ABCD2 is abundant in adipose tissue and opposes the accumulation of dietary erucic acid (C22:1) in fat

Jingjing Liu,* Nadezhda S. Sabeva,* Saloni Bhatnagar,† Xiang-An Li,† Aurora Pujol,§ and Gregory A. Graf†,‡*

Department of Pharmaceutical Sciences and Cardiovascular Research Center,§ Department of Pediatrics,† University of Kentucky, Lexington, Kentucky; Neurometabolic Disease Lab and Institute of Neuropathology,§ Institut d’Investigació Biomèdica de Bellvitge, (IDIBELL), Center for Biomedical Research on Rare Diseases (CIBERER) and Catalan Institution for Research and Advanced Studies (ICREA), Barcelona, Spain

Abstract The ATP binding cassette transporter, ABCD2 (D2), is a peroxisomal protein whose mRNA has been detected in the adrenal, brain, liver, and fat. Although the role of this transporter in neural tissues has been studied, its function in adipose tissue remains unexplored. The level of immunoreactive D2 in epididymal fat is >50-fold of that found in brain or adrenal. D2 is highly enriched in adipocytes and is upregulated during adipogenesis but is not essential for adipocyte differentiation or lipid accumulation in day 13.5 mouse embryonic fibroblasts isolated from D2-deficient (D2−/−) mice. Although no differences were appreciated in differentiation percentage, total lipid accumulation was greater in D2−/− adipocytes compared with the wild type. These results were consistent with in vivo observations in which no significant differences in adiposity or adipocyte diameter between wild-type and D2−/− mice were observed. D2−/− adipose tissue showed an increase in the abundance of 20:1 and 22:1 fatty acids. When mice were challenged with a diet enriched in erucic acid (22:1), this lipid accumulated in the adipose tissue in a gene-dosage-dependent manner. In conclusion, D2 is a sterol regulatory element binding protein target gene that is highly abundant in fat and opposes the accumulation of dietary lipids generally absent from the triglyceride storage pool within adipose tissue.—Liu, J., N. S. Sabeva, S. Bhatnagar, X.-A. Li, A. Pujol, and G. A. Graf. ABCD2 is abundant in adipose tissue and opposes the accumulation of dietary erucic acid (C22:1) in fat. J. Lipid Res. 2010. 51: 162–168.

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Peroxisomes play essential roles in the metabolism of both dietary and endogenously synthesized lipids (1, 2). The synthesis of the etherphospholipids (platelet activating factor and plasmalogens), partial β-oxidation of very-long-chain fatty acids (VLCFAs; C>22), and the α-oxidation of phytanic acid occur within this organelle. A comprehensive integrative map of peroxisomal metabolic pathways can be found at www.peroxisomedb.org (3). The importance of the organelle is best exemplified by the occurrence of peroxisome biogenesis disorders and single-enzyme deficiencies that result in severe metabolic diseases that are often lethal in early childhood. X-linked adrenoleukodystrophy (X-ALD) is the most common peroxisomal disorder characterized by the accumulation of VLCFA in tissues, progressive demyelination, and adrenocortical dysfunction (4). X-ALD is caused by mutations in ABCD1 (D1), an ATP binding cassette (ABC) half transporter that presumably mediates the transport of very-long-chain acyl-CoAs into peroxisomes (5). D1 is the founding member of a quartet of ABC half-transporters that contain peroxisomal targeting sequences and form heterodimers in vitro (6). However, homodimers appear to be preferred in vivo, and each subfamily member has a unique tissue distribution (7, 8).

The closest paralog to D1 is D2, which shares 66% amino acid identity and is expressed in the adrenal, brain, and liver (9). Expression of a D2 transgene in D1-deficient mice partially corrected the phenotype in this mouse

Abbreviations: ABC, ATP binding cassette; ACC, acetyl CoA carboxylase; FID, flame ionization detector; MEF, mouse embryonic fibroblast; qRT-PCR, quantitative real-time PCR; SREBP, sterol regulatory element binding protein; VLCFA, very-long-chain fatty acid; X-ALD, X-linked adrenoleukodystrophy.

†To whom correspondence should be addressed.
e-mail: gregory.graf@uky.edu
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model of X-ALD, indicating that these transporters have some degree of overlapping substrate specificity (10, 11).

In liver, D2 mRNA levels are typically <10–20% of those observed in brain or adrenal (9, 12). However, its expression is induced by fasting, feeding fibrate, and statin treatment, while expression levels in brain remain constant (13–17). More recently, D2 mRNA was shown to be expressed in adipose tissue where its mRNA levels are highest in the fed state, decline in the fasted state, and return during refeeding (17). The promoter of D2 contains a functional sterol response element that interacts with both regulatory element binding protein (SREBP-1a) and -1c, suggesting that it is a component of the lipogenic program (17–19).

Mice deficient in D2 (D2−/−) are characterized by late-onset cerebellar and sensory ataxia, degeneration of dorsal root ganglia, and accumulation of VLCFA in dorsal root ganglia (20). They show evidence of disrupted mitochondrial membranes, consistent with increased oxidative stress. In addition, the adrenal gland of D2−/− mice shows morphological signs of oxidative stress and has increased expression of manganese superoxide dismutase (21). Collectively, the data suggest that D2 opposes the accumulation of VLCFA and oxidative stress in adrenal and brain. A recent report suggests that D2 may play a role in fatty acid metabolism in adipose tissue since VLCFA increased in plasma following a 48 h fast in mice lacking D2 but not in wild-type controls (22). However, the role of D2 in adipocytes and adipose tissue remains unexplored. In this study, we determined the relative abundance of D2 protein in adipose tissue, its role in adipogenesis, and the clearance of dietary erucic acid in adipose tissue. Our results indicate that D2 expression is robust in adipose tissue and upregulated during adipogenesis but is not essential for adipogenesis or lipid storage in vitro. Conversely, erucic acid accumulated in the adipose tissue of mice challenged with a diet enriched in this fatty acid in a gene-dosage-dependent manner, suggesting that the role of D2 in fat is to facilitate the clearance of this, and presumably other, dietary VLCFAs that are generally very low or absent in adipose tissue but present in significant quantities in the diet.

MATERIALS AND METHODS

Reagents and buffers

General chemical reagents were obtained from Sigma (St. Louis, MO). Calnexin and GRP78 antibodies were purchased from StressGen, Nventa (San Diego, CA). CD36 was a generous gift from Dr. Nancy Webb (UK). Actin and GAPDH antibodies were purchased from Sigma. Secondary antibodies and enhanced chemiluminescence reagents were purchased from Pierce (Rockford, IL).

Development of ABCD2 antibody

The cDNA encoding the cytoplasmic domain C-terminal of the ABC of murine D2 (amino acids 366–711) was fused to the large T-antigen of tetanus toxin and a six-histidine tag and cloned into pET28a (+) bacterial expression vector. Expression in the BL21 strain of *Escherichia coli* cells was induced by isopropyl-β-D-1-thiogalactopyranoside and the 6-His fusion protein isolated under denaturing conditions using Ni-NTA agarose beads. The purified peptide was dialyzed against phosphate-buffered 4M urea and quantified. The immunization of rabbits was outsourced to ProSci (Poway, CA). Antiserum was screened for immunoreactivity to D2 by immunoblotting of the antigen. Its utility immunoblotting and immunofluorescence applications was determined in CHO-K1 cells transfected with plasmid harboring cDNA encoding full-length murine ABCD2 and total membrane preparations from adipose tissue of wild-type and D2-deficient mice (see supplementary Fig. I).

Animal husbandry

Mice lacking Abcd2 and their wild-type littermates maintained on the C57BL/6J background were examined at 8 weeks of age. Genotyping experiments to differentiate Abcd2 knockouts from heterozygotes and wild types were done as described (20). Animals were housed in a temperature-controlled room with 12:12 light:dark cycle (6:00 AM to 6:00 PM). All mice were maintained on standard rodent chow (Harlan Teklad 2014S). The erucic acid enriched diet was made by mixing 50 g (37°C) of erucic acid with 1,000 g of powdered diet (see supplementary Tables I and II; #D1001, Research Diets, New Brunswick, NJ) in a standard mixer equipped with a wire whisk. The diet was vacuum packaged after preparation and stored at 4°C to prevent oxidation.

Following a 4 h fast beginning shortly after lights-on, mice were euthanized by exsanguination under ketamine/xylazine anesthesia. Blood was collected from the right ventricle with a 1 cc syringe fitted with a 20 ga hypodermic needle. Serum was separated by centrifugation and stored at −20°C until analysis. Tissues were excised, rinsed with PBS to remove blood, and snap frozen in liquid nitrogen. Tissue samples were stored at −80°C until analyses.

Blood analysis

Blood glucose levels were measured using a standard glucometer from a drop of blood obtained by tail-vein prick following a 4 h fast beginning at lights-on. Total serum cholesterol and triglyceride concentrations were determined by colorimetric enzymatic assays (Wako Chemicals, Richmond, VA).

Lipid analyses

Total fatty acids in adipose tissue were extracted by chloroform/bHT (50 mg/ml), and C17:0 was added after extraction as internal standard. The lipids were esterified following the same procedure as adipose tissue lipid extraction. One microliter of the sample was injected onto a GC system (Agilent 6890 GC oven: 160°C (5 min) to 220°C at 4°C/min; carrier: helium, 1.2 ml/min).

Cell culture

The 3T3-L1 cells were propagated as fibroblasts in subconfluent cultures. Two days postconfluence, the cells were differentiated...
The 75 kDa band was most prominent in adipose tissue.

In addition, much of the immunoreactive D2 migrated as a prominent smear in our SDS-PAGE gels, ranging from 120 to >220 kDa, the largest of our molecular weight markers. We initially presumed these forms to represent protein aggregates of D2 since a number of ABC transporters are prone to aggregation upon boiling in SDS. However, efforts to resolve this material using gentle heating in urea buffer or increasing the relative amount of SDS and β-mercaptoethanol to membrane proteins failed to resolve this protein into a single 75 kDa band (see supplementary Fig. II).

Given the prominent signal observed for D2 in adipose tissue, we next determined the relative abundance of D2 among mouse fat depots by qRT-PCR and immunoblotting (Fig. 2). Whereas the mRNAs for D2 were similar among the epididymal, inguinal, and retroperitoneal fat pads, the expression was low in mesenteric and brown fat (Fig. 2A). For comparative purposes, we evaluated the expression of the related family member, D1. Whereas D1 was similar among each of the white fat pads, its expression was 4-fold greater in brown adipose tissue when compared with epididymal fat. It should be noted that the relative abundance of D1 mRNA among the white fat depots was similar to that of liver. Conversely, the abundance

Protein and RNA analysis

Membrane proteins were prepared and analyzed by SDS-PAGE and immunoblot analysis as previously described (23). The isolation of total RNA and the determination of relative transcript abundance by quantitative real-time PCR (qRT-PCR) for both tissues and cells were conducted as previously described (23).

Statistical analysis

Data were analyzed by ANOVA. Bonferroni posts tests were employed where indicated. All statistical analyses were conducted using GraphPad Prism statistical analysis software.

RESULTS

Previous reports indicated that D2 mRNA was present in adipose tissue and was regulated by fasting and refeeding in mice; however, the presence and relative abundance of D2 protein in adipose tissue is unknown (17). To determine the relative abundance of D2 protein in fat with respect to other tissues, we developed a polyclonal antibody to mouse ABCD2 (see supplementary Fig. I). We then prepared total membranes from tissue homogenates and compared the relative levels of immunoreactive D2 by immunoblot analysis (Fig. 1).

Total membrane preparations were pooled from four male C57BL6/J mice. Epididymal fat from a D2-deficient mouse (D2−/−) was used as a negative control. The antibody detected a 75 kDa band in brain, skeletal muscle, lung, liver, testis, brown fat, and epididymal fat. With longer exposures, D2 was also detected in adrenal (data not shown). Our antibody also cross-reacted with a 65 kDa band present in several tissues that was most prominent in lung, liver, kidney, testis, and brown fat. This band was judged to be nonspecific based on its persistence in blots in each of these tissues in D2−/− mice (data not shown).

Fig. 1. Relative expression of immunoreactive D2 in selected mouse tissues. Total membrane proteins were prepared from tissues of male C57BL6/J mice. Equal amounts of proteins were pooled from each mouse and analyzed by immunoblotting using the antibody directed against D2. Specificity of the immunoreactive D2 bands was confirmed using tissues from D2-deficient mice (D2−/−) mice. Epi., epididymal; NSB, nonspecific band.

Fig. 2. Relative expression of D2 mRNA and protein among selected mouse fat depots. A: The relative levels of D2 and D1 mRNA among fat pads were measured by qRT-PCR and normalized to cyclophilin and epididymal fat using the ΔΔCT method. B: Total membrane proteins were prepared from epididymal (Epi.), inguinal, retroperitoneal, mesenteric, and brown fat pads and analyzed by immunoblotting. Epididymal and brown fat pads from D2−/− mice were also analyzed to ensure specificity of immunoreactive proteins. The blot was reprobed for calnexin to verify equal loading of membrane proteins. Asterisk denotes residual signal from D2 immunoblot upon reblotting with calnexin.
of D2 mRNA in liver was <5% of epididymal fat (data not shown).

Immunoblotting results were consistent with mRNA data indicating abundant expression of D2 in epididymal, inguinal, and retroperitoneal fat pads and much less expression in mesenteric and brown fat (Fig. 2B). As in Fig. 1, visualization of immunoreactive D2 in membrane preparations from mesenteric and brown adipose tissue resulted in overexposure of films for epididymal and other fat pads.

Although the adipocyte is the predominant cell type present in adipose tissue, other cell types are also present and could be the source of immunoreactive D2 in fat. To test the hypothesis that D2 was present in adipocytes within adipose tissue, we compared the expression between adipocytes and the stromal vascular fraction that contains a mixture of preadipocytes, fibroblasts, macrophages, endothelial cells, and others. Following collagenase digestion of adipose tissue, adipocytes were separated from stromal vascular cells by low-speed centrifugation. Whole adipose, adipocytes, and stromal vascular cells were evaluated for abundance of D2 by quantitative PCR and immunoblotting (Fig. 3). The relative abundance of adipocyte markers [FAS and acetyl CoA carboxylase (ACC)], macrophages (CD68), and endothelial cells (CD31) were used as controls in this experiment. Whereas mRNAs for FAS and ACC are enriched in adipocytes, CD68 and CD31 are enriched in the stromal vascular fraction (Fig. 3A). D2 and D1 were enriched in the adipocyte and stromal vascular fractions, respectively. These results were confirmed by immunoblotting (Fig. 3B), which shows that virtually all of the immunoreactive D2 was confined to the adipocyte fraction within adipose tissue. CD36, a protein expressed in adipocytes, macrophages, and endothelial cells was used as a loading control, although it is slightly enriched in adipocytes.

Next, we determined if D2 was upregulated during adipogenesis. For these experiments, we utilized murine NIH3T3-L1 cells (Fig. 4). Cells were cultured to confluence (day 0) and treated with differentiation cocktail beginning on day 2 (see Materials and Methods). Neutral lipids were stained with Oil-Red O on even days of the differentiation protocol (Fig. 4A). Neutral lipid is detectible by day 4, accumulates in a linear fashion until day 8, and remains constant through day 10 (data not shown). The expression of fatty acid binding protein 4 (aP2) was used to assess expression of adipocyte markers (Fig. 4B). Although not nearly as robust as the upregulation of aP2, D2 mRNA increased 4-fold between days 2 and 4 and remained elevated throughout the differentiation protocol. In contrast, the expression of D1 declined between days 0 and 2 and never returned to predifferentiation levels. We also evaluated immunoreactive levels of D2 in cell lysates on even days of the differentiation protocol. Similar to other tissues, a nonspecific band was observed for D2 in NIH3T3-L1 cells. Immunoreactive D2 was first visible on day 4 and accumulated throughout the differentiation protocol.
For increased adiposity in D2 mice, we observed no statistically significant differences due to genotype or suppress lipid accumulation in adipocytes. In fact, observations, the absence of D2 did not prevent adipogenesis, (see supplementary Fig. III). Consistent with our in vivo results, adipose tissue fatty acids mirrored that of the diet, suggesting that there is neither preferential metabolism nor storage in the absence of D2.

GC-MS analysis of adipose tissue revealed significant differences in the fatty acid profiles of these mice (Table 2). We observed an accumulation of C20 and C22 fatty acids in adipose tissue, but not C24 or C26. These results are largely consistent with those observed in neural tissues of D2-deficient mice (20) and suggest that although D2 is a target of SREBP-1a/c and upregulated during adipogenesis, it is not essential to adipogenesis or lipid accumulation in mice maintained on standard rodent chow.

One potential explanation for this result is that adipose tissue is not the predominant lipogenic organ in vivo and that the absence of D2 in adipose tissue is compensated by other mechanisms, perhaps the presence of the related family member D1 in the liver where the majority of endogenous lipids are synthesized. To test the hypothesis that D2 expression is critical for adipogenesis in cultured adipocytes, we isolated day 13.5 MEFs from wild-type and D2−/− mice and differentiated them to adipocytes. Total lipid accumulation was measured by Oil-Red O staining (see supplementary Fig. III). Consistent with our in vivo observations, the absence of D2 did not prevent adipogenesis or suppress lipid accumulation in adipocytes. In fact, there was a significant increase in lipid accumulation in MEF cells from D2−/− mice compared with cells from wild-type controls. These results indicate that although D2 is upregulated during adipogenesis and is regulated by SREBP, it is not essential for adipocyte differentiation or lipid accumulation in vitro or in vivo.

These findings suggest that the role of D2 in adipose is not to facilitate bulk lipid storage but rather to mediate the clearance of fatty acids not typically found in the triglyceride storage droplets of adipocytes, but may be present in the diet. To determine if D2 mediated the clearance of such fatty acids in adipose, we evaluated the effect of a diet enriched in erucic acid (C22:1, n-9; see supplementary Table I). Although it is unlikely that the substrates of D2 are limited to erucic acid, this fatty acid was selected as a model lipid since it accumulates to the greatest extent in adipose and neural tissues of mice maintained on standard rodent chow.

Wild-type, heterozygous, and D2−/− mice were challenged with a diet containing 10% erucic acid for 8 weeks. The fatty acid profile of serum and adipose tissue was examined by GC-MS. Our results indicate that in adipose tissue, the deficiency of D2 has a gene-dosage dependent effect on the accumulation of erucic acid (Fig. 5A). The increase in erucic acid occurred at the expense of 18:1 and 18:2. In D2-deficient mice, the relative abundance of erucic acid among adipose tissue fatty acids mirrored that of the diet, suggesting that there is neither preferential metabolism nor storage in the absence of D2.

Consistent with the observations in adipose tissue, erucic acid accumulated to a greater extent in serum from D2−/− and D2+/− compared with wild-type (D2+/+) mice after 24 h of fasting (Fig. 5B). However, erucic acid was not detected in serum from mice fasted for 4 h (data not shown). These data suggest that in the absence of D2, erucic acid is efficiently stored in adipose tissue and is mobilized from triglyceride stores during fasting. Interestingly, total free fatty acids were greater in fasted D2-deficient mice compared with wild-type controls (Fig. 5B, inset). The significance of this observation awaits further investigation.

**DISCUSSION**

The major findings of this study are 1) expression of ABCD2 protein is much more abundant in adipose tissue than in other tissues in which it is expressed, 2) Consistent with previous reports of its regulation by SREBP, it is upregulated during adipogenesis, 3) Although D2 is a component of the adipogenic program, its expression is not required for adipogenesis or lipid storage in vitro or in vivo, 4) Erucic acid accumulates in adipose of D2−/− and D2−/−, when mice were challenged with an erucic acid-enriched diet, suggesting that D2 facilitates metabolism of this dietary fatty acid in adipose tissue. To the best of our knowledge, this is the first report that establishes a role for D2 in VLCFA metabolism in non-neuronal tissues.
complete oxidation to provide energy. However, acyl-CoA esters containing atypical fatty acids are not the substrates for carnitine palmitoyltransferase (25). These include VLCFAs, branched chain fatty acids, highly unsaturated fatty acids, and some polyunsaturated fatty acids, each of which have been shown to be metabolized in peroxisomes (25–27).

Peroxisomal oxidation of fatty acids yields acetyl-CoA and chain-shortened fatty acids (C8-C20). To exit peroxisomes, medium- and long-chain fatty acids require esterification to carnitine by one of two peroxisomal acyl-carnitine transferases (24). Acyl-carnitine esters can be transported into mitochondria independently of carnitine palmitoyltransferases, effectively bypassing the rate-limiting step in mitochondrial oxidation of fatty acids (24). Alternatively, removal of the carnitine moiety allows for reactivation of fatty acids that can then be elongated and desaturated to generate the more common species stored in triglycerides. Consequently, the transport of atypical fatty acyl-CoAs into adipose tissue peroxisomes would allow for remodeling of dietary fatty acids and facilitate energy storage. A limitation of this study is that the fate of dietary erucic acid in wild-type mice is not known. While it may have been remodeled and stored in adipose tissue, this has not formally been demonstrated and will require additional studies. In addition, this study cannot exclude a role for D2 in nonadipose tissues in this process.

The entry of atypical fatty acyl CoAs into peroxisomes is thought to be dependent on the transporters on the peroxisomal membrane, principally ABCD transporters. Unlike other classes of transport proteins, ABC transporters generally mediate the transmembrane movement of a variety of substrates. Consequently, D2 may facilitate the metabolism of a number of fatty acyl-CoAs. The type and number of atypical lipids dependent upon D2 for adipose tissue metabolism and the consequences of allowing atypical lipid accumulation within the adipose triglyceride storage pool await further investigation.

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REFERENCES


