected by peroxisome proliferators is consistent with the impaired fatty acid oxidation observed in fld/fld hepatocytes. Although many of the altered expression levels may represent secondary effects in response to the defect present in fld/fld cells, some of these proteins may serve as candidates for the fld gene product in future studies.

DISCUSSION

We present here a further characterization of the fatty liver dystrophy mutation by examining the nature of the fatty liver phenotype. Previous studies of the *fld/fld* mouse revealed that during neonatal development the mutant mice exhibit hypertriglyceridemia, reduced expression of lipases involved in the metabolism of triglyceride-rich lipoproteins, and fatty liver (2). Remarkably, these abnormalities resolve completely during the suckling/weanling transition, usually at 13-15 days of age. Thus, the *fld* mutation appears to impair homeostatic mechanisms operating in newborn mice that control the metabolism of triglycerides present at high concentrations in mother's milk that is ingested during the postnatal period. To further elucidate the nature of this defect, we have investigated the alterations in cellular lipid metabolism occurring in the fatty liver of *fld/fld* neonates. We have established that the lipid accumulating in the fatty liver is virtually all in the form of triglyceride. It is clear from examination of both liver sections and isolated hepatocytes that the accumulation of lipid in the liver of neonatal *fld/fld* mice occurs within the parenchymal cells and cannot be attributed to other cell types such as Kupffer cells or fat storing Ito cells. We also demonstrate that isolated neonatal hepatocytes grown in culture maintain several hallmark features of the fatty liver phenotype. These include cytoplasmic lipid droplets, elevated apo A-IV mRNA levels, and reduced hepatic lipase activity.

To identify the metabolic defect that leads to formation of the fatty liver, we investigated several aspects of cellular triglyceride metabolism. We first measured the activity of acid triacylglycerol lipase, an enzyme responsible for hydrolysis of dietary triglycerides entering the liver from lipoprotein remnants in the blood (16). Normal acid lipase activity was detected in liver extracts from *fld/fld* mice of all ages indicating that triglyceride accumulation does not result from a defect in hydrolysis of exogenous triglyceride. A second possible mechanism for triglyceride accumulation is increased triglyceride synthesis in *fld/fld* hepatocytes. Since the livers of *fld/fld* mice appear normal at birth, but are completely fatty and hypertrophied after suckling for a day, it is likely that increased triglyceride synthesis does occur during the initial 24 hours. However, our metabolic labeling experiments performed in 3 day old mice revealed that increased triglyceride synthesis is not required for

maintenance of the fatty liver throughout the neonatal period. Rather than increased rates of fatty acid incorporation into triglyceride, we observed an accumulation of free fatty acid in the liver (see Figs. 2 and 5a). Furthermore, plasma free fatty acid levels were elevated in fld/fld neonates, with levels highest in very young mice and returning to normal by the age of one month. These results indicate that increased triglyceride synthesis is not the underlying defect responsible for the fatty liver, and suggested that a defect in fatty acid oxidation may be involved.

The direct quantitation of fatty acid oxidation in neonatal fld/fld hepatocytes revealed a 60% decrease in the rate of palmitate oxidation. Similar studies performed in fibroblasts also showed a reduced efficiency (40%), although less pronounced than in hepatocytes. The lack of lipid accumulation in fld/fld fibroblasts even though they exhibit impaired fatty acid oxidation is presumably due to the fact that this cell type does not take up triglyceride-rich lipoproteins from the blood, and is consistent with what has been observed in fibroblasts derived from human patients with inborn errors in fatty acid oxidation (17). Interestingly, the fatty liver phenotype closely resembles that of suckling rats which have been treated with fatty acid oxidation inhibitors. Administration of a single dose of a carnitine acyl transferase inhibitor to suckling rats results in fatty liver formation within 18 hours (23). However, treatment of adult rat liver with the same inhibitor results in stimulation of hepatic very low density lipoprotein (VLDL) secretion (24). This difference between neonatal and adult response has been attributed to the fact that the neonatal rodent liver has a limited capacity to secrete VLDL triglyceride (15). Thus, results from our study are consistent with the hypothesis that the fatty liver in fld/fld neonates results from impaired fatty acid oxidation compounded by both high levels of dietary triglyceride derived from mother's milk, and the inability to secrete VLDL. The spontaneous resolution of the fatty liver at the age of weaning corresponds temporally to both a reduction in dietary fat and acquisition of the capacity for VLDL secretion. However, as Langner et al. (2) have previously demonstrated, neither prolonged suckling of fld/fld pups beyond the normal age of weaning nor feeding adult fld/fld mice a triglyceride-rich diet elicit fatty liver formation. Based on these observations, it seems likely that the initiation of VLDL secretion at a particular developmental stage is a major factor contributing to the resolution of the fatty liver.

A somewhat perplexing feature of the *fld* phenotype is the appearance of normal rates of fatty acid oxidation in hepatocytes from adult *fld/fld* mice. It is clear that adult *fld/fld* mice exhibit none of the secondary phenotypes associated with impaired fatty acid oxidation, such as increased fatty acid levels in blood or liver. In adult mice the consumption of a low fat diet together with the capacity for VLDL secretion may not require that fatty acid oxidation be operating at full capacity. In that case, adult hepatocytes that are isolated and subjected to the oxidation assay may be capable of oxidizing the radiolabeled substrate at high efficiency for the 1 hour duration of the assay before the oxidation pathway becomes overburdened. Another possibility is that the capacity for fatty acid oxidation in adult liver is different than in neonatal liver due to developmentally programmed alterations in the expression of fatty acid oxidation enzymes. For example, levels of the acyl-CoA dehydrogenases, enzymes that catalyze the first step of mitochondrial fatty acid oxidation, are low at birth, increase several-fold to peak at postnatal days 10-14, and then diminish to birth levels (25). Thus, these enzymes are critical for maintaining lipid homeostasis during the neonatal period corresponding to the fatty liver stage, and then abruptly decline as the requirement for oxidation of diet derived fatty acids declines when animals are weaned to a low fat diet (days 14-17). Thus, if the *fld* mutation affects a protein, such as an acyl CoA dehydrogenase, whose expression is most important during the neonatal period, a phenomenon similar to that seen with the *fld* fatty liver might occur. Alternatively, expression of proteins with functions that may compensate for that of the mutant protein could be switched on at the time corresponding to resolution of the fatty liver.

The molecular basis for impaired fatty acid oxidation in the *fld/fld* liver is not known. Thus far, only one mutation affecting fatty acid oxidation has been identified in the mouse. This mutation, a deletion in the gene encoding the enzyme butyryl acyl-CoA dehydrogenase (*bcd*), occurred spontaneously in the BALB/cByJ mouse strain (19, 20). The resulting *bcd* deficiency produces a block in the multistep conversion of short-chain fatty acid (butyrate) to acetyl-CoA via β -oxidation. While the *bcd* gene is not linked to the *fld* mutation (E. Birkenmeier, personal communication), mutations in both genes occurred in the same mouse strain. Using a definitive PCR assay to distinguish between native and defective *bcd* alleles, we demonstrated that both *fld/fld* and unaffected littermates of the BALB/cByJ-*fld*

strain are homozygous for the native *bcd* gene. These results indicate that the *fld* mutation must have occurred in the BALB/cByJ strain some time after the isolation and breeding of the *fld* mutants in 1981 (1). Furthermore, the results demonstrate that a deficiency in the *bcd* allele does not contribute to the *fld* phenotype, and that the *fld* defect represents a second mouse mutation leading to impaired fatty acid oxidation.

Some aspects of the neonatal *fld/fld* mouse phenotype resemble disorders in mitochondrial or peroxisomal fatty acid oxidation that occur in humans. The β -oxidation of fatty acids occurs in both mitochondria and peroxisomes via independent and distinct enzyme systems (reviewed in 26, 27, 28). The two organelles also differ in their substrate specificities, with mitochondria serving as the primary site for oxidation of short- (C4-C6), medium- (C8-16) and long-chain (C18-22) fatty acids, whereas peroxisomes catalyze the oxidation of very long-chain (C24-26) fatty acids, dicarboxylic acids, branched chain fatty acids, and prostaglandins. Some general characteristics of human mitochondrial fatty acid oxidation disorders that are also apparent in *fld/fld* mice include fatty liver, hypoglycemia, increased free fatty acid concentration in serum, and reduced fatty acid oxidation in fibroblasts. Furthermore, the degree to which fatty acid oxidation is impaired in *fld/fld* fibroblasts (38%) is similar to that reported for some human fatty acid oxidation disorders using the same assay conditions employed here. For example, human disorders that may each exhibit reductions of 40-85% in oxidation activity include medium-chain acyl CoA dehydrogenase deficiency, long-chain acyl CoA dehydrogenase deficiency, and deficiencies (mild form) in electron transfer flavoprotein and electron transfer flavoprotein dehydrogenase (18). These disorders in mitochondrial oxidation are characterized by the accumulation of specific metabolic intermediates. For example, deficiencies in medium-chain and long-chain acyl CoA dehydrogenases, and electron transfer flavoprotein, are typified by the accumulation of dicarboxylic (adipic, suberic, and sebacic) acids (17, 29). A preliminary examination of the organic acids present in livers of *fld/fld* neonates via mass spectrometry/gas chromatography has revealed normal levels of dicarboxylic acids (K. Reue and L. Sweetman, unpublished observations). In this respect the fatty acid oxidation disorder in the *fld* mouse appears not to mirror the human disorders in mitochondrial fatty acid oxidation mentioned above. However, it should be noted that differences exist

between mice and humans in the efficiency with which fatty acid metabolites are eliminated, and this could produce different biochemical and pathological features for the two species (30).

Peroxisomal fatty acid oxidation disorders have also been described in humans (reviewed in 28, 31). The most severe peroxisomal disorders are those in which peroxisomes are severely diminished or absent, such as Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease (28). Electron micrographs of livers from both neonatal and adult *fld/fld* mice reveals that intact peroxisomes are present in these animals (K. Reue and B. Slavin, unpublished observation) indicating that if a peroxisomal defect is present, it must involve expression or function of a specific peroxisomal enzyme(s) rather than a gross peroxisome assembly defect. In addition to fatty acid oxidation, peroxisomes are involved in the synthesis of bile acids and phospholipids such as plasmalogens, which are abundant in membranes of nervous tissues (28). Thus, many peroxisomal disorders are characterized by neurodegeneration and psychomotor retardation. This symptom is reminiscent of the neuropathy that progressively develops in the *fld/fld* mouse throughout adulthood (3). Under normal conditions, the contribution of peroxisomes to β -oxidation in hepatocytes is minor, estimated to be 10% that in mitochondria (32). However, in conditions in which lipid metabolism is perturbed by starvation, high fat diet, or diabetes, peroxisomal activity is stimulated (33, 34). Increased peroxisome number and metabolic capacity can also be induced by a diverse group of chemicals known as peroxisome proliferators (35, 36). These compounds appear to have a direct effect on hepatic lipid metabolism by stimulating peroxisomal β -oxidation and inducing intracellular lipid accumulation. Interestingly, a majority of the 25 proteins that we have shown to exhibit altered expression levels in liver of *fld/fld* neonates are known to respond to peroxisome proliferators. Furthermore, it has been demonstrated previously that the expression of non-peroxisomal proteins including apo A-IV, hepatic lipase, and lipoprotein lipase is modulated by peroxisome proliferators (36).

Although the mechanism by which peroxisome proliferators exert their effect is not fully understood, a class of proteins known as peroxisome proliferator activated receptors (PPAR) have been identified which appear to play a role in modulating gene expression in response to these agents (36, 37, 38). The PPARs belong to the nuclear hormone receptor gene family and are known to modulate the

transcription of specific genes in response to activators. In addition to peroxisome proliferator chemicals such as fibric acid derivatives, PPAR activators include various fatty acids including linoleic, arachidonic, and lauric acids (37, 38, 39, 40). A current hypothesis linking the action of peroxisome proliferators and fatty acid metabolism has been proposed (36). This hypothesis suggests that the perturbation of intracellular lipid metabolism by peroxisome proliferating chemicals results in the accumulation of fatty acids, leading to the activation of PPARs, and subsequent modulation of gene transcription. Given the observed alterations in fatty acid oxidation and protein expression in the liver of fld/fld neonates, it is tempting to speculate that the fld mutation could affect the activity of one or more PPAR. Since the precise mechanism by which fatty acids activate PPARs is not yet known, the further characterization of the fld mutation may be helpful in elucidating the the peroxisome proliferator signalling pathway.

Ultimately, a complete understanding of the fld mutation requires the identification of the fld gene product itself. In this study we have detected numerous proteins with altered expression in the fatty liver, some of which may be candidates for the fld gene product. Increased expression of most of these proteins are likely to represent secondary responses designed to compensate for the primary defect. Alternatively, the fld ^{mutation} gene-product could be ^{in a} ^{region} a regulatory protein that is required to achieve proper expression levels of this specific set of proteins. Further studies aimed at identifying several of the proteins with altered levels may distinguish between these possibilities and suggest specific candidates for the fld gene product. An approach to cloning the fld gene may also be feasible based on data from the current study. The demonstration that isolated hepatocytes from fld/fld neonates maintain the fld phenotype in culture for several days may provide a means for identifying the fld gene product by complementation. Since the fld mutation is recessive, the expression of exogenously introduced DNA containing sequences encoded by the normal fld gene should allow correction of the phenotype. Complementation should result in loss of lipid from the cells and increased secretion of hepatic lipase, two phenotypes that could be detected in cultured cells. This approach could initially be used to test candidates suggested by our study, including proteins involved in fatty acid oxidation or peroxisome proliferator signalling.

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FOOTNOTES

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³Abbreviations used are: LPL, lipoprotein lipase; apo, apolipoprotein; I.P., intraperitoneal; bcd, butyryl CoA dehydrogenase; 2DE, two-dimensional gel electrophoresis; VLDL, very low density lipoprotein; PPAR, peroxisome proliferator activated receptor.

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FIGURE LEGENDS

FIGURE 1. Light micrographs of liver sections from 9 day and 3 month old flt/flt and wild-type mice. Liver tissue was sectioned and stained with hemotoxylin and eosin and examined by light microscopy. Liver sections from 9 day old flt/flt mice are characterized by extensive accumulation of large intracellular lipid droplets, apparent as poorly stained circular structures within the parenchymal cells (**panel a**), compared to the presence of small, sparse lipid droplets in sections from 9 day old wild-type mice (**panel b**). At 3 months of age, flt/flt (**panel c**) and wild-type (**panel d**) liver sections appear similar with few small lipid droplets apparent. Magnification is 495X.

FIGURE 2. Thin-layer chromatogram of neutral lipids from livers of flt/flt and wild-type mice. Representative thin layer chromatogram of liver homogenates from 7, 14, 30, and 90 day old wild-type (wt) and flt/flt mice. Aliquots containing equivalent amounts of protein were applied to silica gel plates, developed in hexane-ethyl ether-acetic acid (80:20:1), and detected by charring in 3% cupric acetate/8% phosphoric acid. Lipid species were identified by comparison to lipid standards, shown in the lane at far left. Cholesteryl ester (CE), triacylglycerol (TG), fatty acid (FA), free cholesterol (FC), and phospholipid (PL). The band migrating directly below free cholesterol which is visible in 7 and 14 day flt samples represents diacylglycerol.

FIGURE 3. mRNA levels for apo A-IV and apo A-I in cultured hepatocytes prepared from 10 day old flt/flt and wild-type mice. Isolated hepatocytes from 10 day old flt/flt and wild-type (wt) mice were maintained in culture for 4 days and the total RNA isolated. Northern blot analysis was performed with 10 μ g of RNA. Hepatocytes from flt/flt mice (lane 1) exhibit approximately 90-fold higher levels of mRNA for apo A-IV than cells from wild-type littermates (lane 2), although mRNA levels for apo A-I are similar in both.

FIGURE 4. Hydrolysis of [^3H]triolein at acid pH in liver homogenates from fld/fld and wild-type mice. Liver homogenates prepared from mice of indicated ages were incubated with [^3H]triolein at pH 5 to estimate hydrolytic activity due to lysosomal acid lipase (detailed in Methods). Values given represent mU enzyme activity/mg protein. Error bars represent SD of assays performed on samples from at least three mice.

FIGURE 5. Accumulation of free fatty acids in liver and plasma of neonatal fld/fld mice. Pulse label experiments performed with [^3H]oleic acid in 3 day old (**panel a**) and 60 day old mice (**panel b**). [^3H]oleic acid was injected I.P. and livers harvested after a 15 min pulse. Lipids were extracted and separated by thin layer chromatography on silica plates (see Methods and Fig. 2). Regions corresponding to specific lipid species were collected and quantitated by scintillation counting. Plots show the proportion of [^3H]oleic acid label incorporated into lipid species. Values shown represent the average $\pm$ SD for two mice of each phenotype and each age. PL, phospholipid; DAG, diacylglycerol; FFA, free fatty acid; TG, triacylglycerol; CE, cholesteryl ester. (**Panel c**) Free fatty acid levels were determined in plasma samples from mice aged 5, 8, 12, 30, and 90 days. Values shown represent the average $\pm$ SD for 3 mice of each age and genotype, except 8 days of age, which represents the average of 2 mice. *, fld significantly different than wild-type at $p > 0.01$; •, fld significantly different than wild-type at $p > 0.05$.

FIGURE 6. PCR assay for bcd-1 alleles in BALB/c mouse substrains. Genomic DNA from BALB/c substrains BALB/cJ, BALB/cBy and BALB/cByJ as well as fld/fld and wild-type mice from strain BALB/cByJ-fld were amplified with oligonucleotide primers corresponding to exonic sequences of the bcd-1 gene. PCR products were analyzed on a 2% NuSieve agarose gel stained with ethidium bromide. The 870 bp product from the native bcd-1 allele is present in BALB/cJ (lane 1), BALB/cBy (lane 2), and both wild-type (lane 5) and fld/fld mice (lane 6) of the BALB/cByJ-fld strain (upper arrow). The 592 bp product from the mutant bcd-1 allele is present in the BALB/cByJ strain (lane 3, lower arrow). The molecular weight standard shown in lane 4 is ΦX174 digested with Hae III.

FIGURE 7. Representative 2-dimensional gel electrophoresis pattern from liver of 5 day old fld/fld mice. Arrows indicate proteins with altered abundance in fld/fld compared to wild-type liver samples; numbers refer to protein spot numbers as assigned in the Argonne National Laboratory Mouse 2DE Database and correspond to the numbers indicated in Table 2. The gel is oriented with acidic proteins to the left, basic proteins to the right, high molecular weight proteins toward the top, and low molecular weight proteins toward the bottom. All proteins indicated with arrows exhibited elevated levels in fld/fld compared to wild-type mice, except spots 36 and 112, which were diminished in fld/fld liver. Relative levels of all indicated proteins are given in Table 2.

<u>Cell Type</u>	<u>Phenotype</u>	<u>cpm $^3\text{H}_2\text{O}/\mu\text{g}$ protein</u>	<u>pmol $^3\text{H}_2\text{O}/\text{mg}$ protein/hr</u>	<u>fld:wt ratio</u>
Neonatal Hepatocyte	wt	18,312 $\pm$ 480	465 $\pm$ 12	0.36
	fld	6,605 $\pm$ 297	*167 $\pm$ 8	
Neonatal Fibroblasts	wt	5,674 $\pm$ 212	143 $\pm$ 5	0.61
	fld	3,432 $\pm$ 134	*87 $\pm$ 3	
Adult Hepatocyte	wt	4,816 $\pm$ 1516	122 $\pm$ 38	1.10
	fld	5,296 $\pm$ 1436	134 $\pm$ 36	

Table 1. $^3\text{H}_2\text{O}$ formation from [9,10(n) ^3H]palmitate by cultured cells from fld/fld and wild-type mice. The oxidation of labelled palmitate substrate by primary hepatocytes or fibroblasts isolated from fld/fld and wild-type (wt) mice was determined as detailed in Methods. Neonatal hepatocytes and fibroblasts were isolated from 10 day old animals; adult hepatocytes were isolated from 60 day old animals. Hepatocyte data are from experiments performed with cells from at least two individual animals in independent assays performed in triplicate. Fibroblasts data are from experiments performed with cell lines established from a single animal of each genotype in three independent assays performed in triplicate. Activity is presented as both the cpm of $^3\text{H}_2\text{O}$ produced per μg of cellular protein $\pm$ SD, and as the rate of production in pmol/mg protein/hr $\pm$ SD. The relative activity in fld/fld compared to wild-type cells is indicated in the final column. *Significantly different from wild-type ($p < 0.01$).

Spot Number	Molecular Weight	wild-type	fld	fld wt	Peroxisome Prolif. Response
1	72,500	66,133 (17.7%)	105,991 (14.4%)	1.60	+
23	48,000	17,963 (11.3%)	28,204 (18.9%)	1.57	+
29	43,200	18,024 (16.3%)	38,106 (15.1%)	2.11	+
36	35,700	40,683 (12.2%)	14,969 (28.2%)	0.37	+
112	33,500	5221 (17.5%)	2438 (34.8%)	0.47	+
181	47,300	2368 (11.9%)	3388 (17.6%)	1.43	+
185	43,500	4069 (33.6%)	9006 (30.3%)	2.21	+
198	62,000	3822 (13.0%)	6416 (9.0%)	1.68	+
206	34,000	5036 (13.7%)	13,318 (22.7%)	2.64	+
223	62,500	1504 (12.8%)	2305 (10.5%)	1.53	+
227	25,800	2147 (54.0%)	4571 (23.2%)	2.13	+
245	43,000	7306 (34.3%)	53,163 (20.4%)	7.28	-
258	61,700	2262 (6.8%)	3163 (8.7%)	1.40	+
301	38,700	2286 (11.6%)	3149 (11.6%)	1.38	+
316	27,900	2944 (10.4%)	7154 (16.2%)	2.43	+
395	33,400	1612 (29.1%)	3252 (14.9%)	2.02	-
421	49,500	957 (11.2%)	1311 (12.9%)	1.37	n.d.
433	60,600	1849 (12.0%)	3162 (12.2%)	1.71	n.d.
436	39,000	2007 (18.9%)	4384 (30.4%)	2.18	+
485	43,500	1617 (18.3%)	2339 (15.3%)	1.45	n.d.
1038	49,000	1042 (27.3%)	1853 (17.0%)	1.78	n.d.
1061	54,000	947 (32.2%)	1904 (27.7%)	2.01	n.d.
1067	43,400	1611 (33.4%)	3799 (21.9%)	2.36	n.d.
1096	58,000	1427 (47.3%)	2898 (17.2%)	2.03	n.d.

Table 2. Integrated densities and characteristics of proteins with altered expression levels in neonatal fld/fld liver. Protein densities were determined for liver samples from each of 10 wild-type (wt) and 10 fld/fld mice; numbers in parentheses represent the coefficient of variation for determinations of 10 animals of each phenotype. The 25 proteins listed were significantly different in the fld/fld livers ($p < 0.001$). Molecular weight and peroxisome proliferator response data are from the mouse protein database (13). n.d., not determined.

FIG 2

wt fld wt fld wt fld wt fld wt fld
7 day 14 day 30 day 90 day

FIG 3

By/J-fld

wt

fld

By/Jstd

By

J

6

5

4

3

2

1

870

592