FA2H-dependent fatty acid 2-hydroxylation in postnatal mouse brain

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Abstract **2-Hydroxy fatty acids are relatively minor species of membrane lipids found almost exclusively as *N*-acyl chains of sphingolipids. In mammals, 2-hydroxy sphingolipids are uniquely abundant in myelin galactosylceramide and sulfatide. Despite the well-documented abundance of 2-hydroxy galactolipids in the nervous system, the enzymatic process of the 2-hydroxylation is not fully understood. To fill this gap, we have identified a human fatty acid 2-hydroxylase gene (*FA2H*) that is highly expressed in brain. In this report, we test the hypothesis that FA2H is the major fatty acid 2-hydroxylase in mouse brain and that free 2-hydroxy fatty acids are formed as precursors of myelin 2-hydroxy galactolipids. The fatty acid compositions of galactolipids in neonatal mouse brain gradually changed during the course of myelination. The relative ratio of 2-hydroxy versus nonhydroxy galactolipids was very low at 2 days of age (~8% of total galactolipids) and increased 6- to 8-fold by 30 days of age. During this period, free 2-hydroxy fatty acid levels in mouse brain increased 5- to 9-fold, and their composition was reflected in the fatty acids in galactolipids, consistent with a precursor-product relationship. The changes in free 2-hydroxy fatty acid levels coincided with fatty acid 2-hydroxylase activity and with the upregulation of *FA2H* expression. Furthermore, mouse brain fatty acid 2-hydroxylase activity was inhibited by anti-FA2H antibodies. Together, these data provide evidence that FA2H is the major fatty acid 2-hydroxylase in brain and that 2-hydroxylation of free fatty acids is the first step in the synthesis of 2-hydroxy galactolipids.**—Alderson, N. L., E. N. Maldonado, M. J. Kern, N. R. Bhat, and H. Hama. **FA2H-dependent fatty acid 2-hydroxylation in postnatal mouse brain.** ***J. Lipid Res.*** **2006.** 47: **2772–2780.**

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2-Hydroxy fatty acid-containing sphingolipids (2-hydroxy sphingolipids) are present in most living organisms, including yeast, some bacteria, and vertebrates. In humans and other mammals, 2-hydroxy sphingolipids are found in much higher concentrations in myelin and epidermal tissues compared with other tissues. Several lines of evidence suggest that 2-hydroxy sphingolipids may play a crucial role in creating the special characteristics of myelin in humans and other vertebrates.

Myelin consists of ~70% lipids and 30% proteins, compared with 30–50% lipids in most cell membranes, which presumably contributes to its high electrical resistance (1). One of the striking features of myelin is that approximately one-third of all lipids consist of galactosylceramides (GalCers) and sulfatides (3-sulfate ester of GalCer), with half of their amide-linked fatty acids hydroxylated at the C2 position (2-hydroxy fatty acids) (2, 3).

In myelin-forming oligodendrocytes and Schwann cells, GalCer containing both 2-hydroxy and nonhydroxy fatty acids are synthesized by the enzyme UDP-galactose:ceramide galactosyltransferase (CGT) (4). CGT-knockout mice, which lack GalCer and sulfatides, form functionally altered myelin, develop neurological abnormalities, and have a short life span (5, 6). Detailed studies of these mice revealed crucial roles for GalCer and sulfatides in myelination and axo-glial organization (7–9). Myelin of CGT-knockout mice contains 2-hydroxy glucosylceramides, which are not found in normal myelin (5, 6). CGT-transgenic mice also had unstable and uncompacted myelin and developed progressive hind limb paralysis and demyelination (10). Although total galactolipids in these mice were not altered significantly, the ratio of 2-hydroxy GalCer to nonhydroxy GalCer was reduced, indicating that the underlying cause of the unstable myelin was reduced 2-hydroxy GalCer. These studies underscore the importance of 2-hydroxy fatty acid-containing sphingolipids in myelin.

The precursor of all complex sphingolipids is ceramide. For the synthesis of 2-hydroxy galactolipids, CGT uses

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Abbreviations: CGT, UDP-galactose:ceramide galactosyltransferase; ER, endoplasmic reticulum; FA2H, fatty acid 2-hydroxylase protein or gene; GalCer, galactosylceramide; OPC, oligodendrocyte progenitor cell; PLP, proteolipid protein.

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2-hydroxy ceramides, which are formed by the action of fatty acid 2-hydroxylase (also known as fatty acid α-hydroxylase) (11, 12). In 1973, Hoshi and Kishimoto (11) demonstrated fatty acid 2-hydroxylase activity in rat brain. The rat brain fatty acid 2-hydroxylase is a microsomal enzyme (12) that requires molecular oxygen, Mg^{2+}, pyridine nucleotides, cellular cofactors (11), and microsomal electron transfer proteins (13, 14). Despite their extensive efforts, the rat brain fatty acid 2-hydroxylase has not been purified, and its molecular identity remained elusive until recently. We have reported the identification and characterization of the human gene encoding a fatty acid 2-hydroxylase (*FA2H*) that is highly expressed in brain (15). Human FA2H is a highly hydrophobic protein with an N-terminal cytochrome b_5 domain essential for enzyme activity. FA2H also contains a putative catalytic site with the histidine motif conserved among membrane-bound desaturases and hydroxylases [consensus: $HX_{(3-4)}HX_{(7-41)}HX_{(2-3)}HHX_{(61-189)}(H/Q)X_{(2-3)}HH$]. These histidine residues are thought to coordinate the nonheme di-iron cluster at the active site of the enzyme (16). FA2H catalyzes 2-hydroxylation of free fatty acid in vitro, which was dependent on a reconstituted electron transport system (15). Subsequently, Eckhardt et al. (17) reported that the mouse *FA2H* gene was highly expressed in brain during myelination and that *FA2H* mRNA colocalizes with proteolipid protein (PLP) mRNA. These findings provide strong evidence that the *FA2H* gene encodes the fatty acid 2-hydroxylase previously characterized by Kishimoto and colleagues (11–14).

Despite extensive studies on biochemical characteristics of fatty acid 2-hydroxylase, the pathway for the synthesis of 2-hydroxy galactolipids remains unclear, because in vivo substrates of this enzyme are not clearly defined. Based on our data obtained in vitro, we hypothesize that FA2H forms free 2-hydroxy fatty acids in vivo, which serve as the precursors of 2-hydroxy galactolipids. In this report, we present data that are consistent with the pathway shown in **Fig. 1**, in which FA2H-dependent 2-hydroxylation of free fatty acids is the first step in the synthesis of myelin 2-hydroxy galactolipids.

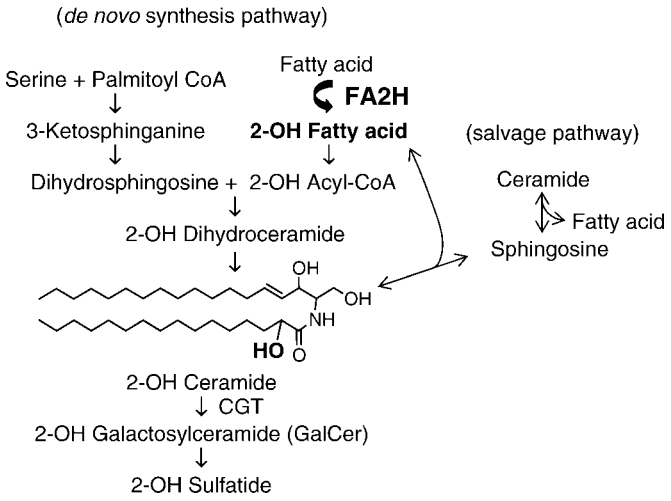

Fig. 1. Proposed biosynthetic pathway for 2-hydroxy sphingolipids. The de novo pathway is identical to the biosynthesis of nonhydroxy sphingolipids, except for the 2-hydroxylation step catalyzed by fatty acid 2-hydroxylase (FA2H). Note that fatty acids with variable chain lengths are incorporated into sphingolipids. UDP-galactose:ceramide galactosyltransferase (CGT) catalyzes the synthesis of galactosylceramide (GalCer). The salvage pathway may contribute to the formation of 2-hydroxy ceramide from 2-hydroxy fatty acid and sphingosine.

EXPERIMENTAL PROCEDURES

Materials

Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). Deuterated tetracosanoic acid [3,3,5,5-D_4] and odd chain fatty acids (C_{15}–C_{25}) were purchased from Larodan Fine Chemicals (Malmö, Sweden). Bovine sulfatides and cerebrosides were purchased from Matreya (Pleasant Gap, PA). The generation of anti-human FA2H polyclonal antibodies has been reported (15). Anti-mouse FA2H polyclonal antibodies were generated in rabbits using a synthetic multiantigen peptide corresponding to the C-terminal 20 amino acids of mouse FA2H (KLWDYFFHTLIPEEAHPKMQ). HRP-linked donkey anti-rabbit IgG (NA934) and the ECL Western Blotting Detection Kit were purchased from Amersham Biosciences (Piscataway, NJ). Anti-actin polyclonal antibodies were purchased from Sigma (St. Louis, MO). Purified human NADPH:cytochrome P450 reductase and NADPH regenerating system solutions were purchased from BD Biosciences Discovery Labware (Bedford, MA).

Animals

A breeding colony of C57BL/6 mice was maintained in animal care facilities of the Medical University of South Carolina with water and food ad libitum. Mice were treated in accordance with the university's Institutional Animal Care and Use Committee-approved procedures.

Cell cultures

CG4 cells (18) were maintained in DMEM containing insulin (10 μg/ml), transferrin (5.5 μg/ml), sodium selenite (40 nM), ethanolamine (2 μg/ml), 1% fetal bovine serum, penicillin-streptomycin, and supplemented with 25% B104 neuroblastoma-conditioned medium as reported previously (19). Oligodendrocyte differentiation was initiated upon removal of B104-conditioned medium.

Primary glial cells were isolated from newborn rat brains as described previously (19, 20). Briefly, cells were grown for 7–10 days in the presence of 10% calf serum. Microglia were separated by shaking the culture flasks for 30 min, plated in six-well dishes, and harvested for immunoblot analysis. Oligodendrocyte progenitor cells (OPCs) were harvested by overnight shaking on a gyratory shaker at 200 rpm, resuspended in 10% calf serum, and subjected to several rounds of attachment/detachment for further enrichment. The final OPC suspension was seeded in six-well dishes or 100 mm dishes coated with poly-D-lysine. Cells were allowed to grow in DMEM supplemented with transferrin (50 μg/ml), insulin (5 μg/ml), sodium selenite (20 nM), triiodo-L-thyronine (30 nM), and 0.5% fetal calf serum. Growth medium included the growth factors basic fibroblast growth factor (10 ng/ml) and platelet-derived growth factor (10 ng/ml), and differentiation medium excluded growth factors. Astrocyte-enriched cultures were prepared by subculturing the original mixed glial culture devoid of most oligodendrocytes and microglia as described previously (21).

Isolation of mouse brain galactolipids

GalCer and sulfatides (2-hydroxy and nonhydroxy) were isolated as described previously with minor modifications (6). Briefly, whole mouse brains were homogenized using a PT1200E Polytron homogenizer with a 7 mm generator, and lipids were extracted with 19 volumes of chloroform-methanol (2:1, v/v) (22). The extracts were washed with 0.2 volume of 0.9% NaCl, and the lower phase was collected. Aliquots (50 mg wet weight equivalent per sample) were used for preparative TLC. For the isolation of sulfatides, aliquots of lipid extracts were subjected to mild alkaline hydrolysis (in 0.5 N KOH for 10 min at 50°C) to remove glycerolipids (5). TLC plates were developed in chloroform-methanol-water (70:30:4, v/v/v) after saturation with solvent vapor for 45 min. Lipid spots were visualized under ultraviolet light after spraying with primuline solution [0.005% primuline in acetone-water (80:20, v/v)]. Galactolipid spots (2-hydroxy and nonhydroxy) were removed from the plates and subjected to alkaline hydrolysis (in 4 N KOH overnight at 80°C). After neutralization with glacial acetic acid, fatty acids were extracted three times with 3 ml of diethyl ether and dried under N_2. Fatty acids were derivatized and quantified as described in the next section.

Fatty acid determination by GC-MS

Whole brains were immediately excised from neonatal mice after careful cervical dislocation and decapitation. The tissue was quickly homogenized in 1.5 ml of ice-cold cell lysis buffer (10 mM Tris-HCl, pH 7.4, 1% glycerol, and 1 mM PMSF) by 30 strokes in a 2 ml Dounce tissue grinder. The tissue homogenate was sonicated (Fisher Sonic Dismembrator model 500; amplitude at 29%; 5 s on, 0.1 s off for 12 cycles), and cell lysates were centrifuged at 1,000 *g* for 5 min to remove unbroken cells and large debris. The supernatant was removed, a small aliquot was taken for immediate protein determination, and the remaining supernatant was immediately frozen at −80°C until analysis. Crude cell lysate (1 ml) was mixed with a set of internal standards (C_{15}, C_{17}, C_{19}, C_{21}, C_{23}, and C_{25} fatty acids), and free fatty acids were extracted three times with 2.5 ml of diethyl ether. Combined diethyl ether extracts were brought to dryness under N_2 for GC-MS analysis. To prepare fatty acid methyl esters, 1 ml of methanolic HCl was added to each sample and incubated at 65°C for 45 min, and samples were brought to dryness under nitrogen. To prepare trimethylsilyl derivatives of hydroxyl groups, 100 μl of Tri-Sil Reagent (Pierce Biotechnology, Rockford, IL) was added to each sample and incubated for 30 min at room temperature. Derivatized samples (1–2 μl) were directly applied to a GC-2010 gas chromatograph (Shimadzu Scientific, Columbia, MD) with the injector in splitless mode. The injection port and transfer line were maintained at 250°C, and analytes were fractionated on a Restek RTX-5 column (5% diphenyl and 95% dimethyl polysiloxane; 0.25 mm inner diameter, 0.25 μm D.F., 30 m). The initial oven temperature was 110°C with no hold time; it was increased to 300°C at 10°C/min. Mass spectral data were obtained on a Shimadzu GC/MS-QP2010 mass spectrometer after electron-impact ionization. Peaks of the target analytes and internal standards were processed using GC-MS Lab Solutions software (Shimadzu Scientific). Calibration curves were constructed by plotting peak area ratios of the target analytes to their respective internal standard against concentration.

Fatty acid 2-hydroxylase assay

Brain FA2H activity was determined as described previously (23). Briefly, in a 50 ml polypropylene tube, crude brain homogenate (50 μg of protein) was added to an assay mixture containing 2.7 mM Tris-HCl, pH 7.6, 1.28 mM $NADP^+$, 3.3 mM glucose 6-phosphate, 3.3 mM $MgCl_2$, 0.2 unit of glucose 6-phosphate dehydrogenase, and 1 μg of human NADPH:cytochrome P450 reductase in a total volume of 1.4 ml. The substrate, 1 μg (2.7 nmol) of [3,3,5,5-D_4] C_{24} fatty acid (stock solution was 10 μg/ml in 1.5 mM α-cyclodextrin), was added at time zero. After gentle mixing by swirling, the assay mixture was incubated at 37°C for 180 min with shaking (100 rpm) to facilitate the diffusion of oxygen. At the end of incubation, 1 pmol of C_{23} fatty acid (an internal standard) and 20 μl of glacial acetic acid were added to each sample, and fatty acids were immediately extracted three times with 2 ml of diethyl ether. The combined diethyl ether extracts were brought to dryness under a stream of nitrogen. Fatty acids were derivatized and quantified as described in the preceding section.

Immunoblot analyses

CG4 and primary glial cells were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM PMSF, and 1% glycerol), a small aliquot was removed for protein determination, and the remaining samples were mixed with an equal volume of 2× SDS-PAGE sample buffer. Total protein levels were quantified with the Pierce BCA Protein Assay Kit. Proteins (20 μg per well) were separated on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were blocked with TBST-milk (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, and 5% nonfat dry milk) overnight at 4°C, incubated with affinity-purified anti-human FA2H polyclonal antibodies (1:1,000) or anti-actin polyclonal antibodies (1:200) in TBST-milk for 2 h, and washed, followed by an incubation with HRP-linked donkey anti-rabbit IgG (1:5,000) (Amersham Biosciences, Piscataway, NJ) for 1.5 h. Membranes were washed, and target proteins were visualized using the ECL Chemiluminescent Detection System (Amersham Biosciences).

Whole brains were immediately excised from adult mice after careful cervical dislocation and decapitation. The brain tissue was quickly homogenized in 1.5 ml of ice-cold cell lysis buffer (10 mM Tris-HCl, pH 7.4, 1% glycerol, and 1 mM PMSF) by 30 strokes in a 2 ml Dounce tissue grinder. The tissue homogenate was sonicated (Fisher Sonic Dismembrator model 500; amplitude at 29%; 5 s on, 0.1 s off for 12 cycles), and cell lysates were centrifuged at 1,000 *g* for 5 min to remove unbroken cells and large debris. The supernatant was removed, a small aliquot was taken for immediate protein determination, and the remaining supernatant was analyzed as described above.

Quantitative RT-PCR

Mouse brain was immediately excised after careful cervical dislocation and decapitation and homogenized using a PT1200E Polytron homogenizer with a 7 mm generator. RNA was isolated using the Qiagen RNeasy Lipid Tissue kit. OPCs were disrupted by QIAshredder, and total RNA was isolated using the Qiagen RNeasy kit. cDNA was generated using the Promega AT Reverse Transcriptase kit. Real-time quantitative RT-PCR was performed on a Bio-Rad MyiQ single-color real-time PCR detection system. The primers used for each gene were as follows: for mouse *PLP*, mPLP-F1 (ggcagatctttggcgactac) and mPLP-R1 (tgagcttgatgttggcctct); for mouse *FA2H*, mFA2H-F1 (gtgttcctgcggctcattct) and mFA2H-R1 (atggtgggccttcatgttgt); for mouse *CGT*, mCGT-F1 (aaaggcatggggatcttgtt) and mCGT-R1 (gccgggttgatccttgtg); and for mouse 18S rRNA, m18S-F1 (gcccgaagcgtttactttga) and m18S-R1 (ggcctcagttccgaaaacc). A standard reaction mixture contained 15 μl of iQ SYBR Green Supermix (Bio-Rad), cDNA template, and 200 nM each of forward and reverse primers in a total volume of 30 μl. The mixture was first heated at 95°C for 3 min, followed

by 40 cycles of two-step amplification (10 s at 95°C and 45 s at 57°C). All reactions were performed in triplicate. For each sample, 18S rRNA was measured as an internal standard. Threshold cycle (C_t) for each target was obtained with Bio-Rad MyiQ Optical System software. The relative abundances of FA2H, CGT, and PLP mRNA were calculated using the $\Delta\Delta C_t$ method (24).

RESULTS

We propose the pathway for the synthesis of 2-hydroxy galactolipids shown in Fig. 1. In this pathway, it is postulated that free fatty acids are converted to 2-hydroxy fatty acids by FA2H, which are incorporated into ceramide, the direct precursor of myelin GalCer. We hypothesize that the main function of FA2H in brain is to provide precursors of myelin 2-hydroxy galactolipids. Thus, FA2H is expected to be present primarily in oligodendrocytes. In fact, *FA2H* mRNA has been shown to colocalize with PLP mRNA in mouse brain (17). To demonstrate that FA2H protein is present in oligodendrocytes, we performed immunoblot analysis of glial cells. FA2H was not detectable in the rat oligodendrocyte cell line CG4 cultured in growth medium (supplemented with platelet-derived growth factor and basic fibroblast growth factor), but distinct bands were visible when cells were grown in differentiation medium (with no growth factors) (**Fig. 2**). Based on the rat genome database, the rat FA2H protein is 80% identical to human FA2H with the same molecular mass (43 kDa). This is consistent with the size of the band on the immunoblot. Similarly, FA2H was present at a low level in rat primary oligodendrocytes maintained in growth medium, and it increased when cultured in differentiation medium. There was no detectable FA2H in primary astrocytes, and a very faint band was visible in primary microglia. These results are consistent with the presumed role of FA2H in myelin 2-hydroxy galactolipid synthesis.

To validate the precursor-product relationship between fatty acids and galactolipids in developing mouse brain, we first determined the fatty acid compositions of myelin galactolipids in neonatal mouse brains and their changes during myelination (**Tables 1**, **2**). It has been reported that in GalCer of rat brain, only a small fraction of total fatty acids are 2-hydroxylated at the onset of myelinogenesis, and the proportion of 2-hydroxy fatty acids increases dramatically during myelination (2). Although the general trend of changes in fatty acid compositions of mouse brain GalCer and sulfatides was similar to that in rat brain GalCer, there were distinct differences in fatty acid species. Most notably, mouse GalCer and sulfatide contained high levels of 2-hydroxy C_{16} fatty acid, which was not found in rat GalCer (2). When normalized against protein contents, changes in total nonhydroxy fatty acids of GalCer and sulfatides were within 1.5- to 2-fold from postnatal daty 2 (P2) to P30. In striking contrast, 2-hydroxy fatty acid contents in GalCer and sulfatides increased 7- and 11-fold from P2 to P20 and remained high at P30. The increase in 2-hydroxy fatty acids resulted in a marked increase in the ratio of 2-hydroxy fatty acids to nonhydroxy fatty acids during myelination (**Fig. 3**). This ratio reached a peak in sulfatides at P20 (4.8-fold higher than at P2) but continued to increase in GalCer to P30 (8.7-fold higher than at P2). As in rat brain GalCer, there was a shift in chain lengths of 2-hydroxy fatty acids in mouse brain GalCer as animals matured. The relative content of nonhydroxy C_{24} fatty acid in GalCer remained consistent at 2–5% of total GalCer-associated fatty acids, whereas 2-hydroxy C_{24} fatty acid content increased from 0.6% to 25% from P2 to P30.

TABLE 1. Fatty acid composition of GalCer in neonatal mouse brain

	C16	C18	C20	C22	C24	C24:1	C26	Total
				nmol/mg protein				
Nonhydroxy fatty acids								
P2	862	841	20.8	11.9	96.9	95.8	6.6	1,935
P17	818	722	20.7	11.9	73.3	111	5.5	1,761
P20	1232	1101	30.2	11.5	105	133	5.0	2,617
P30	757	742	16.7	7.6	61.3	82.9	3.2	1,671
2-Hydroxy fatty acids								
P2	99.3	14.7	0.2	2.2	12.1	14.4	5.2	148
P17	125	65.1	0.2	11.6	347	20.9	5.0	574
P20	417	105	0.2	13.3	464	27.1	9.3	1,036
P30	298	68.8	0.2	10.6	705	22.5	9.0	1,114

Galactosylceramides (GalCers) were purified from whole brain, and their fatty acid compositions were analyzed by GC-MS. P2, postnatal day 2; P17, postnatal day 17; P20, postnatal day 20; P30, postnatal day 30.

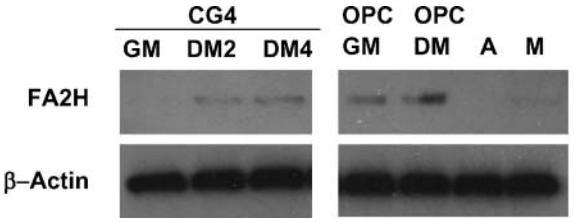

Fig. 2. FA2H is present primarily in oligodendrocytes. Total cell lysates (20 μg of protein) of rat glial progenitor cell line CG4 or rat primary cells were subjected to SDS-PAGE, followed by immunoblot analysis with anti-human FA2H or anti-actin polyclonal antibodies. CG4 cells were grown in a growth medium (GM) or differentiation medium (DM) for 2 days (DM2) or 4 days (DM4). OPC GM, oligodendrocyte progenitors in GM; OPC DM, oligodendrocyte progenitors in DM; A, astrocytes; M, microglia.

TABLE 2. Fatty acid composition of sulfatides in neonatal mouse brain

	C16	C18	C20	C22	C24	C24:1	C26	Total
				nmol/mg protein				
Nonhydroxy fatty acids								
P2	146	120	5.8	4.2	65.4	87.0	1.4	430
P17	172	164	5.3	4.9	78.1	123	1.3	548
P20	298	330	9.4	7.6	159	200	2.7	1,007
P30	279	354	11.2	7.1	107	121	1.7	881
2-Hydroxy fatty acids								
P2	21.2	13.4	0.6	0.9	13.8	0.3	0.8	51
P17	91.7	41.7	0.5	1.2	29.9	0.3	0.8	166
P20	284	151	0.9	4.6	133	0.8	2.1	576
P30	221	102	0.7	3.2	135	1.1	2.7	466

Sulfatides were purified from whole brain, and their fatty acid compositions were analyzed by GC-MS. P2, postnatal day 2; P17, postnatal day 17; P20, postnatal day 20; P30, postnatal day 30.

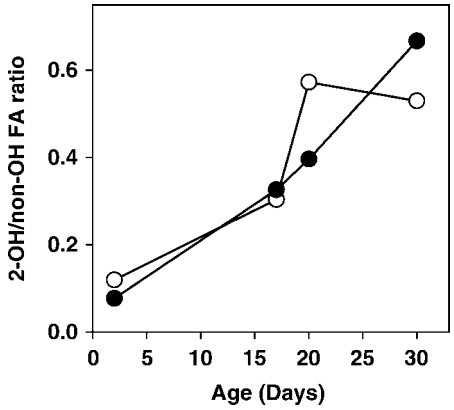

Fig. 3. The 2-hydroxy fatty acid contents in brain GalCer and sulfatide increase during postnatal myelination. Whole brains were isolated from neonatal mice at various ages. GalCer and sulfatides were extracted from whole brain and purified by preparative TLC. Fatty acids were liberated from GalCer and sulfatides by alkaline hydrolysis and quantified by GC-MS. The data are expressed as the ratios of total 2-hydroxy fatty acids to total nonhydroxy fatty acids in GalCer (closed circles) and sulfatide (open circles).

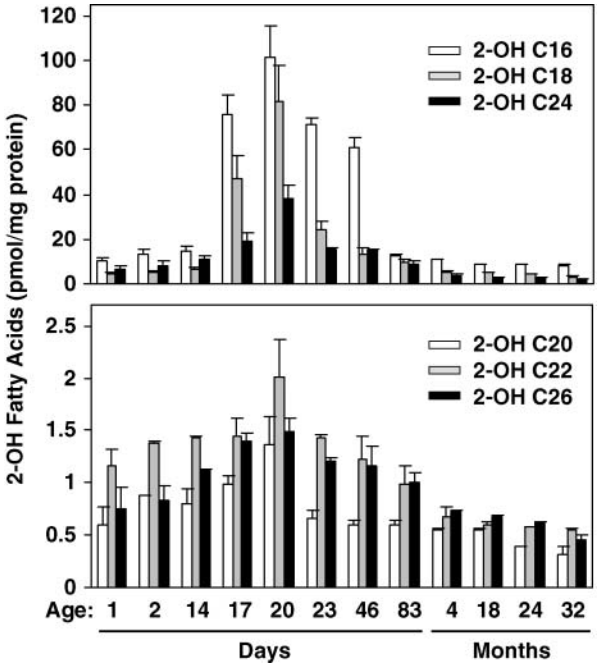

Fig. 4. Free 2-hydroxy fatty acids in postnatal mouse brain. Whole brains were isolated from mice at various ages as indicated. Free fatty acids were extracted by diethyl ether and derivatized for GC-MS analysis. Free 2-hydroxy fatty acid levels were normalized against protein contents. Data are shown as means ± SD (n = 5 for day 1, n = 1 for 24 months, n = 2 for all other time points). Top panel, levels of major 2-hydroxy fatty acids; bottom panel, levels of minor 2-hydroxy fatty acids.

The increase in 2-hydroxy C_{24} fatty acid content in sulfatide was less dramatic (from 3% to 10%).

Cellular 2-hydroxy fatty acids have been described only as components of various sphingolipids, and free 2-hydroxy fatty acid levels have not been reported. If free 2-hydroxy fatty acids are the precursors of 2-hydroxy galactolipids, then free fatty acids are expected to be present in brain with compositions similar to those found in myelin 2-hydroxy galactolipids. To test this possibility, we quantified free 2-hydroxy fatty acids in developing mouse brain (**Fig. 4**). The three major species of free 2-hydroxy fatty acids (2-hydroxy C_{16}, C_{18}, and C_{24}) were the same as in 2-hydroxy GalCer and sulfatides, indicative of a precursor-product relationship. The three major free 2-hydroxy fatty acids increased 5- to 19-fold during postnatal myelination, presumably driving the higher rate of incorporation into myelin galactolipids. The relative ratio among the three major species, however, was not precisely the same. The high levels of C_{24} fatty acid in galactolipids do not reflect free 2-hydroxy C_{24} fatty acids, suggesting a chain-length preference by one or more enzymes in the pathway. The minor 2-hydroxy fatty acid species (C_{20}, C_{22}, and C_{26}) increased ~2-fold during the same period. After the period of peak myelination, by P83, all 2-hydroxy fatty acids decreased to the P1 levels, and they decreased further in aging animals. Nonhydroxy free fatty acid levels increased 2- to 5-fold after birth and remained consistent after P20 (data not shown).

Determination of tissue free fatty acids could be compromised by the hydrolysis of complex lipids by lipases during handling of the tissues. In our measurements, however, we observed a sharp decrease of free 2-hydroxy fatty acids after P20, whereas brain 2-hydroxy galactolipids continued to increase at the same time. Therefore, it is unlikely that free 2-hydroxy fatty acids were generated by the degradation of tissue 2-hydroxy galactolipids. To provide further evidence that the increase in free 2-hydroxy fatty acids represents de novo synthesis, and not degradation of 2-hydroxy galactolipids, mouse brain fatty acid 2-hydroxylase activities were determined using the newly developed GC-MS-based in vitro assay (15, 23). This assay measures the conversion of deuterated C_{24} fatty acid to deuterated 2-hydroxy C_{24} fatty acid. This system allows for the accurate measurement of <1% conversion of the deuterated substrate. As shown in **Fig. 5A**, the mouse brain fatty acid 2-hydroxylase activity increased sharply after P2, and the highest activity (5-fold increase from P1) was observed in P20 mice, which closely paralleled the changes in free 2-hydroxy fatty acids. After P20, the FA2H activity decreased to a "maintenance level" (~30% of peak activity) by P83, which was maintained for most of the life span of the mouse. Thus, it is highly unlikely that a significant portion of our measurements of free 2-hydroxy fatty acids is attributable to the degradation of 2-hydroxy galactolipids. Interestingly, the activity decreased gradually as animals aged, by as much as 40% in 32 month old mice compared with 4 month old mice.

We further tested the hypothesis that FA2H is the major fatty acid 2-hydroxylase responsible for the formation of 2-hydroxy fatty acids in mouse brain during myelination. First, FA2H protein levels in mouse brain were determined by immunoblot analysis. As shown in

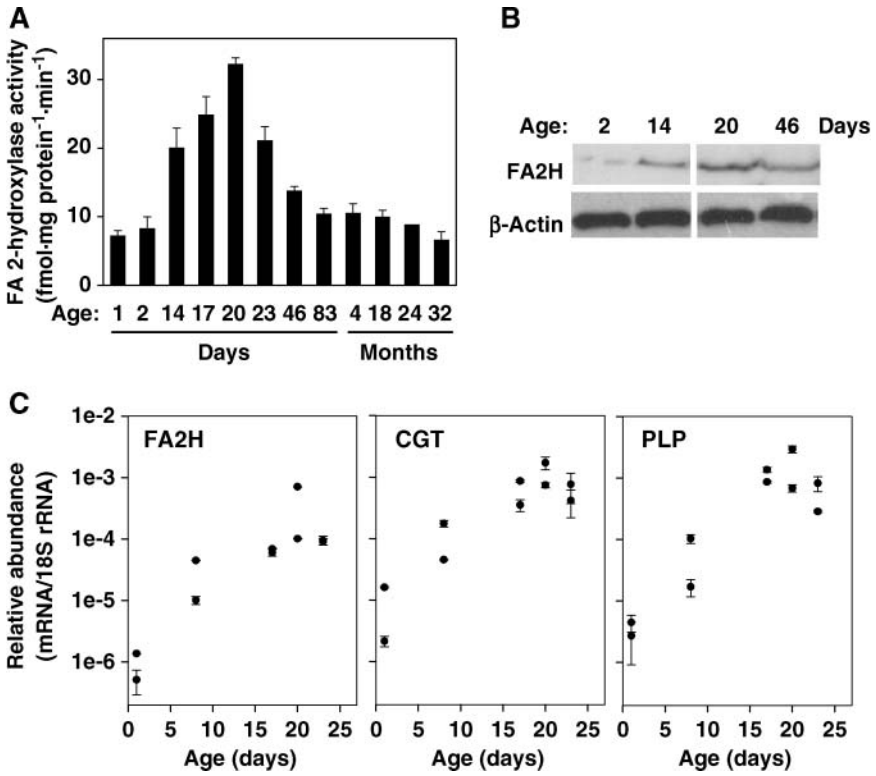

Fig. 5. Fatty acid 2-hydroxylase activity and FA2H expression are upregulated during myelination. A: Total brain homogenates (50 μg of protein) were used for fatty acid 2-hydroxylase assays. The activities are expressed as fmol 2-hydroxy [3,3,5,5-D_4] C_{24} fatty acid formed/mg protein/min. Data are shown as means ± SD (n = 5 for day 1, n = 1 for 24 months, n = 2 for all other time points). B: Crude brain homogenates were subjected to SDS-PAGE followed by immunoblot analysis with anti-human FA2H or anti-actin polyclonal antibodies. C: Total RNA was isolated from neonatal mouse brain, and FA2H, CGT, and proteolipid protein (PLP) mRNA levels were determined by quantitative RT-PCR. Data are normalized against 18S rRNA levels. Circles represent individual animals. Means ± SD from three measurements are shown.

Fig. 5B, a weak but detectable signal was present for FA2H at P2. As in the case of rat FA2H, the mouse FA2H protein was 81% identical to human FA2H, with a molecular mass of 43 kDa, consistent with the size of the band on the immunoblot. FA2H protein increased ~4- and 9.7-fold at P14 and P20, respectively, followed by a slight decrease at P46. The change in FA2H protein levels coincides with the change in brain fatty acid 2-hydroxylase activity, providing evidence that FA2H is responsible for the activity.

Next, we tested whether FA2H is upregulated in parallel with other myelin genes during myelination. It has been reported that *FA2H* mRNA in mouse brain increased by 7-fold from P7 to P14, as determined by Northern blot analysis (17). Because we were interested in the change in *FA2H* expression from the onset of myelination, *FA2H* mRNA was quantified by quantitative PCR from P1 through P23 mice. To compare the change of expression with other myelin genes, *CGT* and *PLP* mRNA levels were also measured by quantitative PCR. *CGT* encodes the enzyme responsible for the conversion of ceramides to GalCer; thus, the expressions of *FA2H* and *CGT* are expected to be coordinately upregulated. PLP is a structural protein of myelin, which serves as an indicator of the progression of myelinogenesis. As shown in Fig. 5C, mouse brain *FA2H* mRNA was present at much lower levels than *CGT* or *PLP* mRNA at P1 and increased exponentially during the neonatal period of myelination, reaching on average 400-fold at P20. The slight decrease in P23 is consistent with the decrease in FA2H activity and free 2-hydroxy fatty acid content shown above. A similar pattern was observed with *CGT* (130-fold average) and *PLP* (800-fold average) mRNA during this time period. These data are consistent with the hypothesis that the upregulation of FA2H is coordinated with *CGT* expression to efficiently incorporate 2-hydroxy fatty acids into myelin galactolipids.

These data show that the brain fatty acid 2-hydroxylase activity correlates with changes in FA2H expression and protein levels. However, there may be more than one fatty acid 2-hydroxylase present in brain. To address this question, we tested whether brain fatty acid 2-hydroxylase activity was inhibited by anti-FA2H polyclonal antibodies. As shown in **Fig. 6A**, fatty acid 2-hydroxylase activity in P20 mouse brain was inhibited by anti-mouse FA2H antibodies in a dose-dependent matter. Importantly, this inhibitory effect was abolished when FA2H antibodies were preincu-

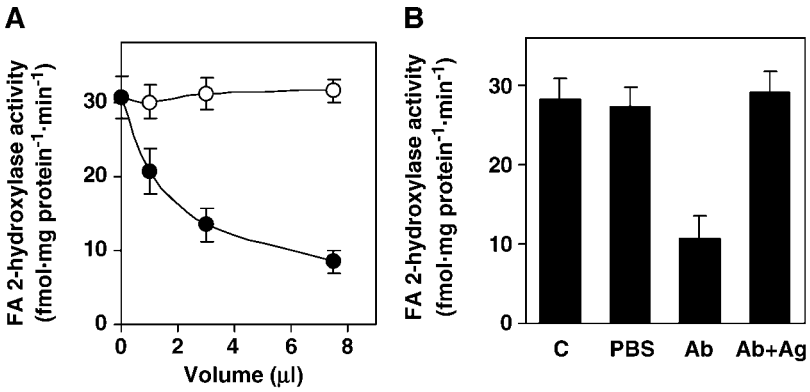

Fig. 6. Brain fatty acid 2-hydroxylase activity is inhibited by anti-FA2H antibodies. Fatty acid 2-hydroxylase assays were performed with brain homogenates (50 μg of protein) from a 20 day old mouse. A: The indicated volumes of PBS (open circles) and affinity-purified anti-mouse FA2H antibodies in PBS (closed circles) were added. B: Assays were performed in the presence of the following: C, no addition; PBS, 7.5 μl of PBS; Ab, 7.5 μl of affinity-purified anti-mouse FA2H antibodies; Ab+Ag, 7.5 μl of affinity-purified anti-mouse FA2H antibodies preincubated with the antigen (a synthetic multiantigen peptide corresponding to the C-terminal 20 amino acids of mouse FA2H). Data are shown as means ± SD from triplicate measurements.

bated with the antigen, indicating that the inhibition was attributable to specific binding of the antibodies to FA2H (Fig. 6B). Antibodies against other myelin proteins [myelin basic protein (MBP) and 2′,3′-cyclic nucleotide 3′-phosphodiesterase] had no effect on the brain fatty acid 2-hydroxylase activity (data not shown). These data clearly demonstrate that FA2H is the primary fatty acid 2-hydroxylase responsible for the formation of 2-hydroxy fatty acids in mouse brain during myelination.

DISCUSSION

In this report, we provide evidence that FA2H is responsible for the formation of 2-hydroxy fatty acids during myelination and that free 2-hydroxy fatty acids are likely precursors of myelin 2-hydroxy galactolipids. We show that *1*) FA2H is present in oligodendrocytes, the myelin-forming cells of the central nervous system; *2*) the fatty acid compositions of mouse brain galactolipids gradually change during myelination, such that increasing proportions of 2-hydroxy fatty acids are incorporated; *3*) the relative ratio of 2-hydroxy versus nonhydroxy galactolipids was very low at the onset of myelinogenesis (~8% of galactolipids contained 2-hydroxy fatty acids) and increased 6- to 8-fold by 30 days of age; *4*) free 2-hydroxy fatty acid levels in mouse brain increased 5- to 9-fold during this period, and their composition was reflected in the fatty acids in galactolipids, consistent with a precursor-product relationship; and *5*) the changes of brain free 2-hydroxy fatty acid levels coincided with FA2H-dependent fatty acid 2-hydroxylase activity and the upregulation of FA2H expression. Together, these data support the postulated pathway for the synthesis of 2-hydroxy galactolipids shown in Fig. 1, in which free fatty acids are converted to 2-hydroxy fatty acids by FA2H and then incorporated into ceramide, the direct precursor of GalCer.

This study, for the first time, provides strong evidence that free fatty acids are in vivo and in vitro substrates of fatty acid 2-hydroxylase. This issue remained unclear despite the extensive biochemical studies of this enzyme in the 1970s and 1980s. In 1973, Hoshi and Kishimoto (11) first reported a rat brain enzyme that converted free fatty acid to 2-hydroxy ceramide, which they named fatty acid α-hydroxylase. The substrates used in most of their assays were free fatty acids, and the products were detectable only as 2-hydroxy ceramides and not as free acids. The assay thus measured the net outcome of at least two reactions, 2-hydroxylation and ceramide synthesis, the latter presumably by acyl CoA-independent reverse ceramidase activity (25). In their in vitro assay, ceramide (lignoceroyl sphingosine) did not serve as a substrate (11). Because free 2-hydroxy fatty acids were not detectable, and ceramide did not serve as a substrate, it was postulated that an unidentified intermediate was 2-hydroxylated (13, 26). Consistent with their data, ceramide (palmitoyl sphingosine) acted neither as a substrate nor as a competitive inhibitor in our assay (N. L. Alderson and H. Hama, unpublished observation). There are possible explanations for the apparent discrepancy between Kishimoto's observations and our data that show the conversion of free fatty acid to free 2-hydroxy fatty acid. It could be attributable to the differences in the detection limits for free 2-hydroxy fatty acids. In our assay, <1% conversion of the deuterated substrate can be accurately measured. Alternatively, in Kishimoto's assay with a fatty acid-Celite complex, the two consecutive reactions (fatty acid 2-hydroxylation followed by ceramide synthesis) might have proceeded at an extremely high efficiency because of the limited diffusion of 2-hydroxy fatty acid intermediates. In our assay, substrates were delivered as α-cyclodextrin inclusion complexes, which are less likely to limit the diffusion of reaction products. It is also possible that both free fatty acid and ceramide serve as substrates in vivo but not under the in vitro assay con-

ditions used in both studies. This study does not address whether ceramide is also a substrate in vivo. Because there is precedence for ceramide 2-hydroxylation in *Tetrahymena* (27) and yeast (28, 29), this issue needs to be clarified further in mammalian cells. Another possible substrate is acyl-CoA, which has been used in previous studies (12–14). In these studies, the acyl chain of the radioactive acyl-CoA was incorporated into 2-hydroxy ceramide. However, 2-hydroxy acyl-CoA was not detected as an intermediate, and it was concluded that acyl-CoA was not the direct substrate of 2-hydroxylase (12). Because acyl-CoA is quickly hydrolyzed in the presence of brain microsomes (30), purified enzyme would be required to clarify this issue.

In various biological systems, there are at least three types of fatty acid 2-hydroxylases known to date. One type is the di-iron-containing monooxygenases, such as FA2H and its homologs. Database searches for homologs indicated that FA2H is a single gene in human and mouse, and Northern blot analysis showed a single mRNA band in all tissues tested (15, 17). Therefore, it is unlikely that there is a second di-iron-containing fatty acid 2-hydroxylase in human and mouse. A second type is cytochrome P450 enzymes found in bacteria (31, 32). This class of enzymes catalyzes the H_2O_2-dependent 2-hydroxylation of fatty acids, which has not been reported in any eukaryotic species. A third type is the 2-oxoglutarate-dependent oxygenase, phytanoyl-CoA 2-hydroxylase. This peroxisomal enzyme is involved in the oxidation of branched-chain fatty acids and does not use straight-chain fatty acids (33). Thus, FA2H is the only likely candidate for the mouse brain fatty acid 2-hydroxylase responsible for the formation of free 2-hydroxy fatty acids. Consistent with this prediction, our data show that the brain fatty acid 2-hydroxylase activity correlates with changes in FA2H expression and protein levels and that the activity was inhibited by anti-FA2H antibodies. Although we cannot exclude the possibility of a second, minor fatty acid 2-hydroxylase, the current data indicate that FA2H is the major fatty acid 2-hydroxylase involved in the synthesis of myelin 2-hydroxy galactolipids.

Interestingly, 2-hydroxy ceramides are the preferred substrate for CGT over nonhydroxy ceramides (4, 34, 35). Other sphingolipids in myelin (sphingomyelin and complex glycolipids) do not normally contain 2-hydroxy fatty acids, even though the enzymes that synthesize these complex sphingolipids are not inherently incapable of incorporating 2-hydroxy substrates. It appears that the endoplasmic reticulum (ER) localization of CGT is partly responsible for the highly selective incorporation of 2-hydroxy fatty acids into GalCer. Sphingomyelin synthases and glucosylceramide synthase are localized in the Golgi apparatus or plasma membrane; therefore, they require ceramide transport from the ER to these organelles. If 2-hydroxy ceramides were efficiently converted to GalCer by CGT in the ER, little or no 2-hydroxy ceramides would be transported out of the ER. Perhaps the expression of FA2H and CGT is precisely coordinated in oligodendrocytes, such that all 2-hydroxy fatty acids generated by FA2H are incorporated into GalCer.

Another factor that might play a role in the selective incorporation of 2-hydroxy fatty acids into GalCer is a lipid flippase that translocates ceramides from the cytoplasmic leaflet to the extracytoplasmic leaflet of the ER membrane. The catalytic sites of FA2H and the yeast *FAH1* gene product are predicted to be at the cytoplasmic face of the ER membrane (15, 36, 37). Similarly, the catalytic sites of ceramide synthases are predicted to be at the cytoplasmic face (38). Therefore, both 2-hydroxy and nonhydroxy ceramides are presumably formed within the cytoplasmic leaflet of the ER membrane. Newly synthesized ceramides then follow one of the following three routes: *1*) extracted by a cytoplasmic protein (CERT) to be transported to the Golgi (39); *2*) transported via the vesicle-mediated mechanism; or *3*) moved to the extracytoplasmic leaflet to be converted to GalCer by CGT (40). In the third route, the transbilayer movement of ceramides could be spontaneous (41, 42) or catalyzed by a hypothetical ceramide flippase (38, 43). Because 2-hydroxy ceramides are less abundant than nonhydroxy ceramides, it seems reasonable to postulate a ceramide flippase with a higher affinity for 2-hydroxy ceramides than for nonhydroxy ceramides for the delivery of all 2-hydroxy ceramides to CGT for incorporation into 2-hydroxy GalCer.

Our data also show that there is distinct chain length specificity for the incorporation of fatty acids into GalCer and sulfatide at different stages of myelination. The most striking was the 40-fold increase of GalCer-associated 2-hydroxy C_{24} fatty acid from P2 to P30. A similar tendency has been reported in neonatal rat brain, although it is less dramatic (2). These observations suggest that there are distinct acyl chain preferences by ceramide synthases, CGT, and GalCer sulfotransferase. Presumably, fatty acid elongases and ceramide synthases are coordinately regulated with FA2H and CGT to achieve the unique fatty acid compositions of myelin galactolipids during development. There are multiple isoforms of fatty acid elongases (44) and ceramide synthases (38), but the specific isoforms involved in myelin 2-hydroxy GalCer synthesis are not clearly defined. Further molecular and biochemical investigations of FA2H, fatty acid elongases, and ceramide synthases are needed to elucidate the biosynthesis of myelin 2-hydroxy galactolipids and their roles in normal myelination as well as in demyelinating diseases.

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