2007

FA2H Dependent Fatty Acid 2-Hydroxylation in the Mammalian Nervous System

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By

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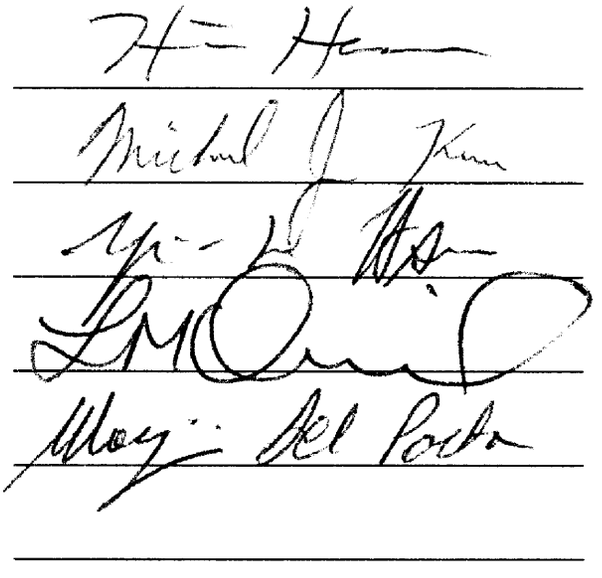
A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies.

Department of Biochemistry and Molecular Biology

2007

Approved by:

Chairman, Advisory Committee



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Acknowledgements

There are many people that have made this body of work possible. To my advisor, Dr. Hiroko Hama, thank you for your endless guidance and support. I am very proud of the work that we have done together during my time in the laboratory. You have made a life-long dream possible for me, and I will always owe you a debt of gratitude.

To my outstanding group of committee members: Dr. Obeid, Dr. Kern, Dr. Del Poeta, Dr. Hsu, and Dr. Luberto. I greatly appreciate all of your time, efforts, and support. All of you have inspired and challenged me to perform at my very best.

To my compadre Eduardo, thank you for all that you have done for me. You are like a brother to me and I will always remember our time in the lab together.

To my former advisors: Dr. Larry Durstine, Dr. John Baynes, and Dr. Susan Thorpe. Thank you for your mentoring, support, and friendship. I will always appreciate you not settling for anything short of perfection.

To my parents, Archie and Pat Alderson, the two of you are my personal heroes. Thank you for teaching me the value of hard work, dedication, and perseverance. It is because of your love and support that I am the person that I am today.

To my beautiful wife Gina, you are the strongest and most courageous person I have ever known. Your drive and determination make me constantly challenge myself to be the best at everything I do. Thank you for your unconditional love and encouragement.

Abstract

NATHAN LANCE ALDERSON. FA2H Dependent Fatty Acid 2-Hydroxylation in the Mammalian Nervous System (Under the direction of Dr. HIROKO HAMA)

Myelin is formed by oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system. This unique membrane is comprised of approximately 70% lipid and 30% protein. The high lipid content is thought to be vital for its insulatory function. Galactosylceramide (GalCer) and sulfatide make up approximately 30% of total myelin lipids, with more than half of these galactolipids containing fatty acids hydroxylated at the C_2 position (2-hydroxy fatty acids). Despite their high abundance, very little is know about the biosynthesis of these 2-hydroxy galactolipids, and specific functions of the 2-hydroxyl group in myelin galactolipids remain speculative. To fill this gap, we recently cloned and characterized a human fatty acid 2-hydroxylase gene, FA2H, that is highly expressed in brain. To study the roles of fatty acid 2-hydoxylase and 2-hydroxy sphingolipids in the brain and other tissues, we have developed highly sensitive methodologies to measure *in vitro* fatty acid 2-hydroxylase and free 2-hydroxy fatty acids by gas chromatography/mass spectrometry (GC/MS). Utilizing these novel methods, here we demonstrate that FA2H is required for the formation of 2-hydroxy fatty acids (precursors of 2-hydroxy galactolipids) in the central and peripheral nervous systems, and 2-hydroxylation of free fatty acids is the first step for the biosynthesis of 2-hydroxy galactolipids. During the course of the study we developed FA2H knockdown using siRNA and shRNA. RNAi against FA2H revealed unexpected cellular phenotypes indicative of altered differentiation. Cells were 2.8-fold more migratory, exhibited increased proliferation, and were strongly resistant to cAMP

induced differentiation. These findings open an exciting area of research involving 2-hydroxy lipids in cell growth, migration, and differentiation.

Introduction

A number of devastating neurodegenerative diseases, including multiple sclerosis (MS) and certain leukodystrophies, are associated with demyelination. Myelin, a lipid-rich membrane that wraps around the axons, facilitates nerve conduction and is formed by oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). The high lipid content of myelin is unique and thought to be critical for insulation and function of neurons [2]. The major myelin lipids are galactosylceramides (GalCer) and sulfatides (3-sulfate ester of GalCer), with approximately one half of the total fatty acids in GalCer and sulfatides hydroxylated at the C_2 position (2-hydroxy fatty acids) [3, 4]. No other mammalian tissues contain such high concentrations of 2-hydroxy fatty acids, suggesting an important role in myelination.

Fatty acid 2-hydroxylase (FA2H) catalyzes the 2-hydroxylation of fatty acids during *de novo* synthesis of ceramide, a precursor of all complex sphingolipids [4, 5]. In myelin, the increased ratio of 2-hydroxy fatty acids to non-hydroxy fatty acids is thought to be critical for membrane compaction, an essential process for normal nerve function [2]. Biophysical studies of 2-hydroxy sphingolipids in model membranes show that the 2-hydroxyl group facilitates membrane lipid-lipid [6-8] and carbohydrate-carbohydrate interactions between GalCer and sulfatides on apposing membranes by an extensive network of hydrogen bonds [9, 10]. Further, in rodent brains, enzyme activities for formation of 2-hydroxy sphingolipids increase after birth in parallel with myelination

and decrease after peak myelination [4, 11]. These studies suggest that 2-hydroxy fatty acids in GalCer and sulfatides have critical structural and functional roles in myelin. Surprisingly, the biosynthetic pathway and regulation of 2-hydroxylation are not well understood. Elucidation of this pathway is imperative for the development of novel therapeutic agents for treatment of demyelinating diseases.

We recently cloned and characterized a human fatty acid 2-hydroxylase gene, FA2H, that is highly expressed in brain [1]. We hypothesized that the FA2H gene product is responsible for the formation of precursors for 2-hydroxy GalCer/sulfatides biosynthesis, and that FA2H expression and activities have a critical role in myelinogenesis.

Review of Literature

2-Hydroxy sphingolipids are found in most living organisms including vertebrate animals, worms, plants, yeast, and some bacterial species. In mammals, 2-hydroxy sphingolipids are uniquely abundant in certain tissues, most notably in the nervous system. Galactosylceramides (GalCer) and sulfatides account for one third of myelin lipids [2, 12]. One half of all fatty acids in these myelin glycosphingolipids are hydroxylated at the C_2 position on the *N*-acyl chain (2-hydroxy fatty acids) [3, 4, 13]. This modification is thought to play a critical role in electrical insulation and nerve conduction.

The 2-hydroxy fatty acid content in postnatal mammalian brain increases with active myelination and reaches stationary levels as animals mature [14-16]. Comparatively, myelin forming cells contain the highest reported levels of 2-hydroxy fatty acids, suggesting these lipids play a unique role in the myelination of the mammalian nervous systems (CNS & PNS) [17]. However, the biosynthetic pathway and regulation of 2-hydroxylation are not well understood. To this end, we recently cloned and characterized a human fatty acid 2-hydroxylase gene, FA2H, that is highly expressed in brain [1].

2-Hydroxy Galactolipids in Vertebrate Nervous Systems.

In myelin-forming oligodendrocytes and Schwann cells, GalCer containing both 2-hydroxy and non-hydroxy fatty acids are synthesized by the enzyme UDP-galactose:ceramide galactosyltransferase (CGT) [18]. CGT-knockout mice, which lack

GalCer and sulfatides, form functionally altered myelin, develop neurological abnormalities, and have a short life span [19-21]. Detailed studies of these mice revealed crucial roles for GalCer and sulfatides in myelination and axo-glial organization [21-23]. Myelin of CGT-knockout mice contains 2-hydroxy glucosylceramides, which are not found in normal myelin [19, 20]. CGT-transgenic mice also had unstable and uncompacted myelin and developed progressive hind limb paralysis and demyelination [24]. Although total galactolipids in these mice were not altered significantly, the ratio of 2-hydroxy GalCer to non-hydroxy 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.

Biophysical Studies on 2-Hydroxy Sphingolipids.

Several biophysical studies [6, 8, 9] demonstrated that the 2-hydroxyl group in sphingolipids has a profound effect in the lipid organization within model membranes because of its hydrogen-bonding capability. The participation of the 2-hydroxyl group in hydrogen bonds with neighboring lipids was shown by analysis of the crystal structure of synthetic glycosphingolipids with 2-hydroxy octadecanoic acid [9] and by analysis of the phase transition temperature [8]. The monolayer behavior of synthetic ceramides showed that the 2-hydroxyl group promotes condensation to a close-packed arrangement [6]. These studies provide a physical basis for the effects of 2-hydroxy sphingolipids in biomembranes.

Characterization of Fatty Acid 2-Hydroxylases.

In mammals, fatty acid 2-hydroxylation is believed to occur during *de novo* synthesis of ceramides, the common precursors of all glycosphingolipids and sphingomyelin.

Hoshi and Kishimoto first reported rat brain fatty acid 2-hydroxylase activities in 1973 [4]. The rat brain fatty acid 2-hydroxylase is a microsomal enzyme [5] that requires molecular oxygen, Mg^{2+}, pyridine nucleotides, cellular cofactors [4], and microsomal electron transfer proteins [25, 26]. Despite their extensive efforts, the rat brain fatty acid 2-hydroxylase has not been purified, and biochemical study of this enzyme has not been reported since 1990. The biosynthetic pathway and coordination with other myelin enzymes is still not fully understood.

Identification of theYeast FAH1 gene (SCS7).

In the yeast *Saccharomyces cerevisiae,* most sphingolipids contain 2-hydroxy fatty acids. The 2-hydroxylation is dependent on the *FAH1* (also known as *SCS7*) gene, which has been identified as a gene containing a cytochrome b_5-like sequence [27] and as a suppressor of the Ca^{2+}-sensitive phenotype of *csg2* mutants [28]. Yeast *fah1* mutants show increased resistance to pore-forming antifungal agents, presumably because of altered plasma membrane properties [29]. Yeast Fah1p is a member of the membrane-bound desaturase/hydroxylase family with the conserved histidine motif ($HX_{(3-4)}HX_{(7-41)}HX_{(2-3)}$-$HHX_{(61-189)}(H/Q)X_{(2-3)}HH$), which is thought to coordinate a non-heme di-iron cluster at an active site [30]. The reactions catalyzed by the enzymes in this family require electron donors and molecular oxygen [30]. For yeast Fah1p, the terminal electron donor is likely the intramolecular cytochrome b_5 domain. It is notable that the predicted properties of yeast Fah1p are consistent with the biochemical properties of rat brain fatty acid 2-hydroxylase. Therefore, it is a reasonable assumption that mammalian fatty acid 2-hydroxylases are encoded by *FAH1* homologues.

Identification of Mammalian FA2H genes.

Previously, we cloned and characterized a human gene encoding a fatty acid 2-hydroxylase (FA2H) that is highly expressed in human brain [1]. BLAST sequence analyses identified several human cDNA sequences that had significant similarity to yeast *FAH1* (also known as SCS7), a gene required for 2-hydroxylation of sphingolipid-associated very long chain fatty acids [27, 28, 31]. All of the cDNA clones were derived from the same gene located in human chromosome 16 (NCBI locus identification, 79152), which we named FA2H for fatty acid 2-hydroxylase. The FA2H gene product is a 372-amino acid protein (42.8 kDa) that has 36% identity and 46% similarity to yeast Fah1p. Yeast Fah1p contains an N-terminal cytochrome b_5 domain (NCBI Conserved Domain Database accession no. pfam00173), four potential transmembrane domains, and the characteristic histidine motif conserved among membrane bound desaturases/hydroxylases (consensus:HX(3–4)HX(7–41)HX(2–3)-HHX(61-89)(H/Q)X (2–3)HH) (13,29,31). These histidines are thought to coordinate the non-heme di-iron cluster at an active site [30] (32). Similarly, human FA2H protein contains a cytochrome b_5 domain at its N terminus (amino acids 1–92). Within this domain is the conserved heme-binding domain (His-Pro-Gly-Gly), suggesting that it is functional as an electron carrier. The cytochrome b_5 domains of yeast Fah1p and human FA2H lack a membrane anchor, which is present in the microsomal cytochrome b_5. The C-terminal three quarters of FA2H are highly homologous to the sphingolipid fatty acid hydroxylase domain (NCBI Conserved Domain Database accession no. pfam04116). This domain is found in previously identified *FAH1* homologues in *Schizosaccharomyces pombe*, *Arabidopsis thaliana*, and *Caenorhabditis elegans* [27], as well as in hypothetical proteins in the fungi

Magnaporthe grisea and Neurospora crassa, the malaria mosquito Anopheles gambiae, Drosophila melanogaster, the nematode C. briggsae, Xenopus laevis, Mus musculus, and Rattus norvegicus (NCBI conserved domain architecture retrieval tool). All of these proteins contain the eight conserved histidines and an N-terminal cytochrome b_5 domain with the exception of the *A. thaliana* homologue, which lacks a cytochrome b_5 domain [27].

Chapter 1

Development of a Novel Fatty Acid 2-Hydroxylase Assay

Introduction

Sphingolipids are a large class of lipids ubiquitously present in eukaryotic cell membranes. The structural diversity of sphingolipids stems from over three hundred distinct head groups, as well as various modifications on hydrocarbon chains of the hydrophobic ceramide moiety. One of the major modifications of ceramide structure is 2-hydroxylation of the amide-linked fatty acids. The 2-hydroxylation of the *N*-acyl chain occurs during *de novo* synthesis of ceramide and is catalyzed by fatty acid 2-hydroxylase (also known as fatty acid alpha-hydroxylase). Sphingolipids containing 2-hydroxylated *N*-acyl chains (2-hydroxy sphingolipids) are found in various organisms, including plants, yeast, worms, and vertebrate animals. In mammals, 2-hydroxy sphingolipids are especially abundant in the nervous system, as the major myelin lipids, galactosylceramides and sulfatides (3-sulfate ester of galactosylceramide), contain a high proportion (~50%) of 2-hydroxy fatty acids [3, 4, 13]. The roles for GalC and sGalC in myelination and axo-glial organization have been demonstrated in studies of knockout mice that lack these lipids [21-23], and the significance of 2-hydroxylation of these myelin lipids has long been speculated [32]. Mammalian epidermal tissues contain a unique set of very long chain ceramides with 2-hydroxy fatty acids, which are thought to be critical for the permeability barrier function of the epidermis [33, 34]. A number of studies also showed 2-hydroxy sphingolipids in liver and kidney, epithelia of digestive

tract, and other tissues (for example [35-38]). Despite the prevalence of 2-hydroxy sphingolipids, fatty acid 2-hydroxylase has not been studied in extraneural tissues until recently, and physiological roles for extraneural 2-hydroxy sphingolipids are poorly understood.

The brain fatty acid 2-hydroxylation activity was first demonstrated by the conversion of [1-^{14}C] tetracosanoic acid to 2-hydroxy tetracosanoic acid in sphingolipids when injected into rat brains [39]. Subsequently, an *in vitro* fatty acid 2-hydroxylase assay was developed using [1-^{14}C] tetracosanoic acid as a substrate, brain homogenate as an enzyme source, and assay products analyzed by thin-layer chromatography [40]. With this method, biochemical properties of this enzyme have been studied in rat and mouse brains [5, 11, 25, 26, 32, 41]. The rat brain fatty acid 2-hydroxylase requires molecular oxygen, Mg^{2+}, pyridine nucleotides (NADPH or NADH), and microsomal electron transport proteins [25, 26]. The rat brain fatty acid 2-hydroxylase was insensitive to carbon monoxide, indicating that it was not a P-450 enzyme but another type of mixed function oxygenase [41]. Although the brain enzyme was successfully characterized, the sensitivity of the previous assay method was limited, and no activities were detected in other tissues containing 2-hydroxy sphingolipids [41]. It was also unclear whether free fatty acids were the substrate of the enzyme, because the assay products were detected only as a component of 2-hydroxy ceramides, and not as free 2-hydroxy fatty acids.

In a recent study we showed that the human FA2H gene encodes a fatty acid 2-hydroxylase that is highly expressed in the human brain [1]. Another study showed that mouse FA2H protein was localized in the endoplasmic reticulum and highly expressed in the brain during active myelination, suggesting that FA2H is the enzyme responsible for

the formation of 2-hydroxylated ceramide in oligodendrocytes of the mammalian brain [42]. In the course of the study, we developed an improved *in vitro* fatty acid 2-hydroxylase assay using a stable isotope and gas chromatography-mass spectrometry. With the new assay, we showed that human FA2H converted a free fatty acid to a corresponding free 2-hydroxy fatty acid [1]. Here we report the details of the assay and an application of the method to a benchtop quadrapole mass spectrometer for convenient measurements of fatty acid 2-hydroxylase activities in various biological specimens.

Materials And Methods

Materials

Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). Tricosanoic acid was purchased from Matreya (catalog number 1186, Pleasant Gap, PA). Deuterated tetracosanoic acid [3,3,5,5-D_4] (catalog number 71-2404-7) was purchased from Larodan Fine Chemicals (Malmö, Sweden). Purified human NADPH:P-450 reductase (catalog number 456078) and NADPH regenerating system solutions (catalog numbers 451220 and 451200) were purchased from BD Biosciences Discovery Labware (Bedford, MA). α-Cyclodextrin was purchased from Sigma (St. Louis, MO). Methanol, acetyl chloride, and diethyl-ether (all HPLC grade) were purchased from VWR (West Chester, PA) and the Tri-Sil Reagent (catalog number 49001) was purchased from Pierce Biotechnology (Rockford, IL).

Cell culture

COS7 and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g/L glucose and L-glutamine, sodium pyruvate, 10% fetal bovine serum, and 0.1% Pen-Strep. Cells were maintained at 5% CO_2 at 37°C. A549 cells

(human lung carcinoma) were cultured in media containing 50% Dulbecco's modified Eagle's medium (as above) and 50% RPMI Medium 1640 supplemented with L-glutamine. When indicated, COS7 cells were transfected with pcDNA3-hFA2H [1] using FuGene 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN).

Preparation of microsomal fractions from tissue culture cells

Approximately 1 x 10^6 cells were harvested by trypsin-EDTA treatment and washed twice with ice-cold Hanks Balanced Salt Solution followed by two washes with ice-cold 10 mM PBS (pH 7.4). The cell pellets were re-suspended in 1 ml lysis buffer (10 mM Tris-HCl, pH 7.4; 1% glycerol; 1 mM PMSF) and lysed by freeze-thawing, followed by sonication (Fisher Sonic Dismembrator Model 500, amplitude 29%, 5 sec on, 0.1 sec off, 12 cycles). Cell lysates were centrifuged at 1,000 x g for 5 min to remove unbroken cells and large debris. The supernatants were centrifuged at 100,000 x g for 2 hr in a Sorvall M120SE ultracentrifuge with an S-100 AT4 rotor to pellet microsomal fractions. The supernatant was discarded and the membrane pellets were resuspended in 1 ml of the ice-cold lysis buffer by brief sonication in a Bransonic B1510MT bath sonicator. A small aliquot was removed for protein determination, and all samples were immediately frozen at –70°C until analyses. Protein was quantified using a Pierce BCA Protein Assay Kit (Rockford, IL).

Preparation of postnuclear fractions from postnatal mouse brain

Whole brains were immediately excised from adult mice following euthanasia then decapitation. The brain tissue was quickly homogenized in 1.5 ml ice-cold cell lysis buffer (10 mM Tris-HCl, pH 7.4; 1% glycerol; 1 mM PMSF) by 30 strokes in a 2-ml Dounce tissue grinder. All samples were immediately frozen at –70°C until the time of

analyses. After thawing, homogenized tissue was sonicated (Fisher Sonic Dismembrator Model 500, amplitude 29%, 5 sec on, 0.1 sec off, 12 cycles) and cell lysates were centrifuged at 1,000 x g for 5 min to remove unbroken cells and large debris. The supernatant was removed, placed on ice, and an aliquot taken for immediate protein determination.

2-Hydroxylation activity measurement

In a 50-ml polypropylene tube, microsomal fractions (25 to 100 μg protein) or crude brain homogenate (50 μg protein) were 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; 1 μg human NADPH:cytochrome P-450 reductase; and a substrate, 1 μg (2.7 nmoles) of [3,3,5,5-D_4]-tetracosanoic acid (stock solution was prepared as 10 μg/ml in 1.5 mM α-cyclodextrin) in a total volume of 1.5 ml. The substrate was added at time-zero of activity measurement. Following gentle mixing by swirling, the assay mixture was incubated at 37°C with shaking (100 rpm) to facilitate the diffusion of oxygen. At the end of incubation, 1 pmole of tricosanoic acid (C23 fatty acid) was added as an internal standard to each sample, and fatty acids were immediately extracted three times with 2 ml diethyl ether. Each extraction consisted of vortex mixing and subsequent centrifugation. The combined diethyl ether extracts were brought to dryness under a stream of nitrogen in 13 x 100 mm screw cap test tubes (Fisher Scientific, catalog number 14-959-25A). Fatty acids were derivatized and quantified as described under "Measurement of fatty acid 2-hydroxylase assay product by GC/MS".

Measurement of fatty acid 2-hydroxylase assay product by GC/MS

The methyl esters of fatty acids were prepared as previously described [1]. Briefly, anhydrous methanolic HCl was prepared by drop-wise addition of acetyl chloride to methanol and 1 ml added to each sample. The samples were tightly capped, incubated for 45 min at 65°C, and dried under a stream of nitrogen. The 2-hydroxy groups were further derivatized to trimethylsilyl (TMS) ethers by the addition of 125 µl Tri-Sil reagent and incubated at room temperature for 30 min tightly capped.

Derivatized samples (1-2 µl) were analyzed by one of the two GC/MS systems. For activities in transfected COS7 cells (Figs. 2, 3, and 4), the samples were directly applied to a Hewlett-Packard 5890 gas chromatograph with injector in splitless mode. The analytes were fractionated on a Restek RTX-5 column (5% diphenyl/95% dimethyl polysiloxane, 0.25 mm I.D., 0.25 µm D.F., 30 m) (Bellefonte, PA) and the injection port and the transfer line were maintained at 250°C. The initial oven temperature was 110°C with no hold time and increased to 300°C at 10°C/min. Mass spectra data were obtained on a VG-70S magnetic sector mass spectrometer following electron impact (EI) ionization. Peaks of the target analytes and internal standard were processed using the Opus software system (Micromass Information Systems, Modesto, CA).

Activities in tissue culture cells (without transfection) and mouse brain were analyzed using a bench-top GC/MS system. Derivatized samples (1-2 µl) were directly applied to a Shimadzu GC-2010 (Shimadzu Scientific, Columbia, MD) gas chromatograph with injector in splitless mode. The analytes were fractionated on a Restek RTX-5 column (5% diphenyl/95% dimethyl polysiloxane, 0.25 mm I.D., 0.25 µm D.F., 30 m) and the injection port and the transfer line were maintained at 250°C. The initial oven

temperature was 110°C with no hold time and increased to 300°C at 10°C/min. Mass spectra data were obtained on a Shimadzu GCMS-QP2010 mass spectrometer following electron impact (EI) ionization. Peaks of the target analytes and internal standard were processed using the GC/MS Lab Solutions software (Shimadzu Scientific, Columbia, MD).

With both GC/MS systems, calibration curves were constructed by plotting peak area ratios of the target analytes to the internal standard against concentration, using linear regression analysis. The ion monitored for the internal standard was 368, corresponding to the molecular ion for $C:_{23}$ FA methyl ester. The ions monitored for 2-hydroxy [3,3,5,5-D_4]-tetracosanoic acid had a mass of 415 and 459, corresponding to m-15 and m-59, respectively. The activities were calculated as pmoles 2-hydroxy [3,3,5,5-D_4]-tetracosanoic acid per mg protein per min.

Results And Discussion

Electron transport system for the FA2H-dependent fatty acid 2-hydroxylation

Fatty acid 2-hydroxylase activity was first demonstrated *in vitro* using a rat brain homogenate [41]. The rat brain enzyme required molecular oxygen, Mg^{2+}, and pyridine nucleotides, which suggested that the enzyme was a mixed function oxidase [41]. Cytochrome P-450 enzymes were not implicated in this reaction, since the activity was not inhibited by carbon monoxide [41]. In modified assays with microsomal fractions, the rat brain fatty acid 2-hydroxylase was shown to require a microsomal electron transfer system [41]. All of these characteristics are consistent with the predicted properties of the human FA2H gene product [1]. The nucleotide sequence of the FA2H gene indicates that FA2H protein (372 amino acid) is a member of membrane-bound

desaturase/hydroxylase family with the conserved non-heme di-iron-binding motif ($HX_{(3-4)}HX_{(7-41)}HX_{(2-3)}HHX_{(61-189)}(H/Q)X_{(2-3)}HH$). The enzymes in this family catalyze diverse reactions (desaturation, hydroxylation, epoxidation, etc.) with hydrophobic substrates (fatty acids, sterols, sphingolipids, etc.) using molecular oxygen [30]. FA2H protein also contains an N-terminal cytochrome b_5 domain, which presumably serves as an electron carrier that feeds electrons to the putative catalytic di-iron site of the enzyme. Based on these findings, we postulated that fatty acid 2-hydroxylation by FA2H was coupled with microsomal electron transfer systems involving NADH:cytochrome b_5 reductase or NADPH:cytochrome P-450 reductase. Since NADPH is a more effective electron donor than NADH for the rat brain enzyme [26], our assay system included purified recombinant human NADPH:cytochrome P-450 reductase and an NADPH regeneration system ($NADP^+$ + glucose 6-phosphate + glucose 6-phosphate dehydrogenase). These components are commonly used for studies of cytochrome P-450 enzymes and are available from commercial sources. With these components, electron transport would occur in the following order: NADPH → NADPH:cytochrome P-450 reductase →

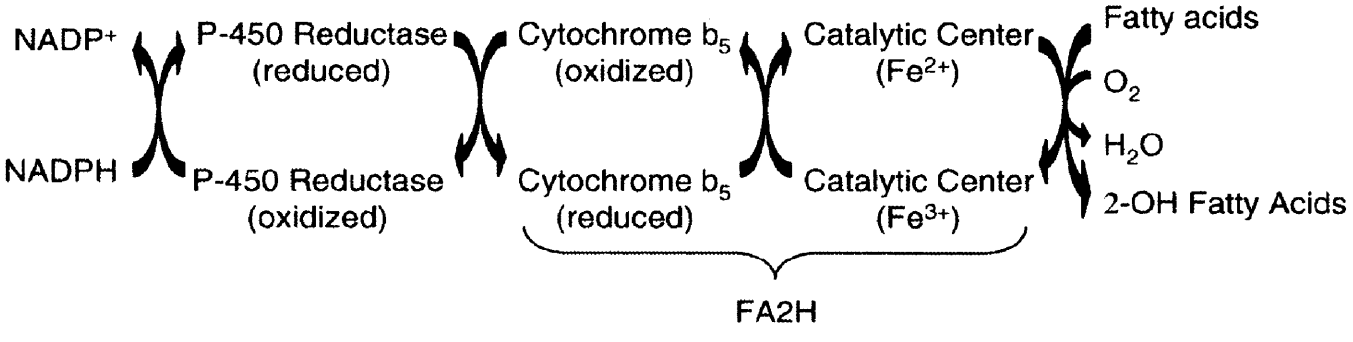

Fig. 1-1. An electron transport system reconstituted in the *in vitro* fatty acid 2-hydroxylase assay. The arrows indicate the flow of electrons. The intramolecular cytochrome b_5 domain is necessary for the activity of FA2H [1], which is believed to serve as a component of an electron transfer system to provide electrons to the putative catalytic iron atoms. Components of the microsomal electron transport system *in vivo* remain to be determined.
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cytochrome b_5 domain of FA2H → the catalytic site of FA2H (Fig. 1-1).

It is of interest that the rat brain enzyme was activated by a heat stable, water soluble cofactor [26], which was later shown to be glucose 6-phosphate [40]. Presumably, glucose 6-phosphate aided in regenerating NADPH from $NADH^+$ in the previous assay system as well.

Detection of 2-hydroxy [3,3,5,5-D_4]-tetracosanoic acid by GC/MS

Rat and mouse brain fatty acid 2-hydroxylase activities were previously measured using [1-^{14}C] tetracosanoic acid as a substrate, and the reaction product was separated from other lipids in the samples and byproducts by multiple chromatographic processes prior to quantification [11, 41]. To simplify the assay, we used a deuterated fatty acid [3,3,5,5-D_4]-tetracosanoic acid (C24:0) as a substrate and a high resolution GC/MS system for identification and quantification of the reaction product. Tetracosanoic acid was a preferred substrate by the rat brain enzyme [41]. A difficulty associated with this substrate was the delivery of this highly hydrophobic molecule to the enzyme. The rat brain fatty acid 2-hydroxylase was sensitive to detergents, and the substrate was coated on the surface of Celite (diatomaceous earth) in the previous assay system [41]. In our assay, deuterated tetracosanoic acid was dissolved in α-cyclodextrin solution, which was known to effectively solubilize fatty acids and ceramides without interfering with enzymatic reactions [43]. Microsomes of FA2H-transfected COS7 cells were a convenient source of the enzyme to establish the assay system.

One μg of deuterated tetracosanoic acid (f.c. 1.8 μM) was incubated with the microsomes, an NADPH regeneration system, and NADPH:cytochrome P-450 reductase. Fatty acids were extracted after adding 1 pmole of an internal standard, tricosanoic acid

(C23:0), which was not present in the microsomes at detectable levels. GC/MS analysis of the fatty acids in assay mixtures showed a new lipid compound that co-eluted with TMS ether of 2-hydroxy tetracosanoic acid methyl ester. The molecular ion of this compound had a mass of 474, that was 4 mass units larger than TMS derivative of 2-hydroxy tetracosanoic acid. Fragmentation of this compound generated two distinct ions with a mass of 459 and 415, respectively, which are also 4 mass units larger than the corresponding ions generated from the non-deuterated 2-hydroxy tetracosanoic acid derivative (Fig. 1-2).

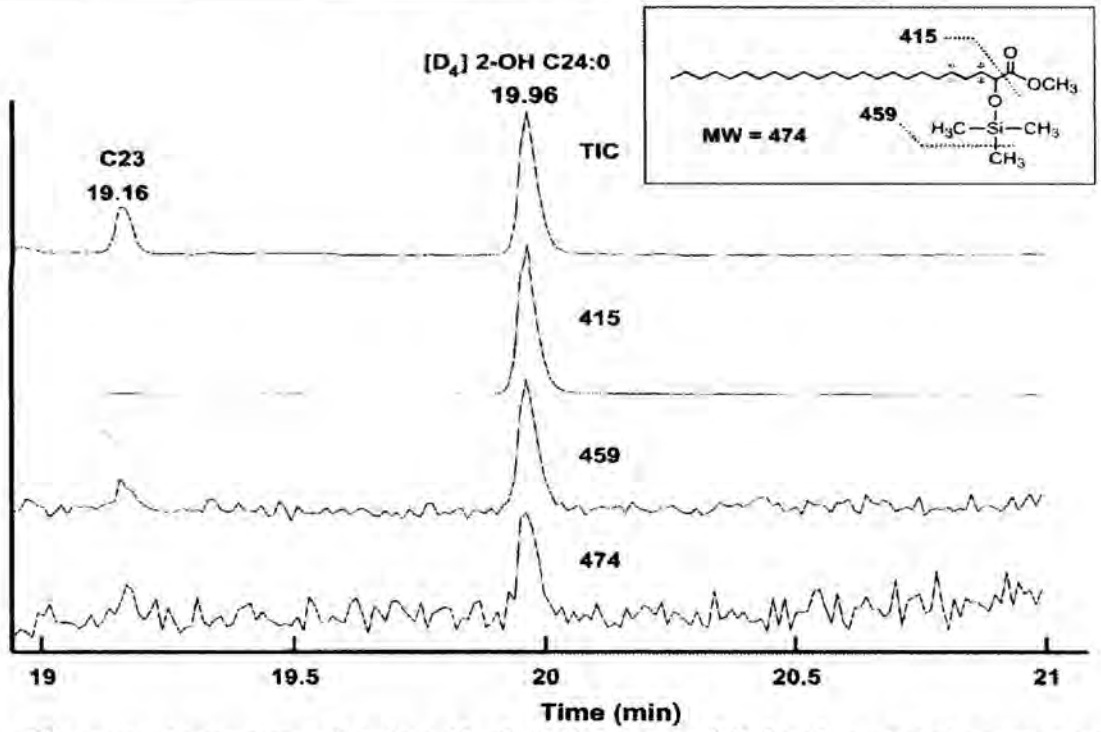

Fig. 1-2. Chromatograms of trimethylsilyl ether of 2-hydroxy tetracosanoic acid methyl ester formed in an *in vitro* reaction. Total ion chromatogram and selected ion chromatograms corresponding to MW=474, m-15, and m-59 are shown. The inlet shows the fragmentation of TMS ether of [3,3,5,5-D_4]-2-hydroxy tetracosanoic acid methyl ester. The asterisks indicate the positions of deuterium.
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Other hydroxy fatty acids were distinguishable from 2-hydroxy fatty acids based on unique fragmentation patterns. As shown in Fig. 1-3, TMS derivatives of 2-hydroxy, 3-hydroxy, and 2,3-dihydroxy hexadecanoic acid methyl esters and 20-hydroxy eicosanoic

acid methyl ester occurred at different positions, generating unique fragments. Based on the retention time and the unique fragmentation pattern, we concluded that the lipid species shown in Fig. 1-2 was a TMS ether of deuterated 2-hydroxy tetracosanoic acid methyl ester. In the initial phase of this study, a high-resolution magnetic sector mass spectrometer was used to attain the lowest detection limit to ensure the detection of low levels of reaction products. The instrument used in this study had a limit of detection at the sub-fmole level.

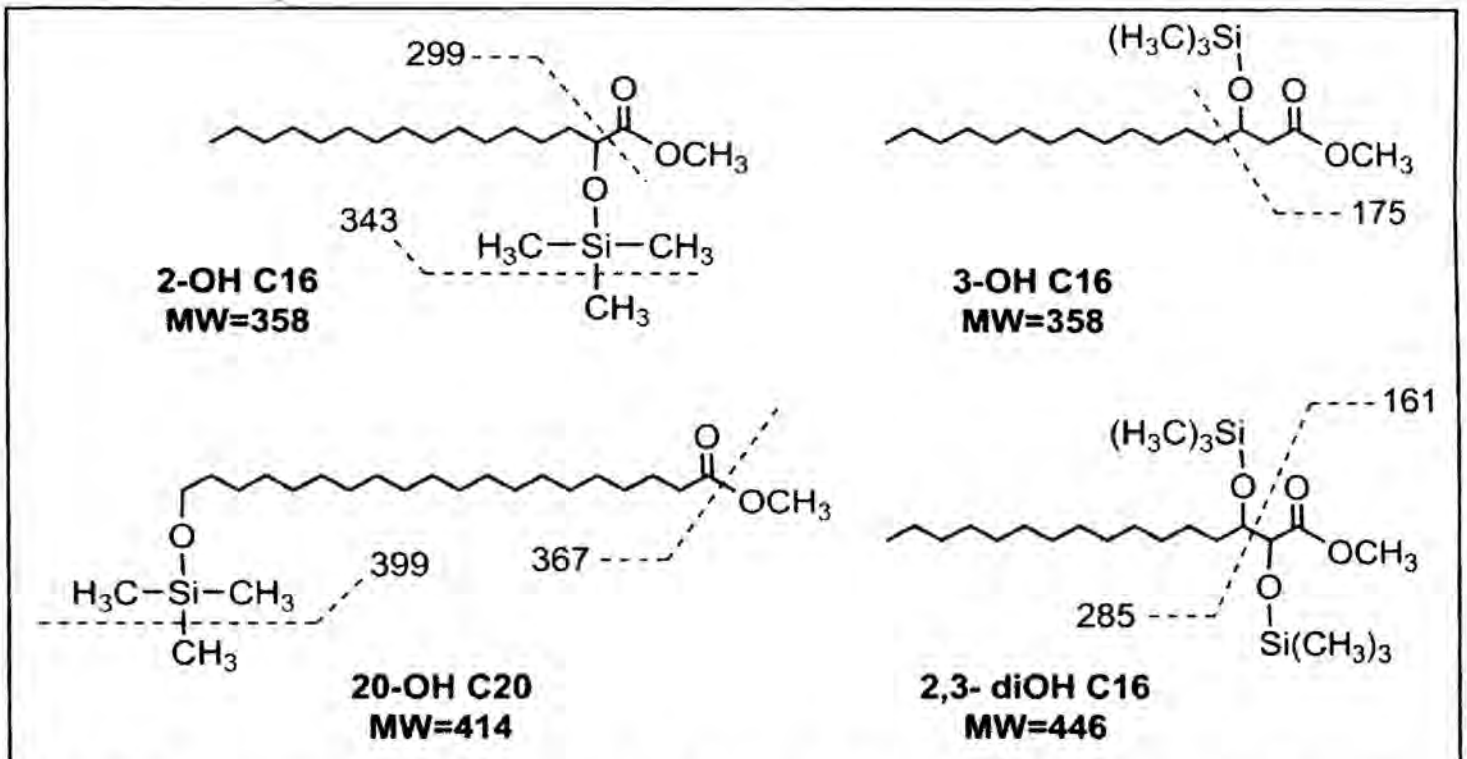

Fig. 1-3. Fragmentation patterns of hydroxy fatty acid derivatives. The masses of unique fragments used for GC/MS quantification are shown.
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With this detection method, fatty acid 2-hydroxylase activity assay was established using microsomes of COS7 cells transiently transfected with pcDNA-FA2H. These cells highly express the human fatty acid 2-hydroxylase and served as a convenient enzyme source to determine the assay condition. In the presence of 50 μg microsomal proteins,

formation of deuterated 2-hydroxy tetracosanoic acid was linear up to 3 hr at a substrate conversion rate of 0.25% per hr (Fig. 1-4). It should be noted that free 2-hydroxy fatty acids were detected as products in this assay. In the previous studies, the product was detected only as a component of ceramide, not as a free fatty acid [41], and the direct substrate of the enzyme remained unclear [25, 32, 41]. Although we have not tested

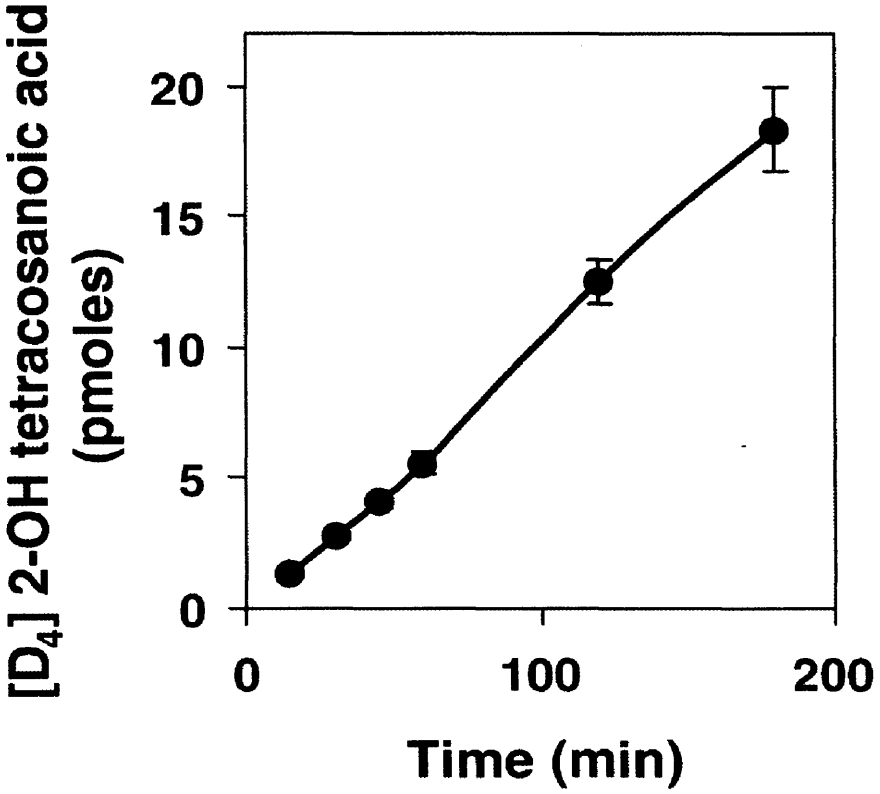

Fig. 1-4. Time-course of fatty acid 2-hydroxylation. Microsomal fraction was prepared from COS7 cells transfected with pcDNA3-hFA2H. The assay mixtures contained 50 μg microsomal proteins in the presence of an NADPH regeneration system and human NADPH:P-450 reductase. Data are shown as mean ± SD of duplicate measurements.
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ceramide 2-hydroxylation by FA2H, the time-course shown in Fig. 1-4 provides strong evidence that FA2H utilizes free fatty acids to generate free 2-hydroxy fatty acids. Based on the previous studies mentioned above, it appears that accumulated free 2-hydroxy fatty acids could be converted to 2-hydroxy ceramides, presumably by ceramide synthases present in microsomes. Thus, a highly sensitive detection system is necessary

to measure free 2-hydroxy fatty acids below the levels that could be utilized by ceramide synthases.

2-Hydroxy tetracosanoic acid formation was also proportional to protein concentration up to 100 μg of microsomes (Fig. 1-5). It should be noted that quantification of the reaction product was performed by injecting 1-2 μl out of 100 μl of derivatized samples into the GC/MS, which corresponds to 0.25 to 1 μg protein. The high sensitivity of this assay is very useful for analysis when sample quantities are limited.

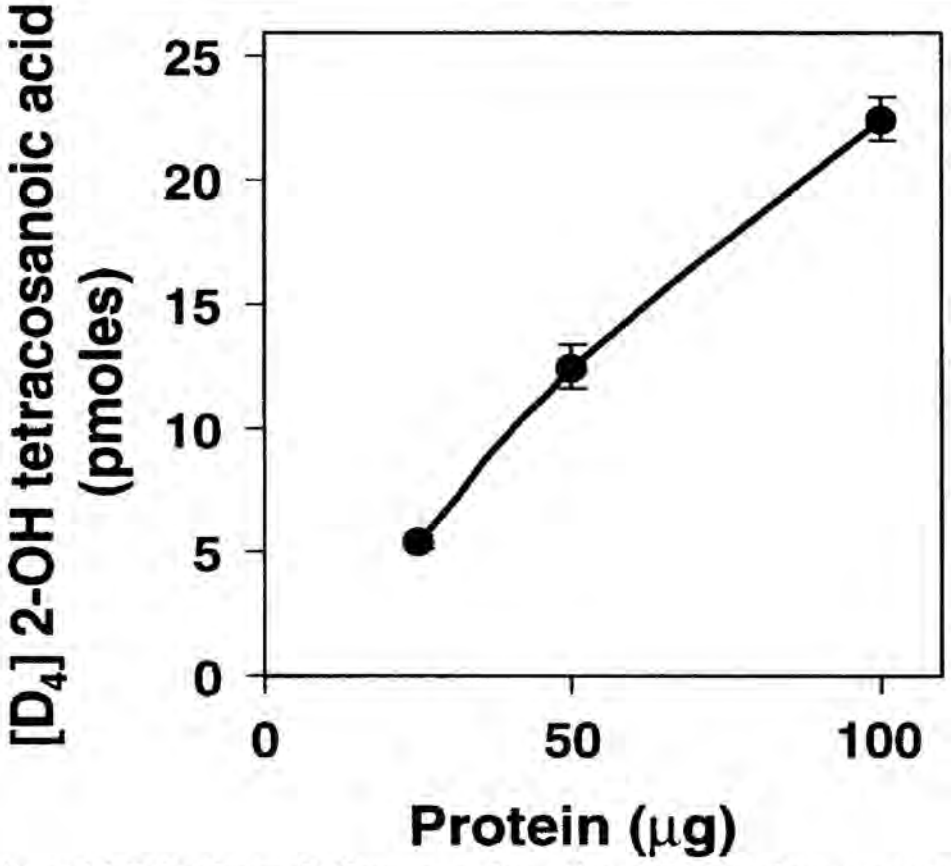

Fig. 1-5. Fatty acid 2-hydroxylation as a function of protein concentration. Microsomal fractions were prepared from COS7 cells transfected with pcDNA3-hFA2H. The assay mixtures contained indicated amount of microsomal proteins in the presence of an NADPH regeneration system and human NADPH:P-450 reductase. Reaction mixtures were incubated for 120 min at 37 °C. Data are shown as mean ± SD of duplicate measurements.
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The pH profile shown in Fig. 1-6 indicated that fatty acid 2-hydroxylase activity of FA2H-transfected COS7 microsomes was the highest at pH 7.6-7.8, which is consistent with the optimum pH for the rat brain enzyme [41]. It should be noted that this assay is a multi-component system involving three enzymes (FA2H, NADPH:cytochrome P-450 reductase, and glucose 6-phosphate dehydrogenase). The linear correlation between protein concentration and activities (Fig. 1-5) shows that FA2H is the limiting component at pH 7.6. It is possible that the rate of electron transport could have become sub-optimal at other pH.

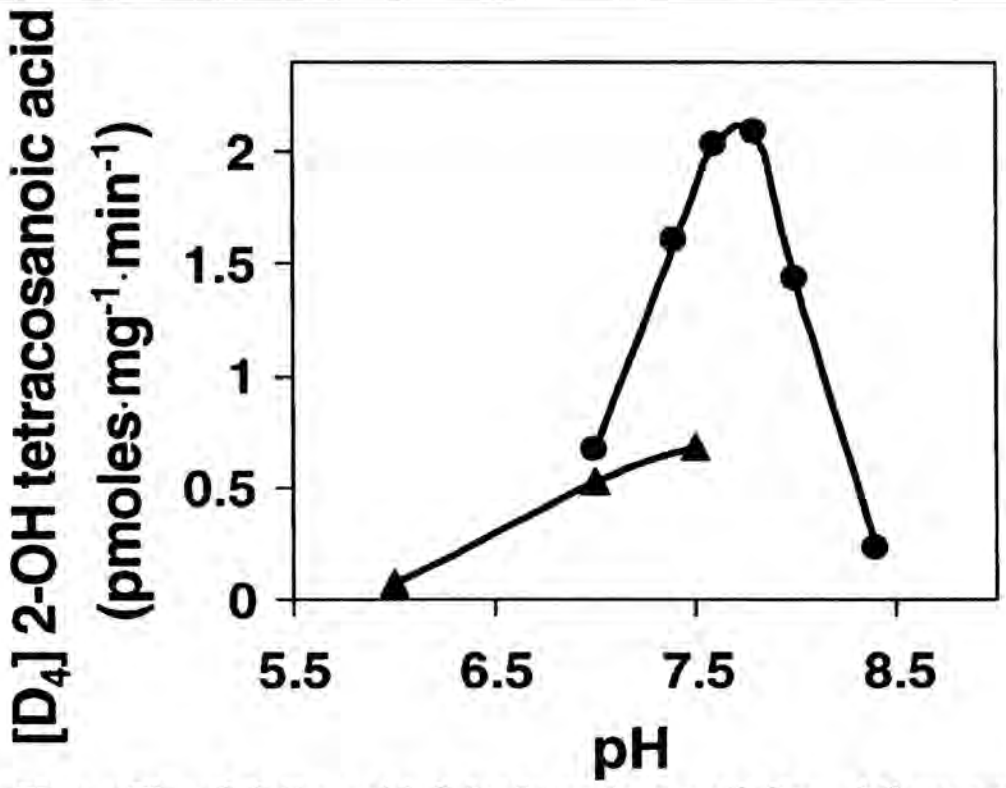

Fig. 1-6. pH profile of fatty acid 2-hydroxylase activity. Microsomal fractions were prepared from COS7 cells transfected with pcDNA3-hFA2H. The reaction mixtures contained 50 μg microsomal proteins in the presence of an NADPH regeneration system, human NADPH:P-450 reductase, and 2.7 mM potassium phosphate (pH 6.0, 7.0, or 7.5) (triangle) or 2.7 mM Tris-HCl (pH 7.0, 7.4, 7.6, 7.8, 8.0, or 8.4) (circle). Reaction mixtures were incubated for 120 min. The pH of the reaction mixture remained unchanged at the end of incubation.

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With crude biological samples, endogenous lipids could interfere with the fatty acid 2-hydroxylase assay. Free fatty acids would compete with the deuterated substrate, and other membrane lipids and storage lipids would affect the delivery of the deuterated substrate to the microsomal fatty acid 2-hydroxylase. When 0.18-1.8 μM of deuterated tetracosanoic acid were added in the assay with 50 μg of microsomal proteins of FA2H-transfected COS7 cells, formation of deuterated 2-hydroxy tetracosanoic acid was consistent at all substrate concentrations, indicating that the presence of microsomal fatty acids and other lipids did not interfere with the assay (Fig. 1-7). This result also indicates

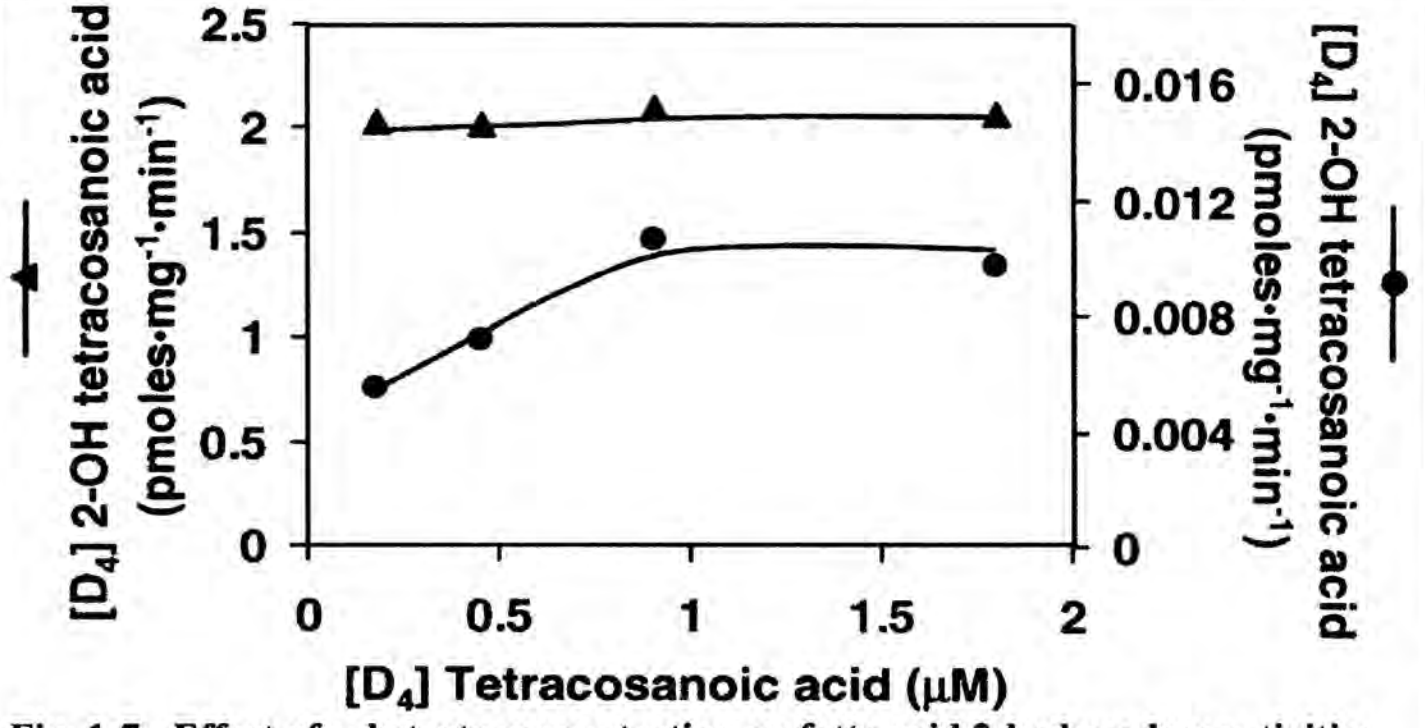

Fig. 1-7. Effect of substrate concentration on fatty acid 2-hydroxylase activities. The reaction mixtures contained 50 μg microsomal proteins from FA2H-transfected COS7 cells (triangle) or murine brain homogenates (circle) and were incubated for 120 min and 180 min, respectively. The substrate conversion was 4.5% (at 0.18 μM with FA2H-transfected COS7 microsomes) or less.
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that apparent K_m for tetracosanoic acid is below 0.18 μM, which is significantly lower than the reported K_m for the rat brain enzyme (4.2 μM) [41]. These values are not

directly comparable since different methods of substrate delivery were used (Celite complexes versus cyclodextrin inclusion complexes). Further, the reaction product in the previous assay was detected as 2-hydroxy ceramide, which was generated by multiple enzymatic reactions.

When crude murine brain homogenates (50 μg protein) were used as enzyme sources, 2-hydroxylation was not saturated at substrate concentrations below 0.9 μM, presumably due to a relatively high concentration of lipids in the brain (Fig. 1-7). The substrate concentration used in this report (1.8 μM) is appropriate for the samples we have tested. However, other samples with higher lipid contents may require a higher concentration of the deuterated substrate.

Fatty acid 2-hydroxylase activity measurement by a benchtop GC/MS

The assay described above was initially developed using a high-resolution magnetic sector mass spectrometer. Although this instrument provides an unsurpassed sensitivity, the highly specialized instrument is not commonly used in research laboratories and not practical for routine analyses. We therefore applied the same method to a benchtop system with a quadrapole mass spectrometer. Although the limit of detection on the benchtop instrument was approximately 100-fold higher (at the fmole level) compared to the magnetic sector mass spectrometer, the activity in FA2H-transfected COS7 cells was readily detectable with this instrument (not shown). To test whether this instrument was useful for various biological samples, we first measured fatty acid 2-hydroxylase activities in mouse brains. When whole brain homogenates (50 μg protein) were incubated with the assay mixture as described above, deuterated 2-hydroxy tetracosanoic acid was reproducibly formed (Table 1-1). Subsequently, activities in commonly used

cell lines, COS7, HeLa, and A549, were measured. To our knowledge, fatty acid 2-hydroxylase activities have never been shown in tissue culture cells. As shown in Table I, all cell lines measured had relatively high fatty acid 2-hydroxylase activities. Consistent with this result, we have observed free 2-hydroxy fatty acids in these cells (not shown). It is likely that fatty acid 2-hydroxylase and 2-hydroxy sphingolipids have been overlooked in many tissues and cell lines due to their low abundance. The new assay will allow us to re-evaluate the presence of fatty acid 2-hydroxylase and 2-hydroxy sphingolipids in various biological samples.

TABLE 1-1

Fatty acid 2-hydroxylase activities in tissue culture cells and mouse brain homogenates

Samples[#]	FA 2-hydroxylase activity (pmoles•mg^{-1}•min^{-1})
Adult mouse brain	$(9.9 \pm 0.9) \times 10^{-3}$
COS7	0.27 ± 0.02
HeLa	0.28 ± 0.03
A549	0.45 ± 0.02

[#] For the measurement of activities in mouse brains, whole brain homogenates were used as enzyme sources. Assay mixtures were incubated with 50 μg proteins for 180 min. For the measurement of activities in tissue culture cells, microsomal fractions were used as enzyme sources. Assay mixtures were incubated with 100 μg proteins for 120 min. Data are shown as the mean ± standard deviation of triplicate measurements.

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Chapter 2

Fatty Acid 2-Hydroxylation in the Central Nervous System

Introduction

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 to 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 approximately 70% lipids and 30% proteins, as compared to 30–50% lipids in most cell membranes, which presumably contributes to its high electrical resistance [2]. One of the striking features of myelin is that approximately one-third of all lipids consist of galactosylceramides (GalCer) and sulfatides (3-sulfate ester of GalCer) with half of their amide-linked fatty acids hydroxylated at the C_2 position (2-hydroxy fatty acids) [3, 4, 13, 41].

In myelin-forming oligodendrocytes and Schwann cells, GalCer containing both 2-hydroxy and non-hydroxy fatty acids are synthesized by the enzyme UDP-galactose:ceramide galactosyltransferase (CGT) [18]. CGT-knockout mice, which lack GalCer and sulfatides, form functionally altered myelin, develop neurological abnormalities, and have a short lifespan [19, 20]. Detailed studies of these mice revealed

crucial roles for GalCer and sulfatides in myelination and axo-glial organization [21-23]. Myelin of CGT-knockout mice contains 2-hydroxy glucosylceramides, which are not found in normal myelin [19, 20]. CGT-transgenic mice also had unstable and uncompacted myelin and developed progressive hindlimb paralysis and demyelination [24]. While total galactolipids in these mice were not significantly altered, the ratio of 2-hydroxy GalCer to non-hydroxy 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 2-hydroxy ceramides, which are formed by the action of fatty acid 2-hydroxylase (also known as fatty acid α-hydroxylase) [5, 41]. In 1973, Hoshi and Kishimoto demonstrated fatty acid 2-hydroxylase activity in rat brain [41]. The rat brain fatty acid 2-hydroxylase is a microsomal enzyme [5] that requires molecular oxygen, Mg^{2+}, pyridine nucleotides, cellular cofactors [41], and microsomal electron transfer proteins [25, 26]. 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 (FA2H) encoding a fatty acid 2-hydroxylase that is highly expressed in brain [1]. 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 non-heme di-iron cluster at the active site of the enzyme

[30]. FA2H catalyzes 2-hydroxylation of free fatty acid *in vitro*, which was dependent on a reconstituted electron transport system [1]. Subsequently, Eckhardt et al. reported that the mouse FA2H gene was highly expressed in brain during myelination, and that FA2H mRNA colocalizes with *PLP* mRNA [42]. These findings provide strong evidence that the FA2H gene encodes the fatty acid 2-hydroxylase previously characterized by Kishimoto and colleagues.

Despite the extensive studies on biochemical characteristics of fatty acid 2-hydroyxlase, 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

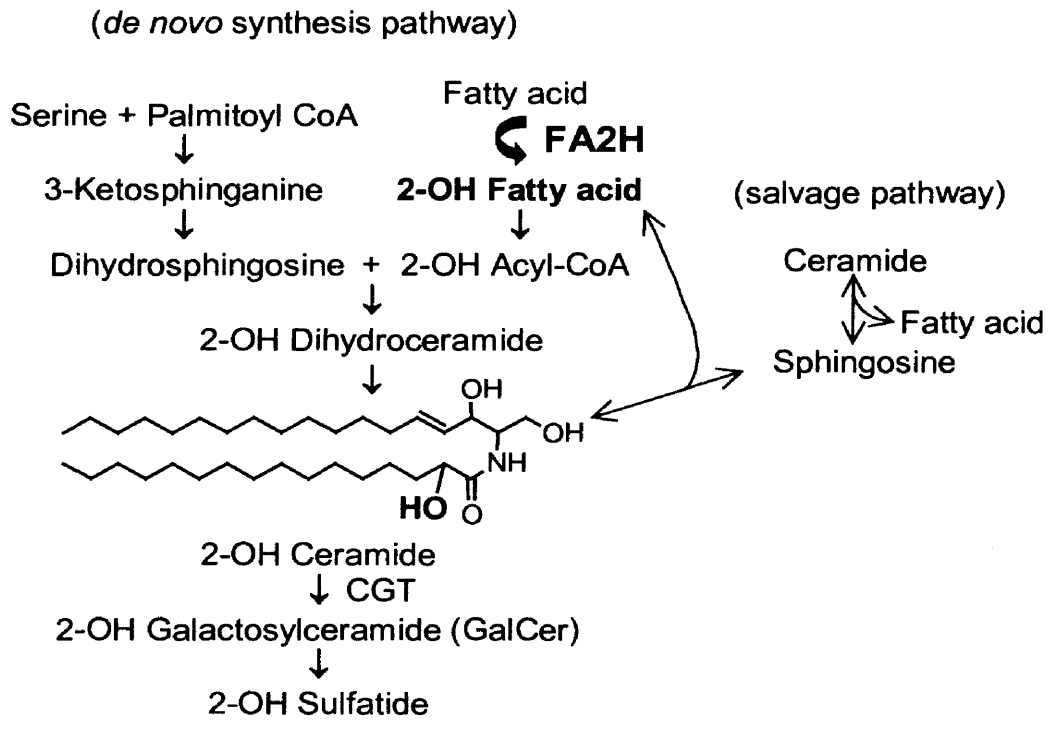

Fig. 2-1. Proposed biosynthetic pathway for 2-hydroxy sphingolipids. The *de novo* pathway is identical to the biosynthesis of non-hydroxy sphingolipids, except for the 2-hydroxylation step catalyzed by FA2H. Note that fatty acids with variable chain lengths are incorporated into sphingolipids. UDP-galactose:ceramide galactosyl transferase (CGT) catalyzes the synthesis of galactosyl ceramide (GalCer). The salvage pathway may contribute to the formation of 2-hydroxy ceramide from 2-hydroxy fatty acid and sphingosine.
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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. 2-1, in which FA2H-dependent 2-hydroxylation of free fatty acids is the first step in the synthesis of myelin 2-hydroxy galactolipids.

Materials and Methods

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). Generation of anti-human FA2H polyclonal antibodies has been reported [1]. Anti-mouse FA2H polyclonal antibodies were generated in rabbits using a synthetic multi-antigenic peptide corresponding to the C-terminal 20-aa 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 (Saint Louis, MO). Purified human NADPH:P-450 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 (MUSC) with water and food *ad libitum*. Mice

were treated in accordance with the MUSC Institutional Animal Care and Use Committee (IACUC) approved procedures.

Cell Cultures

CG4 cells [44] 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 before [45]. Oligodendrocyte differentiation was initiated upon removal of B104-conditioned medium.

Primary glial cells were isolated from newborn rat brains as described previously [45, 46]. 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 6-well dishes, and harvested for immunoblot analysis. Oligodendrocyte progenitor cells (OPCs) were harvested by overnight shaking on a gyratory shaker at 200 rpm and resuspended in 10% calf serum and subjected to several rounds of attachment/detachment for further enrichment. The final OPC suspension was seeded in 6-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 media (GM) included the growth factors bFGF (10 ng/ml) and PDGF (10 ng/ml), and differentiation media (DM) excluded growth factors. Astrocyte-enriched cultures were prepared by subculturing the original mixed glial culture devoid of most oligodendrocytes and microglia as described previously [47].

Isolation of Mouse Brain Galactolipids

GalCer and sulfatides (2-hydroxy and non-hydroxy) were isolated as described previously with minor modifications [20]. Briefly, whole murine brains were homogenized using a PT1200E Polytron homogenizer with a 7-mm generator, and lipids extracted with 19 volumes of chloroform/methanol (2:1, v/v) [48]. The extracts were washed with 0.2 vol of 0.9% NaCl, and the lower phase 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 [19]. 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 UV after spraying with primuline solution (0.005% primuline in acetone/water, 80:20 v/v). Galactolipid spots (2-hydroxy and non-hydroxy) were removed from the plates and subjected to alkaline hydrolysis (in 4 N KOH for overnight at 80 °C). Following neutralization with glacial acetic acid, fatty acids were extracted three times with 3 ml diethyl ether, and dried under N_2. Fatty acids were derivatized and quantified as described in the next section.

Fatty Acid Determination by Gas Chromatography/Mass Spectrometry (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 sec on, 0.1 sec off for 12 cycles), and cell lysates were centrifuged at 1,000 x 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 (TMS) derivatives of hydroxyl groups, 100 µl 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, and increased to 300°C at 10 °C/min. Mass spectra data were obtained on a Shimadzu GC/MS-QP2010 mass spectrometer following electron impact ionization. Peaks of the target analytes and internal standards were processed using the 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 [49]. 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, 1 μg of human NADPH:cytochrome P-450 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 "Fatty Acid Determination by Gas Chromatography/Mass Spectrometry (GC/MS)" section.

Immunoblot Analyses

CG4 and primary glial cells were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.6; 1 mM PMSF; 1% glycerol), a small aliquot removed for protein determination, and remaining samples were mixed with an equal volume of 2x SDS-PAGE sample buffer. Total protein levels were quantified with a Pierce BCA Protein Assay Kit (Rockford, IL). 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; 5% nonfat dry milk) overnight at 4°C, incubated with affinity purified anti-human FA2H polyclonal antibodies (1:1000) or anti-actin polyclonal antibodies (1:200) in TBST-milk for 2 hr, washed, followed by an incubation with HRP-linked donkey anti-rabbit IgG (1:5,000) (Amersham Biosciences,

Piscataway, NJ) for 1.5 hr. Membranes were washed and target proteins 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 sec on, 0.1 sec off for 12 cycles), and cell lysates were centrifuged at 1,000 x 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 above.

Quantitative RT-PCR (qPCR)

Murine 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 PCR (Q-PCR) was performed on a BioRad 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; for mouse 18S rRNA, m18S-F1 gcccgaagcgtttactttga and m18S-R1 ggcctcagttccgaaaacc. A standard reaction mixture contained 15 μl iQ SYBR Green Supermix (BioRad), 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 2-step amplification (10 sec at 95°C and 45 sec at 57°C). All reactions were performed in triplicate. For each sample, 18S rRNA was measured as an internal standard. Threshold cycle (Ct) for each target was obtained by BioRad MyiQ Optical System Software. Relative abundance of FA2H, CGT, and PLP mRNA were calculated using the $\Delta\Delta C_t$ method [50].

Results And Discussion

We propose the pathway for the synthesis of 2-hydroxy galactolipids as shown in Fig. 2-1. 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 proteolipid protein (PLP) mRNA in mouse brain [42]. 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 growth factors PDGF and bFGF), but distinct bands were visible when cells were grown in differentiation medium (with no growth factors) (Fig. 2-2).

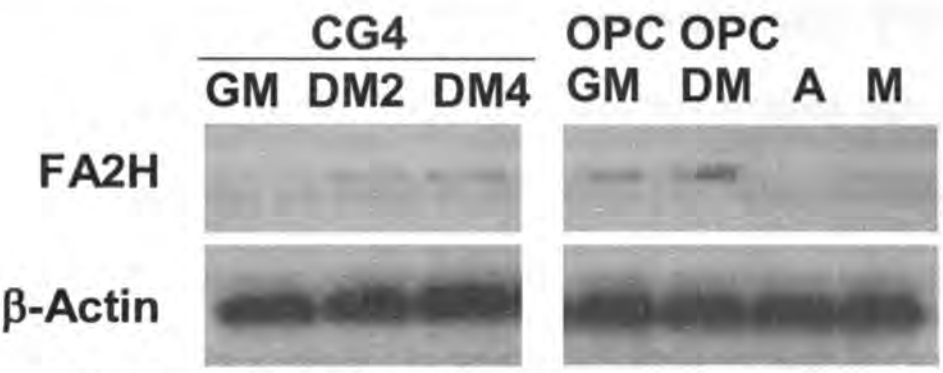

Fig. 2-2. FA2H is present primarily in oligodendrocytes. Total cell lysates (20 μg protein) of rat glial progenitor cell line CG4 or rat primary cells were subjected to SDS-PAGE, followed by immunoblot with anti-human FA2H or anti-actin polyclonal antibodies. CG4 cells were grown in a growth medium (GM) or differentiation medium for 2 days (DM2) or 4 days (DM4). OPC GM, oligodendrocyte progenitors in GM; OPC DM, oligodendrocyte progenitors in DM; A, astrocytes; M, microglia.
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Based on the rat genome database, the rat FA2H protein is 80% identical to human FA2H with the same molecular weight (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 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.

In order 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 (*Table 2-1*, *Table 2-2*). 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 [4]. While

the general trend of changes in fatty acid compositions of mouse brain GalCer and sulfatides was similar to 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 [4]. When normalized against protein contents, changes in total non-hydroxy fatty acids of GalCer and sulfatides were within 1.5- to 2-fold from 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.

Table 2-1

Fatty acid composition of GalCer in neonatal mouse brain.

	Non-hydroxy Fatty Acids (nmol/mg protein) [a]							Total non-hydroxy
Age	C16	C18	C20	C22	C24	C24:1	C26	FA
P2	862	841	20.8	11.9	96.9	95.8	6.6	1935
P17	818	722	20.7	11.9	73.3	111	5.5	1761
P20	1232	1101	30.2	11.5	105	133	5.0	2617
P30	757	742	16.7	7.6	61.3	82.9	3.2	1671

	2-Hydroxy Fatty Acids (nmol/mg protein) [a]							Total 2-hydroxy
Age	C16	C18	C20	C22	C24	C24:1	C26	FA
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	1036
P30	298	68.8	0.2	10.6	705	22.5	9.0	1114

[a] GalCer were purified from whole brain, and their fatty acid compositions analyzed by GC/MS.

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Table 2-2

Fatty acid composition of sulfatides in neonatal mouse brain.

	Non-hydroxy Fatty Acids (nmol/mg protein) [b]							Total non-hydroxy
Age	C16	C18	C20	C22	C24	C24:1	C26	FA
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	1007
P30	279	354	11.2	7.1	107	121	1.7	881

	2-Hydroxy Fatty Acids (nmol/mg protein) [b]							Total 2-hydroxy
Age	C16	C18	C20	C22	C24	C24:1	C26	FA
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

[b] Sulfatides were purified from whole brain, and their fatty acid compositions analyzed by GC/MS.

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The increase in 2-hydroxy fatty acids resulted in a marked increase in the ratio of 2-hydroxy fatty acids to non-hydroxy fatty acids during myelination (Fig. 2-3). This ratio reached a peak in sulfatides at P20 (4.8-fold higher than P2), but continued to increase in GalCer to P30 (8.7-fold higher than 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 non-hydroxy C_{24} fatty acid in GalCer remained consistent at 2-5% of

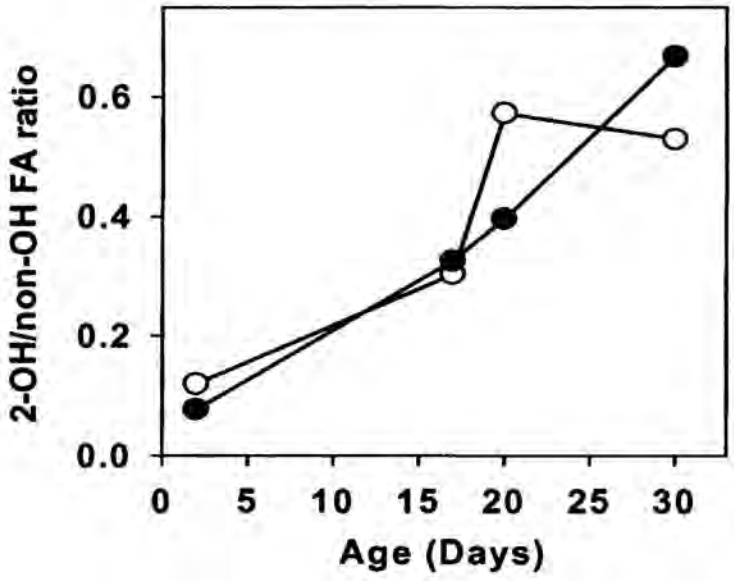

Fig. 2-3. The 2-hydroxy fatty acid contents in brain GalCer and sulfatide increase during postnatal myelination. Whole brains were isolated from neonatal mice at various ages. GalCer and sulfatides were extracted from whole brain and purified by preparative TLC. Fatty acids were liberated from GalCer and sulfatides by alkaline hydrolysis, and quantified by GC/MS. The data are expressed as the ratio of total 2-hydroxy fatty acids to total non-hydroxy fatty acids in GalCer (filled circle) and sulfatide (open circle).
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total GalCer-associated fatty acids, whereas 2-hydroxy C_{24} fatty acid content increased from 0.6% to 25% from P2 to P30. 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 previously 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 similar compositions found in myelin 2-hydroxy galactolipids. To test this possibility, we quantified free 2-hydroxy fatty acids in developing mouse brain (Fig. 2-4). 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 approximately 2-fold during the same period. Following the period of peak myelination, by P83 all 2-hydroxy fatty acids decreased to the P1 levels, and further decreased in aging animals. Non-hydroxy 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 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, while brain 2-hydroxy galactolipids continue to increase at the same time. It is, therefore, unlikely that free 2-hydroxy fatty acids were generated by degradation of tissue 2-hydroxy galactolipids. To

provide further evidence that the increase in free 2-hydroxy fatty acids represent *de novo* synthesis, and not degradation of 2-hydroxy galactolipids, mouse brain fatty acid 2-

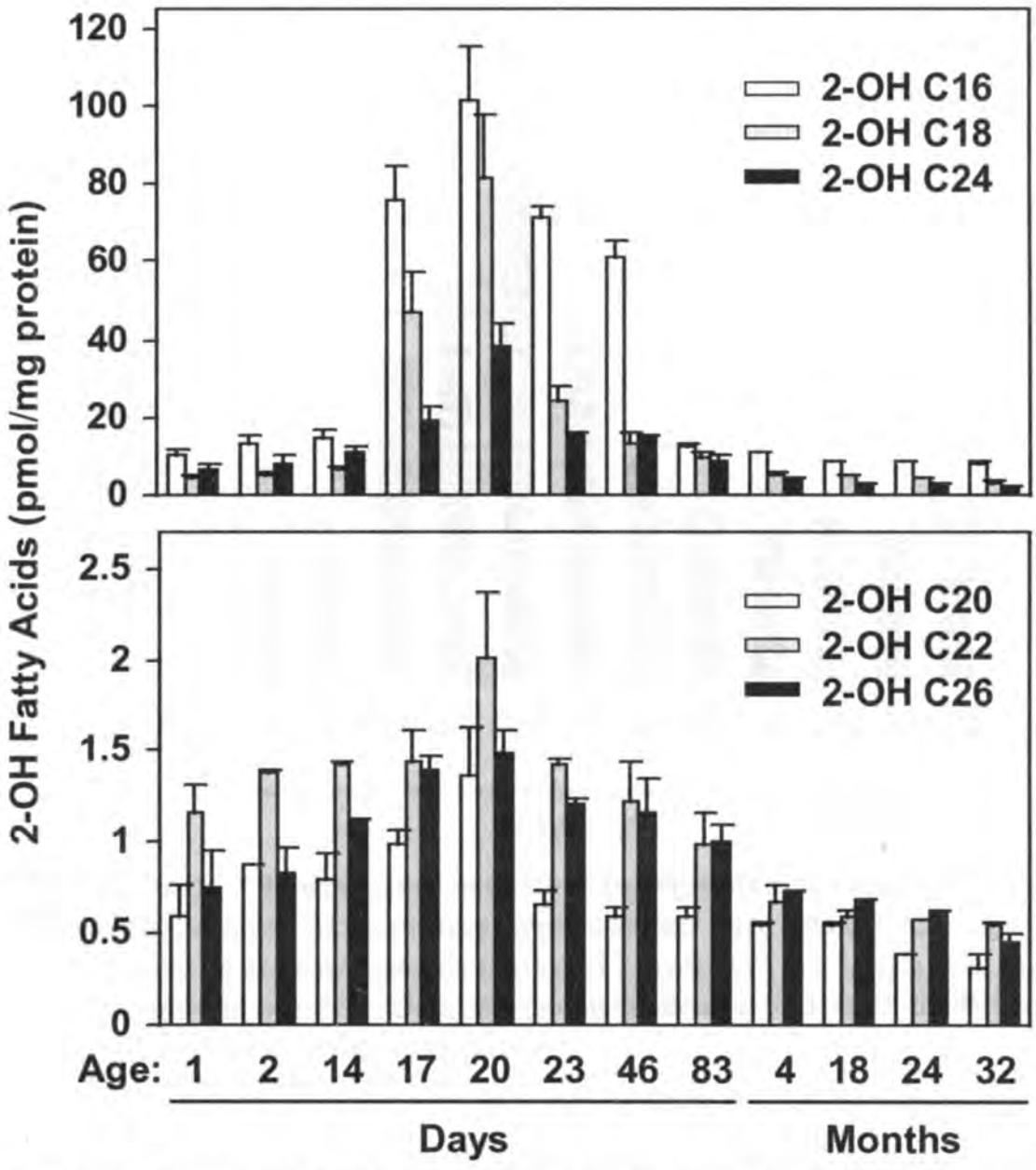

Fig. 2-4. Free 2-hydroxy fatty acids in postnatal mouse brain. *Whole brains were* isolated from mouse at various ages as indicated. Free fatty acids were extracted by diethyl ether, and derivatized for GC/MS analysis. Free 2-hydroxy fatty acid levels were normalized against protein contents. Data are shown as mean and S.D. (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.
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hydroxylase activities were determined using the newly developed GC/MS-based *in vitro* assay [1, 49]. The 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 less than 1% conversion of the deuterated substrate. As shown in Fig. 2-5, the mouse

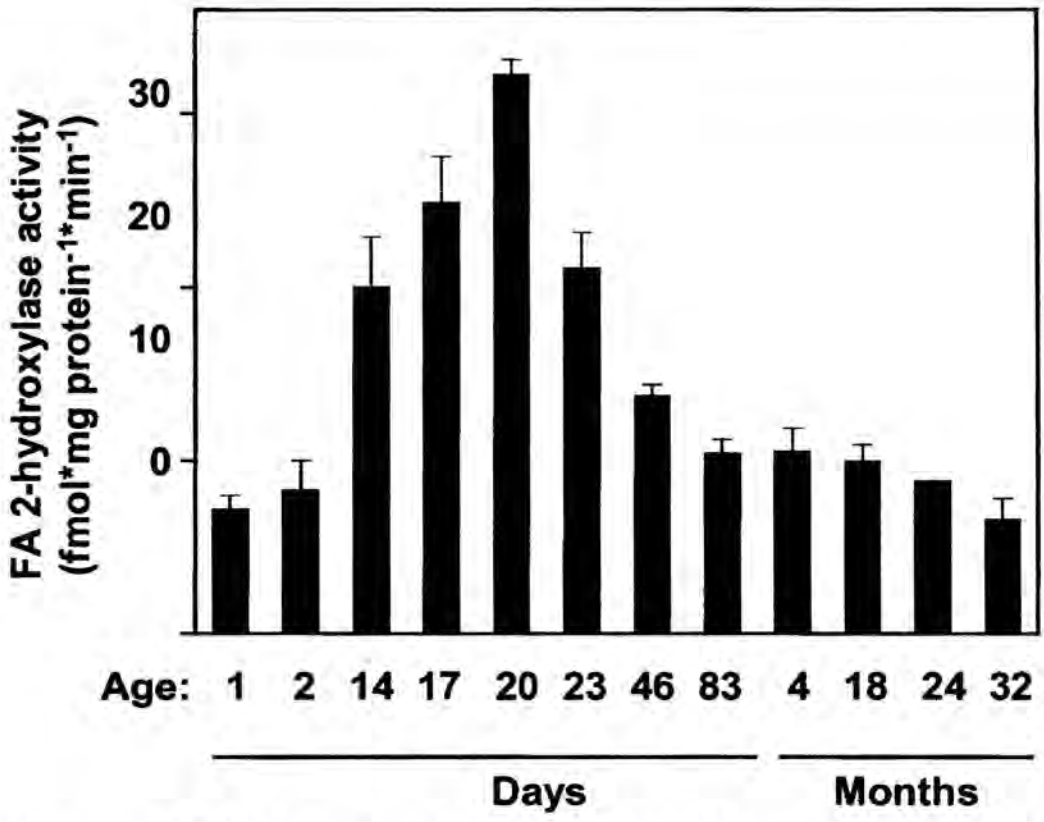

Fig. 2-5. Fatty acid 2-hydroxylase activity is upregulated during myelination. Total brain homogenates (50 μg protein) were used for fatty acid 2-hydroxylase assays. The activities are expressed as fmoles of 2-hydroxy [3,3,5,5-D_4] C_{24} fatty acid formed per mg protein per min. Data are shown as mean ± S.D. (n=5 for P1, n=1 for 24 months, and n=2 for all other time points).
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brain fatty acid 2-hydroxylase activity sharply increased 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 due to degradation of 2-hydroxy galactolipids. Interestingly, the activity gradually decreased as animals aged, by as much as 40% in 32-month-old mice, compared to 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. As shown in Fig. 2-6, a weak but detectable signal was present for FA2H at P2. As in the case of rat FA2H, the mouse FA2H protein is 81% identical to human FA2H with a molecular weight of 43 kDa, which is consistent with the size of the band on the immunoblot. FA2H protein increased approximately 4-fold 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.

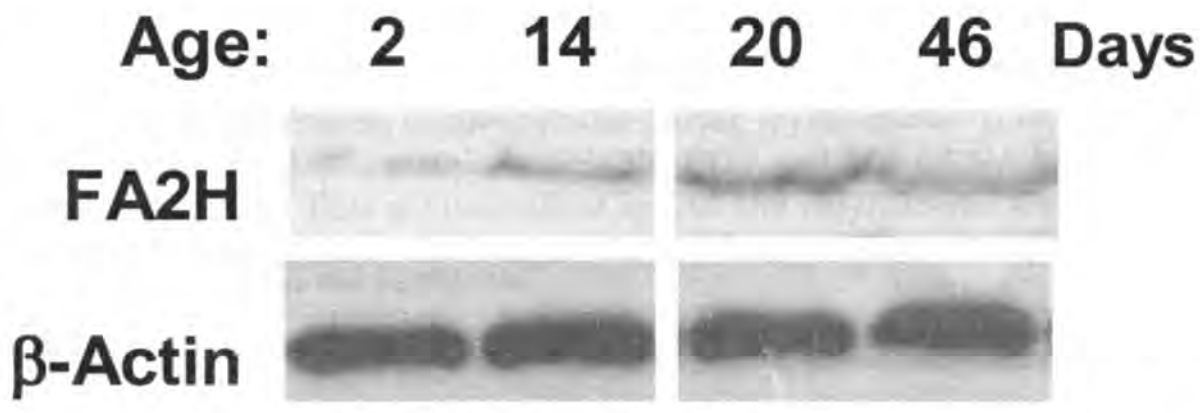

Fig. 2-6. FA2H protein levels are upregulated during myelination. Crude brain homogenates were subjected to SDS-PAGE, followed by immunoblot with anti-human FA2H or anti-actin polyclonal antibodies.
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Next we tested if FA2H is up-regulated in parallel with other myelin genes during myelination. It has been reported that FA2H mRNA in mouse brain increased 7-fold

from P7 to P14, determined by Northern blot analysis [42]. Since we were interested in the change in FA2H expression from the onset of myelination, FA2H mRNA was quantified by qPCR from P1 through P23 mice. To compare the change of expression to other myelin genes, *CGT*, and *PLP* mRNA levels were also measured by qPCR. *CGT* encodes the enzyme responsible for the conversion of ceramides to GalCer, and thus the expression of FA2H and *CGT* are expected to be coordinately up-regulated. PLP is a structural protein of myelin, which serves as an indicator of the progression of myelinogenesis. As shown in Fig. 2-7, mouse brain FA2H mRNA was present at much

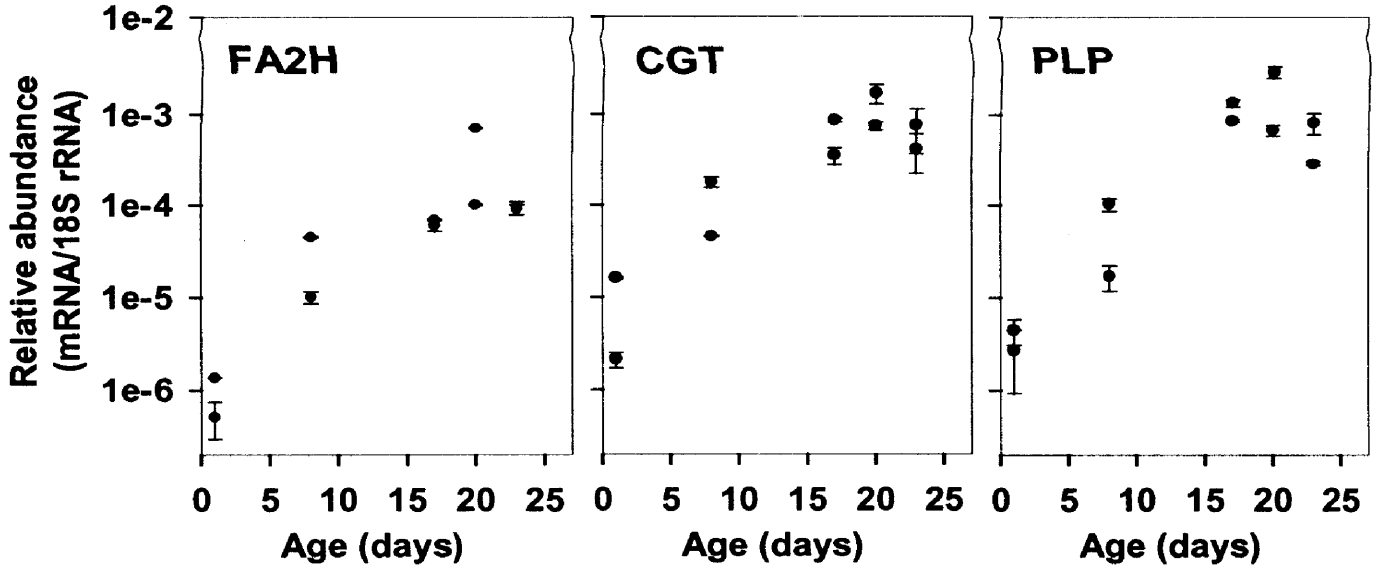

Fig. 2-7. FA2H expression is upregulated during myelination. Total RNA was isolated from neonatal mouse brain, and FA2H, CGT, and PLP mRNA levels were determined by qPCR. Data are normalized against 18S rRNA levels. Circles represent individual animals. The mean ± S.D. of three measurements are shown.
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lower levels than *CGT* or *PLP* mRNA at P1, and exponentially increased during the neonatal period of myelination, reaching on average 400-fold over P1 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* mRNA (800-fold average) during this time period. These data are consistent

with the hypothesis that up-regulation of FA2H is coordinated with *CGT* expression to efficiently incorporate 2-hydroxy fatty acids into myelin galactolipids.

The above 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-hydroxyxlase activity was inhibited by anti-FA2H polyclonal antibodies. As shown in Fig. 2-8A, fatty acid 2-hydroxylase activity in P20 murine brain was inhibited by anti-mouse FA2H antibodies in a dose dependent matter. Importantly, this

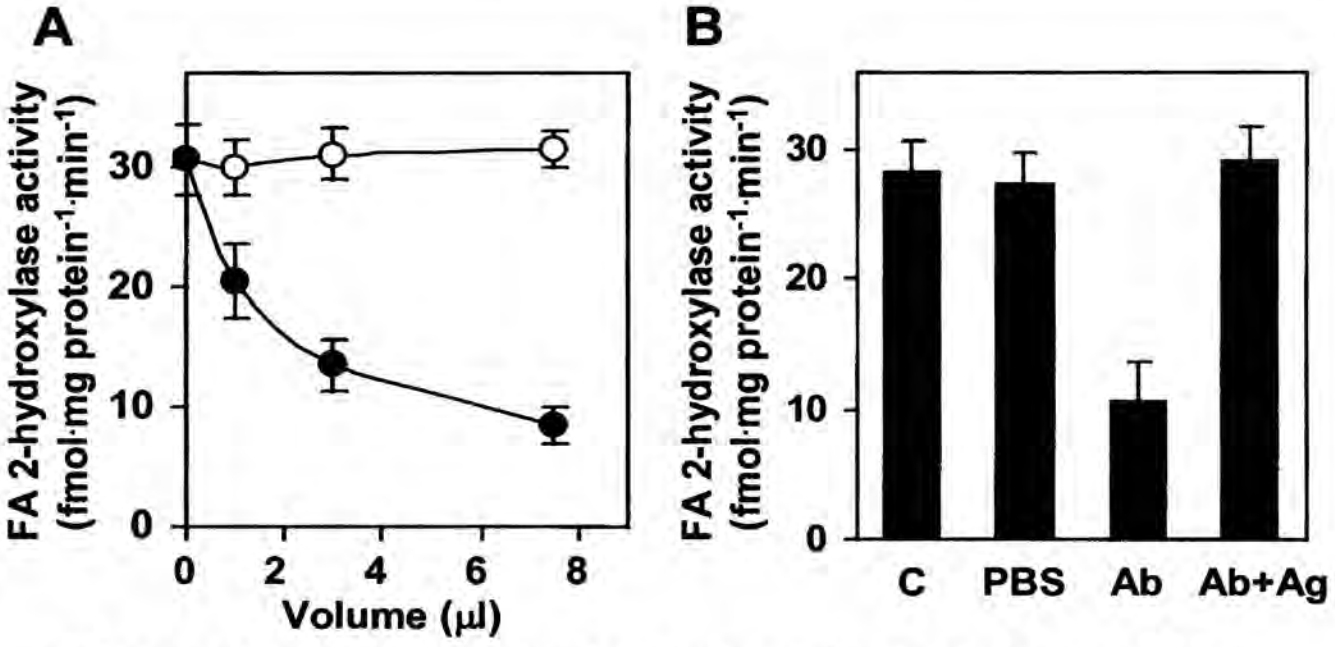

Fig. 2-8. Brain fatty acid 2-hydroxylase activity is inhibited by anti-FA2H antibodies. Fatty acid 2-hydroxylase assays were performed with brain homogenates (50 µg protein) of a 20-day old mouse. A. Indicated volume of PBS (open circle) or affinity purified anti-mouse FA2H antibodies in PBS (filled circle) 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-FA2H antibodies; Ab+Ag, 7.5 µl of affinity purified anti-mouse FA2H antibodies pre-incubated with the antigen (a synthetic multi-antigenic peptide corresponding to the C-terminal 20-aa of mouse FA2H). Data are shown as mean ± S.D. of triplicate measurements.
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inhibitory effect was abolished when FA2H-antibodies were preincubated with the antigen, indicating that the inhibition was due to specific binding of the antibodies to FA2H (Fig. 2-8B). Antibodies against other myelin proteins (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.

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 showed 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 non-hydroxy galactolipids was very low at the onset of myelinogenesis (approximately 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 on the fatty acids in galactolipids, consistent with a precursor-product relationship; 5) the changes of brain free 2-hydroxy fatty acid levels coincided with FA2H-dependent fatty acid 2-hydroxylase activity and upregulation of FA2H expression. Altogether, these data support the postulated pathway for the synthesis of 2-hydroxy galactolipids shown in Fig. 2-1, 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 first reported a rat brain enzyme that converted free fatty acid to 2-hydroxy ceramide, which they named fatty acid α-hydroxylase [41]. 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 [51]. In their *in vitro* assay, ceramide (lignoceroyl sphingosine) did not serve as a substrate [41]. Since 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 [25, 32]. Consistent with their data, ceramide (palmitoyl sphingosine) acted neither as a substrate nor as a competitive inhibitor in our assay (Alderson and Hama, unpublished observation). There are possible explanations for the apparent discrepancy between Kishimoto's observations and our data that show conversion of free fatty acid to free 2-hydroxy fatty acid. It could be due to the differences in the detection limits for free 2-hydroxy fatty acids. In our assay, less than 1% conversion of the deuterated substrate can be accurately measured. Alternatively, in Kishimoto's assay with fatty acid-Celite complex, the two consecutive reactions (fatty acid 2-hydroxylation followed by ceramide synthesis) might have proceeded at an extremely high efficiency due to 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 conditions used in both studies. Our current study does not address whether ceramide is also a substrate *in vivo*. Since there is precedence for ceramide 2-hydroxylation in Tetrahymena [52] and yeast [53, 54], this issue needs to be further clarified in mammalian cells. Another possible substrate is acyl CoA, which has been used in previous studies [5, 25, 26]. 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 [5]. Since acyl CoA is quickly hydrolyzed in the presence of brain microsomes [55], 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 homologues. Database searches for homologues indicated that FA2H is a single gene in human and mouse, and Northern blot showed a single mRNA band in all tissues tested [1, 42]. Therefore, it is unlikely that there is a second di-iron-containing fatty acid 2-hydroyxlase in human and mouse. A second type is cytochrome P-450 enzymes found in bacteria [56, 57]. This class of enzymes catalyzes 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 (2OG)-dependent oxygenase, phytanoyl-CoA 2-hydroxylase. This peroxisomal enzyme is involved in oxidation of branched-chain fatty acids, and does not use straight-chain fatty acids [58]. 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 for a second, minor fatty acid 2-hydroxylase, current data indicates 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 non-hydroxy ceramides [18, 59, 60]. 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 ER localization of CGT is partly responsible for the highly selective incorporation of 2-hydroxy fatty acids into GalCer. Sphingomyelin synthases and GlcCer synthase are localized in the Golgi apparatus or plasma membrane, and therefore 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, 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 [1, 27, 31]. Similarly, the catalytic site of ceramide synthases are predicted to be at the cytoplasmic face [61]. Therefore, both 2-hydroxy and non-hydroxy ceramides

are presumably formed within the cytoplasmic leaflet of the ER membrane. Newly synthesized ceramides then follow one of the three routes; 1) extracted by a cytoplasmic protein CERT to be transported to the Golgi [62], 2) transported via the vesicle-mediated mechanism, or 3) move to the extracytoplasmic leaflet to be converted to GalCer by CGT [63]. In the third route, the transbilayer movement of ceramides could be spontaneous [64, 65], or catalyzed by a hypothetical ceramide flippase [61, 66]. Since 2-hydroxy ceramides are less abundant than non-hydroxy ceramides, it seems reasonable to postulate a ceramide flippase with a higher affinity for 2-hydroxy ceramides than for non-hydroxy ceramides for delivery of all 2-hydroxy ceramides to CGT for incorporation into 2-hydroxy GalCer.

Our data also showed that there is distinct chain length specificity for 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 less dramatic [4]. 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 [67] and ceramide synthases [61], 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.

Chapter 3

Fatty Acid 2-Hydroxylation in the Peripheral Nervous System

Introduction

Myelin is produced by oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system. This unique membrane is comprised of approximately 70% lipids and 30% proteins. The high lipid content is thought to play an integral role in insulation and salutatory nerve conduction [2]. Although myelin proteins have been extensively studied, still very little is known about the role of lipids in myelinogensis. Galactosylceramides (GalCer) and sulfatides (3-sulfate ester of GalCer), comprise approximately 25-30% of total myelin lipids [2, 12]. Interestingly, 50% of GalCer and sulfatides contain fatty acids hydroxylated at the C_2 position (2-hydroxy fatty acids) [3, 4]. No other mammalian tissues contain such high concentrations of 2-hydroxy fatty acids, suggesting an important role in myelination.

Our laboratory has published detailed studies demonstrating FA2H dependent fatty acid 2-hydroxylation in the central nervous system during developmental myelination in rodents [68]. Previously, others have demonstrated that fatty acid 2-hydroxylase (FA2H) catalyzes the 2-hydroxylation of fatty acids during *de novo* synthesis of ceramide, a precursor of all complex sphingolipids [4, 5]. Although biochemical studies have demonstrated that PNS myelin contains a uniquely high content of 2-hydroxy galactolipids [69], still very little is known about the biosynthetic pathway and functional

role for these lipids in the peripheral nervous system. Here we demonstrate that 1) FA2H is highly up-regulated upon Schwann cell differentiation; 2) FA2H is highly expressed in rat sciatic nerve during postnatal myelination and exhibits an expression pattern consistent with other known myelin associated genes (eg. CGT, P_0); 3) FA2H is the major 2-hydroxylase in the rat Schwannoma cell line D6P2T and is required for the formation of 2-hydroxy galactolipids; 4) FA2H may be involved in very early myelinogenic processes including Schwann cell migration and differentiation.

Materials and Methods

Materials

Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). Bovine sulfatides, cerebrosides (kerasin, phrenosin) and glucocerebrosides were purchased from Matreya (Pleasant Gap, PA). Deuterated tetracosanoic acid [3,3,5,5-D4]-and odd chain fatty acids (C_{15}-C_{25}) were purchased from Larodan Fine Chemicals (Malmö, Sweden). Solvents used were HPLC grade and purchased from EMD, Canada and EM Science, Germany. Silica gel 60 TLC plates were from Merck. Recombinant heregulin-$\beta 1_{177-244}$ was from Genentech (South San Francisco, CA). Forskolin, dibutyryl-cAMP was purchased from Sigma (St. Louis, MO).

Animals

Sprague-Dawley rats Fisher rats were maintained in animal care facilities of the Medical University of South Carolina and University of Miami Miller School of Medicine, respectively, with water and food *ad libitum*. Rats were treated in accordance

with the Institutional Animal Care and Use Committee approved procedures at these institutions.

Cell Cultures

Rat Schwann cells were obtained from sciatic nerves of 3-month old Fisher rats by the method of Morrissey [70] with minor modifications. Nerve segments were explanted in DMEM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated FBS (Hyclone) and depleted of fibroblasts by sequential transplantation (2-3 times) to new plastic dishes. After two weeks, tissue explants were dissociated with 0.25% dispase (Roche) and 0.05% collagenase (Worthington) and the resulting cell suspension plated on dishes coated with poly-L-lysine (PLL; 200 μg/ml). Cells were grown in DMEM containing 10% FBS supplemented with a mixture of mitogens [2 μM forskolin, 20 μg/ml bovine pituitary extract (Biomedical Tech., Stoughton, MA), and 2.5 nM recombinant heregulin-$\beta 1_{177\text{-}244}$ (Genentech)]. After 1 week in culture, cells were trypsinized and remaining fibroblasts removed by a 30-min incubation with anti-Thy 1.1 antibodies (conditioned medium from mouse hybridoma cells, ATCC) followed by addition of rabbit complement (ICN). Primary Schwann cells from passage 1 to 4 (2-8 population doublings) were routinely cultured on PLL-coated dishes in medium containing mitogens. Isolated Schwann cells were >98% pure based on immunostaining with anti-S100 (Dako), a protein expressed specifically in Schwann cells. Differentiation was stimulated by plating on 10 cm PLL-laminin coated dishes ($2x10^6$ cells / dish) in DMEM containing 1% FBS and 1 mM dibutyryl-cAMP (Sigma) for 3 days, as described by Harrisingh et al. [71].

Rat Schwannoma D6P2T cells were purchased from ATCC and grown in DMEM containing 10% FBS as recommended by the supplier and collected at approximately 60%-70% confluency.

Migration Assay

D6P2T cells (50,000 per well) were placed on the top surface of filter inserts (8 μm pore) with serum-free media in 24-well culture dishes containing the same media with 10% FBS. After 4 hr, non-migratory cells were removed with a cotton swab, and migratory cells on the bottom surface of the filter were stained with bromophenol blue and counted using light microscopy.

Isolation of Rat Sciatic Nerve Galactolipids

Rats at postnatal days 4, 7, 10, 15, 20, 30 and 60 were euthanized according to guidelines established by the institutional IACUC committee. Sciatic nerves were exposed through a lateral incision in the thigh and sectioned immediately. A minimum of three sciatic nerves (P15, P20, P30, and P60) and a maximum of 16 nerves (P4) were pooled to generate separate samples. At least three samples were analyzed per time point except for P4 that consisted of only one pool. Whole nerves were homogenized in PBS using a PT1200E Polytron with a 7-mm generator. GalCer and sulfatides (2-hydroxy and non-hydroxy) were isolated as described by Coetzee et al [20], with minor modifications. Lipids were extracted with 19 volumes of chloroform:methanol mixtures (2:1, v/v) [48]. The extracts were washed with 0.2 vol of 0.9% NaCl, the lower phase collected and dried under a N_2 stream. Dry lipid extracts were subjected to mild alkaline hydrolysis (0.5N KOH for 10 min at 50°C) to remove glycerolipids [20]. TLC plates were saturated with solvent vapors for 45 min prior to development in chloroform:methanol:water (70:30:4).

Lipid spots were visualized under UV after spraying with primuline solution (0.005% primuline in acetone/water, 80:20 v/v) and identified using the corresponding standards. Hydroxy and non-hydroxygalactolipids were removed from the plates, internal standards added (C_{15}, C_{17}, C_{19}, C_{21}, C_{23}, C_{23} and C_{25} fatty acids), and samples were subjected to alkaline hydrolysis (4N, KOH, overnight at 80°C). Lipids were neutralized using glacial acetic acid, fatty acids extracted three times with 3 mL diethyl ether, and dried under N_2.

Fatty acid determination by Gas Chromatography/Mass Spectrometry (GC/MS)

Fatty acid methyl esters were prepared by adding 1 mL of methanolic HCl to each sample and incubating at 65°C for 45 min and brought to dryness under N_2. To prepare trimethylsilyl (TMS) derivatives of hydroxyl groups, 100 µL Tri-Sil Reagent (Pierce Biotechnology, Rockford, IL) was added and incubated for 30 min at room temperature. Derivatized samples were 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. 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, and increased to 300°C at 10°C/min. Mass spectra data were obtained on a Shimadzu GC/MS-QP2010 mass spectrometer following electron impact ionization. Peaks of the target analytes and internal standards were processed using the 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

FA2H activity was determined in rat sciatic nerves and in D6P2T cells using the method described previously by Alderson et al. [49]. Briefly, crude sciatic nerve or D6P2T homogenates (50 μg of protein) were added to an assay mixture containing 2.7 mM Tris-HCl, pH 7.6, 1.20 mM $NADP^+$, 3.3 mM glucose 6-phosphate, 3.3 mM $MgCl_2$, 0.2 unit of glucose 6-phosphate dehydrogenase, 1 μg of human NADPH:cytochrome P-450 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: 10 μg/mL in 1.5 mM α-cyclodextrin), was added at time zero. After gentle mixing by swirling, the assay mixture was incubated by shaking (100 rpm) at 37°C for 180 min. At the end of the incubation, 1 pmol of C_{23} fatty acid was added to each sample as an internal standard, and samples neutralized by addition of 20 μL of glacial acetic acid. Fatty acids were extracted three times with 2 mL of diethyl ether and combined diethyl ether extracts brought to dryness under N_2. Fatty acids were derivatized and quantified as described in the "Fatty acid determination by Gas Chromatography/Mass Spectrometry (GC/MS)" section.

Quantitative RT-PCR (Q-PCR)

Rats at postnatal days 4, 7, 10, 15, 20, 30 and 60 were euthanized according to guidelines established by the institutional IACUC committee. Sciatic nerves were exposed through a lateral incision in the thigh and sectioned immediately. Whole nerves were homogenized in PBS using a PT1200E Polytron with a 7-mm generator. RNA was isolated using the QIAGEN RNeasy Lipid Tissue kit. Schwann cells were disrupted using QIAshredder mini spin columns (QIAGEN) and RNA was isolated using the QIAGEN RNeasy kit. RNA was quantified using a NanoDrop ND-1000

Spectrophotometer (Nanodrop Technologies, Inc.). cDNA was generated using the Bio-Rad iScript cDNA Synthesis kit. Real-time quantitative PCR (Q-PCR) was performed on a BioRAD MyiQ single-color real-time PCR detection system. The primers used for each gene were as follows: for rat *FA2H* rFA2H-F1: cca tta cta cct gca ctt tgg and rFA2H-R1: tct gga atg agg gtg tgg a; rat *CGT* rCGT-F1 gtt cat ggg tcc agc ttg tg and rCGT-R1 ctg gcc ggc ttt gtt agg; for rat *P_0* rP_0-F1 ctg cac tgc tcc ttc tgg t and rP_0-R1 cct tgg cat agt gga aga ttg; for rat *18S rRNA* r18S-F1 ggc ccg aag cgt tta ctt and r18s-R1 cgg ccg tcc ctc tta atc.

PCR reactions were performed in a 96 well plate with a standard reaction mixture containing 15 μL iQ SYBR Green Supermix (Bio-Rad), cDNA template, and 200 nM each of forward and reverse primers in a total volume of 30 μL. All reactions were performed in triplicate. The thermal cycling conditions were set at 95°C for 3 min, followed by 40 cycles of 2-step amplification (10 sec at 95°C and 45 sec at 57°C). 18S rRNA was measured as the housekeeping gene. Data were analysed using the MyiQ software. Relative abundance of FA2H, CGT and P_0 were calculated using the ΔΔCt method [50].

Construction of hairpin shRNA expression plasmids

FA2H short hairpin RNA (shRNA) or control shRNA expression plasmids were constructed for the target sequence for rat FA2H aagagattattcacttgtggt, or Control #5 from Ambion (Ambion, Austin, TX). Each insert contained a *Bam*HI linker, sense strand, a loop sequence (TTCAAGAGA), antisense strand, RNA Pol III terminator, and a *Hin*dIII linker. Double-stranded DNA inserts were prepared by annealing 63-base complementary synthetic oligonucleotides and cloned into *Bam*HI and *Hin*dIII sites of

pSilencer 5.1-U6 Retro (Ambion). The correct inserts were confirmed by sequencing. Plasmids were transfected into D6P2T using Nucleofector kit T. (amaxa, Gaithersburg, MD).

Transfection of D6P2T cells

D6P2T cells were transfected with double stranded siRNA (2μg) or shRNA expression plasmids (2.5μg) using Nucleofector kit T. (amaxa, Gaithersburg, MD) siRNA was purchased commercially (Ambion, Austin, TX), and shRNA was constructed as described above in "Construction of hairpin shRNA expression plasmids". Transfections were performed according to the manufacturers instructions (Program T-20). Briefly, cells were collected using trypsin-EDTA at 60-70 % confluency. Cells were counted and approximately 2 x 10^6 cells were transfected for each treatment condition, and immediately plated in 75cm^2 flasks to propagate for 48 hours. Transfection efficiency was monitored for each experiment using pmaxGFP and visualization with fluorescence microscopy. Typical experiments averaged approximately 80-90% transfection efficiency with high viability.

[^{14}C] Acetate Metabolic Labeling in D6P2T Cells

D6P2T cells were transfected with 2.5μg of scrambled shRNA or FA2H-shRNA expression plasmids using amaxa Nucleofector kit T (amaxa). After 48 hours, puromycin (1 μg/ml) was added to the culture media and resistant cells selected for two weeks. Cells were grown to approximately 50% confluency and metabolically labeled with [^{14}C] acetate (2 μCi/ml) for 48 hours. Lipids were extracted with 19 volumes of chloroform:methanol mixtures (2:1, v/v) [48]. The extracts were washed with 0.2 vol of

0.9% NaCl, the lower phase collected and dried under a N_2 stream. Dried lipid extracts were subjected to mild alkaline hydrolysis (0.5N KOH for 10 min at 50°C) to remove glycerolipids [20]. TLC plates were saturated with solvent vapors for 45 min previous to being developed in chloroform:methanol:water (70:30:4). A small aliquot was removed for determination of total [^{14}C] counts per sample. Equal counts were loaded for each sample on TLC plates. Non-hydroxy and 2-hydroxy galactolipids standards were run as a cold carrier. Lipid spots were visualized under UV after spraying with primuline solution (0.005% primuline in acetone/water, 80:20 v/v) and identified using the corresponding standards. GalCer were isolated by scraping 2-hydroxy and non-hydroxy [^{14}C]-GalCer separately, and samples were measured by liquid scintillation counter.

Results and Discussion

Myelin is formed by oligodendrocytes in the CNS and Schwann cells in the PNS. We have previously demonstrated that FA2H is expressed in oligodendrocytes of in the central nervous system [68]. We further demonstrated that FA2H gene expression was up-regulated approximately 400-fold during postnatal myelination in parallel to other myelin associated genes. In this report, we examined the expression of FA2H during postnatal myelination of the peripheral nervous system utilizing quantitative-PCR (q-PCR). In order to demonstrate the relative change in gene expression, other well-characterized PNS myelin genes were also measured. CGT encodes the galactolipid biosynthetic enzyme that catalyzes the formation of GalCer from ceramides. As both genes are involved in the same biosynthetic pathway, FA2H and CGT should be coordinately expressed during myelination. The gene P_0, encodes for the major structural protein in PNS, thus serves as a good marker of the myelinogenic process. As shown in

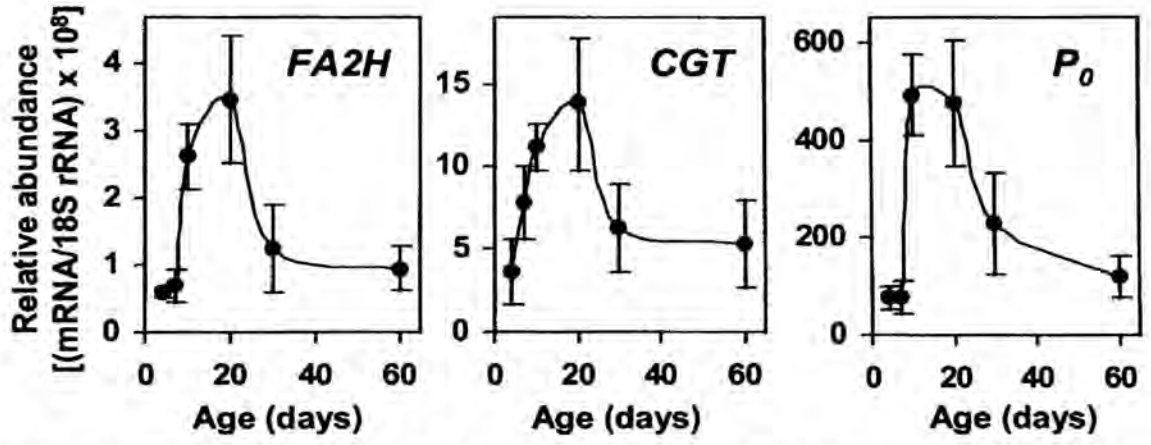

Fig. 3-1. *FA2H* expression in rat sciatic nerve is highly upregulated during myelination in parallel with other myelin genes. *FA2H* , *CGT*, and *P0* mRNA levels in rat sciatic nerve were determined by quantitative RT-PCR. Data were normalized against 18S rRNA levels and expressed as mean ± SD (n=5 for P7, P10, P20, P30, and P60, n=1 for P4).

Fig. 3-1, FA2H was expressed at relatively lower levels than either CGT or P_0 respectively. However, the expression pattern and relative fold increase for FA2H was comparable to the other genes. FA2H expression increased approximately 6-fold during the peak myelination period, from postnatal day 4 (P4) to postnatal day 20 (P20). CGT and P_0 increased 3.7-fold and 6.3 fold respectively during this same period. The expression of all three genes quickly returned to levels measured at P4. The changes in FA2H closely paralleled two previously characterized myelin genes, thus FA2H may be integral for proper developmental myelination of the PNS by generating 2-hydroxy precursors that are efficiently incorporated into myelin by CGT.

We next examined the FA2H enzyme activity in the sciatic nerve during postnatal PNS myelination utilizing a previously published method developed in our laboratory [49]. This assay specifically measures the conversion of deuterated C_{24} free fatty acids to deuterated C_{24} 2-hydroxy fatty acids by GC/MS. FA2H activity increased progressively to P20, reaching approximately 700% of activity levels measured at P4 (Fig. 3-2). There

was a slight decline in enzyme activities (~20%) after this peak myelination period, however this decrease did not directly parallel the decline observed in FA2H gene expression. Thus, FA2H protein may remain relatively stable following peak myelinogenesis in order to continue to produce precursors in the maintenance of abundant myelin lipids. These data also provide *in vitro* evidence that rat FA2H can use free fatty acids as a substrate, however, we cannot exclude the possibility that the enzyme may utilize another (or multiple) substrates *in vivo*.

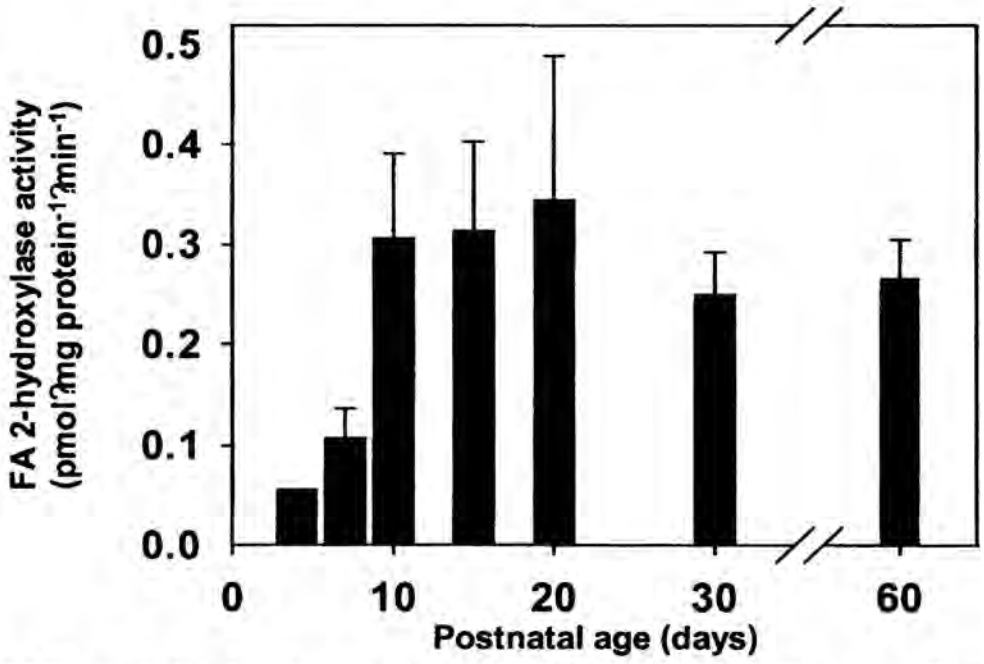

Fig. 3-2. FA2H activity in the sciatic nerve increases during myelination. Fatty acid 2-hydroxylase activity in sciatic nerve homogenates were determined using the GC/MS-based activity assay. The mean and S.D. of data from 3-5 animals are shown, except at day 4, which represents a pool of sciatic nerve from 4 animals.

Since the activity of FA2H remained relatively high following the peak myelination period, this raised the possibility that 2-hydroxy lipids may continue to accumulate in myelin after this time. To address this question we quantified the non-hydroxy and 2-hydroxy fatty acids associated with GalCer and sulfatides isolated from sciatic nerve

during postnatal myelination. Interestingly, GalCer associated C_{16} and C_{18} fatty acids were relatively high compared to other longer chain fatty acids at P4 - P20 (Fig. 3-3, *Left*). However, after P20, longer chain fatty acids (C_{22} – C_{26}) continually accumulate to

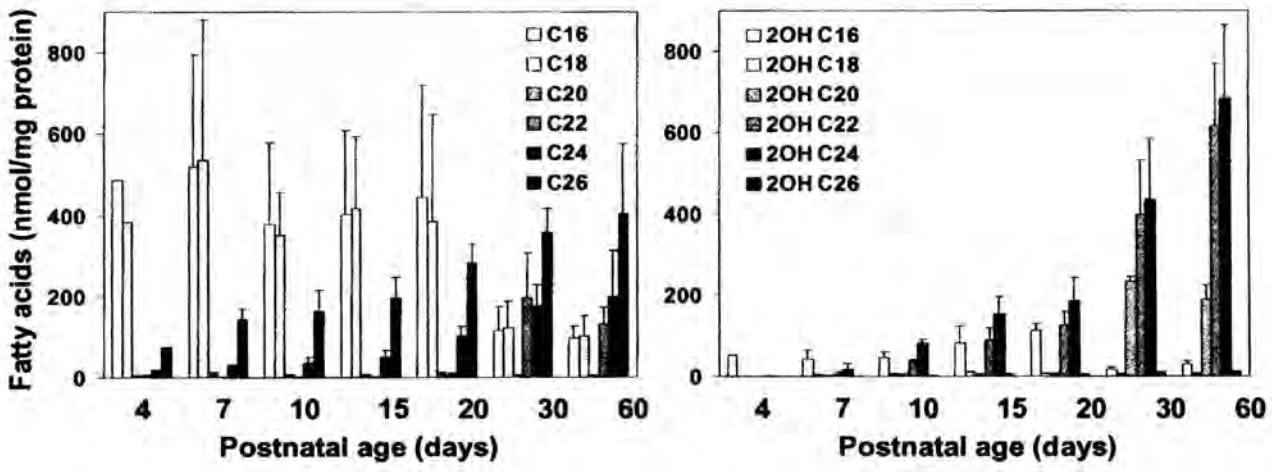

Fig. 3-3. The 2-hydroxy fatty acid content of GalCer increases during myelination. Total lipids were extracted from sciatic nerve. GalCer were purified by TLC and fatty acid compositions determined by GC/MS. Average and S.D. of 3-4 animals are shown, except at day 4, which represents a pool of sciatic nerve from 4 animals.

P60. In contrast, GalCer-associated 2-hydroxy fatty acids were comparatively low in the first twenty days after birth (Fig. 3-3, *Right*). After P20, there is a marked increase in long chain 2-hydroxy fatty acids in GalCer, primarily C_{22}-C_{26}. Taken together, these data indicate that certain fatty acid elongases (ELOVL genes) and ceramide synthases (LASS genes) may be coordinately up-regulated during postnatal myelination to generate preferred chain length substrates in the biosynthesis of GalCer and sulfatides. ELOVL6 (formerly MASR) is the major fatty acid desaturase involved in PNS myelination and may play an integral role in fatty acid elongation during developmental myelination [72]).

The fatty acids associated with sulfatides demonstrated a similar pattern as observed for GalCer, with some interesting differences. Non-hydroxy C_{16} and C_{18} remained relatively high compared to all other non-hydroxy fatty acids except C_{22}, which quantitatively increased after P20 (Fig. 3-4, *Left).* The relative chain lengths of 2-

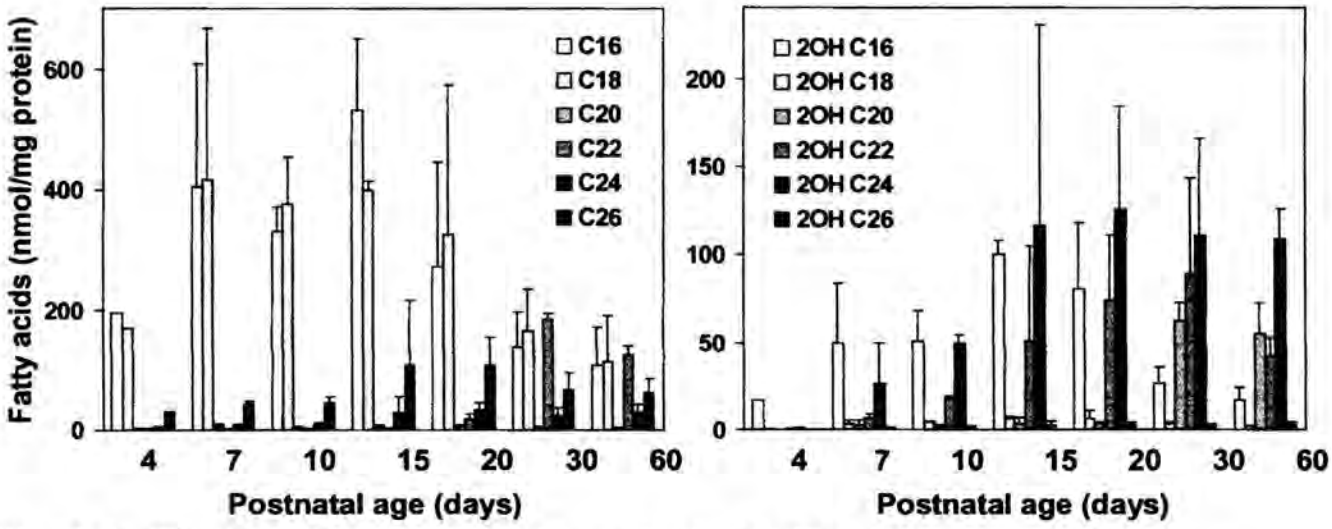

Fig. 3-4. The 2-hydroxy fatty acid content of sulfatides increases during myelination. Total lipids were extracted from sciatic nerve. Sulfatides were purified by TLC and fatty acid compositions determined by GC/MS. Average and S.D. of 3-4 animals are shown, except at day 4, which represents a pool of sciatic nerve from 4 animals.

hydroxy fatty acids were also different compared to GalCer, with C_{22} and C_{24} being the prominent lipid species after P20 (Fig. 3-4, *Right*). The differences observed in fatty acid chain length among GalCer and sulfatides offers direct evidence that the enzyme that catalyze the conversion of GalCer to sulfatides in the biosynthetic pathway, GalCer sulfotransferase, most likely has a substrate chain length specificity.

The relative total abundance of non-hydroxy and 2-hydroxy fatty acids associated with GalCer and sulfatides changed dramatically during the postnatal myelination period.

GalCer associated 2-hydroxy fatty acids accounted for only 5-6 % of total fatty acids at P4 (Fig 3-5, *Left*), and increased to over 60% by P60. In comparison, 2-hydroxy fatty

acids also were relatively low in abundance in sulfatides at P4, and increased to approximately 35% by P60 (Fig. 3-5, *Right*). The percentages reported here are in direct agreement to previously published data on the fatty acid content of rat PNS [69].

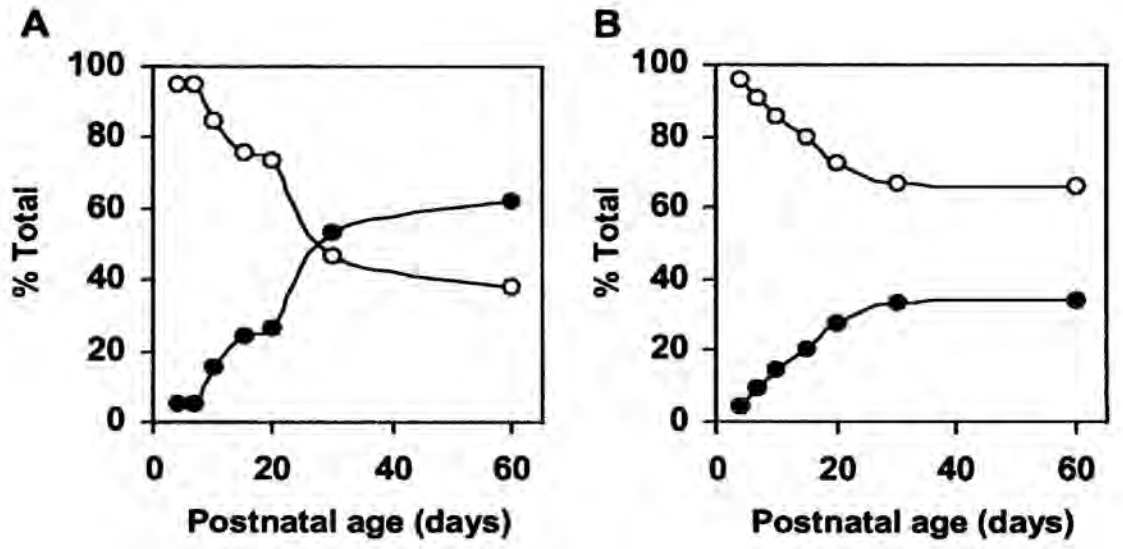

Fig. 3-5. Proportion of 2-OH fatty acids in galactolipids increases during myelination. Individual fatty acids in TLC-purified GalCer (A) and sulfatide (B) were determined by GC/MS. The total of all detectable non-OH (*open circle*) and 2-OH fatty acids (*closed circle*) in galactolipids were used as 100%.

During myelination of the PNS, Schwann cells are derived from the neural crest and must migrate to the appropriate site on the axon prior to the production of myelin. Prior to myelinogenesis, the Schwann cell must senesce, make axonal contact, and undergo differentiation. To determine the role of FA2H in differentiation, we isolated and cultured rat primary Schwann cells. After stimulation to differentiate with addition of cAMP analogues, FA2H gene expression was quantified by q-PCR and compared to CGT and P_0. FA2H was expressed at very low levels prior to differentiation when compared to the relative expression of CGT and P_0. (Fig. 3-6) However, FA2H was increased approximately 90-fold following differentiation. In contrast, CGT and P_0 were

stimulated only 3-4 fold. These results demonstrate that FA2H may play an important role in cAMP mediated Schwann cell differentiation.

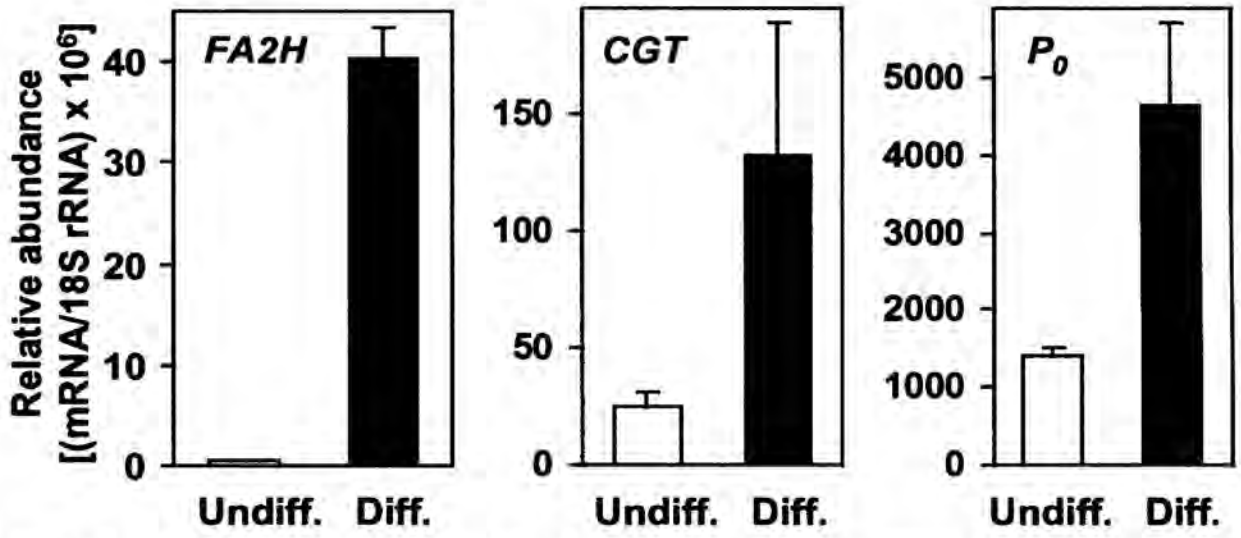

Fig. 3-6. FA2H expression is highly up-regulated during rat Schwann cell differentiation. Total RNA was isolated from adult rat whole sciatic nerves. FA2H, CGT and P_0 mRNA levels were determined by quantitative RT-PCR. Data are normalized against 18SrRNA. Mean and SD from three measurements are shown.

D6P2T cells, a previously characterized rat Schwannoma cell line [73], have been previously shown to synthesize non-hydroxy and 2-hydroxy galactolipids, and exhibit a relatively differentiated phenotype. To determine if FA2H was responsible for 2-hydroxy galactolipid formation, we performed a series of experiments to demonstrate that 1) FA2H is the major 2-hydroxylase in the PNS and 2) FA2H is required for the biosynthesis of 2-hydroxy galactolipids. D6P2T cells were treated by RNAi against FA2H using commercially available rat FA2H siRNA (Ambion) or a shRNA plasmid construct made in our laboratory. The siRNA and shRNA each targeted the same region of FA2H mRNA.

The shRNA plasmid construct was used for long-term knock-down of FA2H, as RNAi effects by siRNA typically last approximately 72 hours. Cells were selected for puromycin resistance by addition of puromycin (1 μg/ml) to the media for two weeks, and the cells were then propagated and harvested. Total non-hydroxy and 2-hydroxy

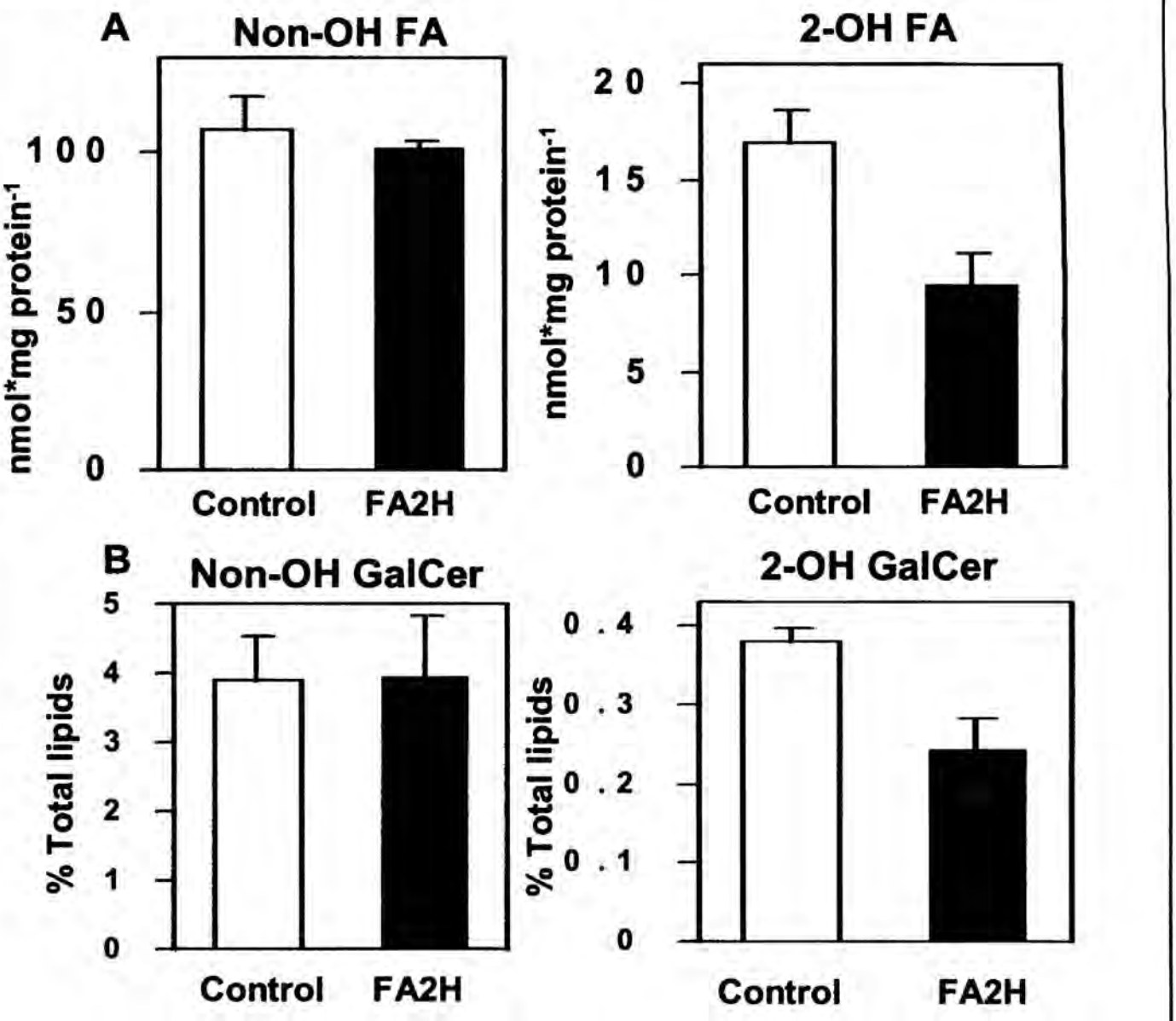

Fig. 3-7. FA2H is required for the biosynthesis of 2-hydroxy galactolipids D6P2T cells were transfected with scrambled shRNA (Control) or shRNA:FA2H (FA2H). After 48 hours, 1 μg/ml puromycin was added to culture media and resistant cells selected for two weeks. (A) Cell lysates were hydrolyzed overnight and the major non-hydroxy and 2-hydroxy fatty acids (C_{16}, C_{18}, C_{24}) were quantified by GC/MS. (B) GalCer were measured by metabolic labeling with [^{14}C] actetate. GalCer was isolated by TLC, visualized by primuline, scraped, and counted by liquid scintillation counter. Data are mean + SD of triplicate samples.

fatty acids were measured by GC/MS following alkaline hydrolysis. A separate experiment was conducted using [^{14}C] acetate metabolic labeling to measure galactolipids. As shown in Fig. 3-7A, cells transfected with shRNA directed against rat FA2H (shRNA:FA2H) had approximately 60% lower levels of total 2-hydroxy fatty acids compared to the scrambled control treated cells (Control). After metabolic labeling with [^{14}C] acetate, there was no difference in the amount of synthesized non-hydroxy GalCer in shRNA:FA2H versus control (Fig. 3-7B). However, 2-hydroxy GalCer was decreased approximately 35-40%. It is important to emphasize that these data are newly synthesized lipids, thus we did not expect to see complete elimination of 2-hydroxy galactolipids.

To confirm that results obtained in 3-7 were not an artifact of retroviral elements in the shRNA plasmid construct, we transfected D6P2T cells with siRNA targeted against a homologous region of the FA2H mRNA. RNAi decreased FA2H activity by 40% when compared to control siRNA (Fig. 3-8, A). Accordingly, total 2-hydroxy fatty acids were decreased approximately 60% compared to control (Fig. 3-8, B). These results are in close agreement with results obtained with shRNA treatment (Fig. 3-7A), demonstrating that the effects of shRNA are not retroviral or puromycin dependent. Together, these data demonstrate that FA2H is the major, if not the only, fatty acid 2-hydroxlyase present in the cell line D6P2T. In addition, FA2H is required for the formation of 2-hydroxy galactolipids.

As shown in Fig. 3-6, FA2H is highly up-regulated during differentiation. Prior to differentiation, Schwann cells are highly migratory and changes in the plasma membrane lipid composition may alter migratory processes. We performed cell migration assays to determine if down-regulation of FA2H expression would affect migration of D6P2T cells. Surprisingly, cells treated with siRNA:FA2H increased cell migration by 2.8 fold compared to control treated cells (Fig. 3-8, C). These data suggest that FA2H may be

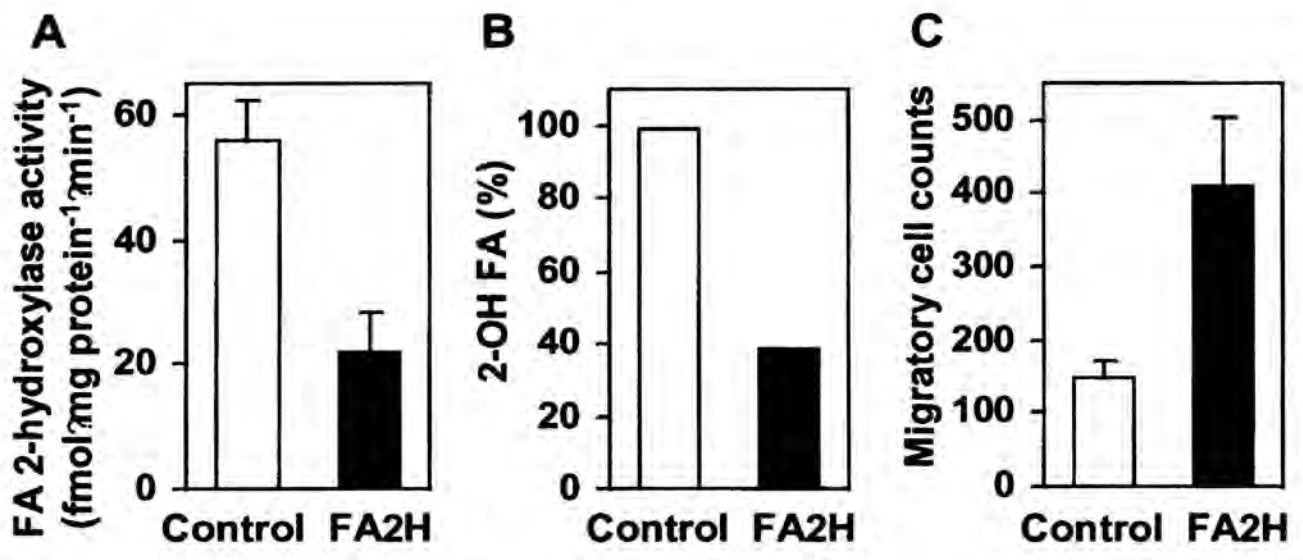

Fig. 3-8. FA2H is the major 2-hydroxylase in D6P2T cells and FA2H RNAi enhances cell migration. Cells were harvested 72 hr after transfection with control or FA2H siRNA. (A) Fatty acid 2-hydroxylase activities in whole cell lysates. The mean and range of two measurements are shown. (B) Free 2-hydroxy fatty acid contents were determined by GC/MS. The data include three major 2-hydroxy fatty acids (2-OH C16, 2-OH C18, and 2-OH C24). (C) Cells were allowed to migrate for 4 hr before staining. The mean and S.D. of cell counts in three different views are shown.

involved in regulating certain cell surface receptors involved in Schwann cell migration, or FA2H may serve as a negative regulator of cell cycle arrest and differentiation. Thus, we hypothesized that down-regulation of FA2H would alter the cellular phenotype toward a less differentiated state and these cells may be prone to remain undifferentiated.

Finally, to determine if FA2H alters cellular differentiation, D6P2T cells were treated with shRNA:FA2H or control and selected against puromycin for two weeks. Cells were treated for 24 hours with the 100 μM of the cAMP analogue dibutyryl cAMP (db-cAMP). Cells were labeled with calcein-AM and visualized by fluorescence microscopy. As shown in Fig. 3-9, control cells treated with db-cAMP exhibited retarded growth (59%), characteristic of senescence and early differentiation processes. Cells treated with shRNA:FA2H continued to proliferate with db-cAMP treatment, with only a 17% decline in growth. These data suggest that FA2H and 2-hydroxy lipids may serve as a regulator of differentiation in D6P2T cells, possible by removing cells from the cell cycle. This is the first demonstration that 2-hydroxy lipids may have roles in myelination other than structural components of the myelin sheath.

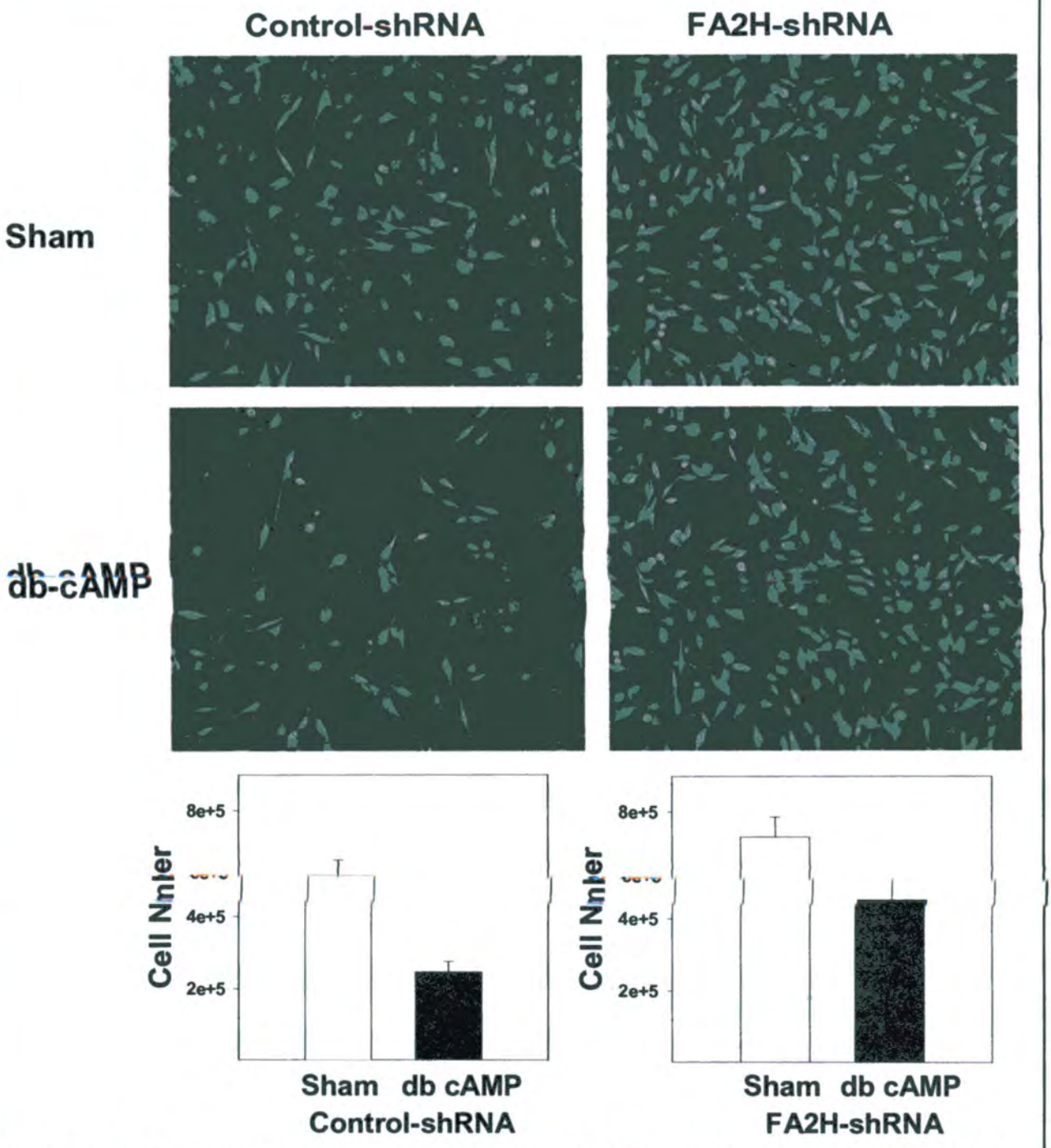

Fig. 3-9. FA2H RNAi delays cAMP induced cellular differentiation. D6P2T cells were transfected with scrambled shRNA (Control) or shRNA:FA2H (FA2H). After 48 hours, 1 μg/ml puromycin was added to culture media and resistant cells selected for two weeks. (Top) Cells were treated with dibutyryl cAMP (db-cAMP) or Sham (media) for 24 hours, and visualized by fluorescence microscopy following addition of calcein-AM (2 μg/ml). (Bottom) Cells were trypsinized, stained with bromophenol blue, and counted by hemocytometer. Data are mean + SD of triplicate samples.

Summary and Conclusions

Galactosylceramides (GalCer) and sulfatides account for one third of myelin lipids [2, 12]. Fifty percent of all fatty acids in these myelin glycosphingolipids are hydroxylated at the C_2 position on the *N*-acyl chain (2-hydroxy fatty acids) [3, 4, 13]. Although the high content of 2-hydroxy lipids in myelin have been known to exist for over fifty years, still very little is known about 2-hydroxy lipid biosynthesis, regulation, and functional role in myelination.

The brain fatty acid 2-hydroxylation activity was first demonstrated by the conversion of [1-^{14}C] tetracosanoic acid to 2-hydroxy tetracosanoic acid in sphingolipids when injected into rat brains [39]. Subsequently, an *in vitro* fatty acid 2-hydroxylase assay was developed using [1-^{14}C] tetracosanoic acid as a substrate, brain homogenate as an enzyme source, and assay products analyzed by thin-layer chromatography [40]. With this method, biochemical properties of this enzyme have been studied in rat and mouse brains [5, 11, 25, 26, 32, 41]. The rat brain fatty acid 2-hydroxylase requires molecular oxygen, Mg^{2+}, pyridine nucleotides (NADPH or NADH), and microsomal electron transport proteins [25, 26]. The rat brain fatty acid 2-hydroxylase was insensitive to carbon monoxide, indicating that it was not a P-450 enzyme but another type of mixed function oxygenase [41]. Although the brain enzyme was successfully characterized, the sensitivity of the previous assay method was limited, and no activities were detected in other tissues containing 2-hydroxy sphingolipids [41]. It was also unclear whether free

fatty acids were the substrate of the enzyme, because the assay products were detected only as a component of 2-hydroxy ceramides, and not as free 2-hydroxy fatty acids.

In Chapter 1 we demonstrate the development of novel and highly sensitive GC/MS-based assays for the quantification of fatty acid 2-hydroxylase activity and 2-hydroxy fatty acid content of biological samples. The activity assay utilizes a deuterated free fatty acid as substrate and quantitatively measures the production of deuterated free 2-hydroxy fatty acid. This allows for the precise determination of enzyme activity by using a substrate that is identifiable in an assay mixture containing high levels of endogenous fatty acids. The current activity assay is over 1000-fold more sensitive than previous methods, however the method is not without limitations. This assay system is not suitable for true enzymological studies or high throughput testing of various substrates and assay conditions. It is likely that purification of the enzyme and a simple high-throughput assay will be necessary to address these issues in the future. In its current form, the assay has allowed detailed study of the enzyme properties and measurement of fatty acid 2-hydroxylase activities in several tissues and cell lines. Further, this assay has provided *in vitro* evidence that FA2H can utilize free fatty acids as a substrate [49]. Development of this assay represents a significant contribution to the study of 2-hydroxy lipids.

In 1973, Hoshi and Kishimoto reported fatty acid 2-hydroxylase activities rat brain [4]. However, despite exhaustive efforts for two decades by Kishimoto and colleagues, the enzyme has not been purified and still very little is known regarding the biosynthetic pathway for 2-hydroxy lipids. We have reported the identification and characterization of the human gene (FA2H) encoding a fatty acid 2-hydroxylase that is highly expressed

in brain [1]. Human FA2H is a highly hydrophobic 42.8 kDa 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 non-heme di-iron cluster at the active site of the enzyme [30]. FA2H catalyzes 2-hydroxylation of free fatty acid *in vitro*, which was dependent on a reconstituted electron transport system [1]. Subsequently, Eckhardt et al. reported that the mouse FA2H gene was highly expressed in brain during myelination, and that FA2H mRNA colocalizes with *PLP* mRNA [42]. These findings provide strong evidence that the FA2H gene encodes the fatty acid 2-hydroxylase previously characterized by Kishimoto and colleagues.

In Chapter 2 we demonstrate the role of FA2H during developmental myelination of the central nervous system. FA2H is expressed primarily in oligodendrocytes and protein content increases during OPC differentiation. FA2H dependent 2-hydroxylase activity increased during the period of developmental myelination in parallel with increases in FA2H protein and gene expression. These data demonstrate that FA2H is likely controlled at the transcriptional level. Free 2-hydroxy fatty acids were measurable during myelination and quantitatively changed in parallel to FA2H 2-hydroxylase activity, providing *in vivo* evidence that free fatty acids are the likely substrate for FA2H. The lipid composition of brain GalCer and sulfatides were measured by GC/MS. 2-Hydroxy fatty acid species associated with galactolipids were very similar to the composition of 2-hydroxy free fatty acids, providing further evidence *in vivo* of a precursor-product relationship. In addition, we performed antibody blocking experiments to demonstrate

that FA2H is the major, if not only, fatty acid 2-hydroxylase in the mammalian central nervous system.

Myelin is produced by oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system. Biochemical studies have demonstrated that PNS myelin contains a high content of 2-hydroxy galactolipids similar to CNS [69]. However, there are differences in the proteins found in each system. P_0 is the major protein in the PNS, however this protein is absent in the central nervous system. Differences in the composition among 2-hydroxy chain lengths have been reported between the CNS and PNS [74]. Taken together, it seems likely that there may be differences between the two systems with regard to myelin formation, structure, and function.

In Chapter 3, we demonstrate that FA2H is highly up-regulated upon Schwann cell differentiation and during postnatal myelination of the sciatic nerve. Fatty acid 2-hydroxylase activity increased during myelination in parallel to the gene expression. The gene expression pattern was consistent with other known myelin associated genes (eg. CGT, P_0), suggesting that FA2H may play an integral role in PNS myelination. The composition of 2-hydroxy fatty acids associated with GalCer and sulfatides changed during myelination, with longer chain 2-hydroxy fatty acids accumulating. These data suggest that fatty acid elongases (ELOVL) may be up-regulated during myelination to provide preferred substrates for GalCer and sulfatide synthesis. We further demonstrate that FA2H is the major 2-hydroxylase in the rat Schwannoma cell line D6P2T and is required for the formation of 2-hydroxy galactolipids. Interestingly, we also discovered that down-regulation of FA2H increases D6P2T cell migration and delayed cAMP-induced differentiation. These data suggest that FA2H and 2-hydroxy lipids appear to

play a significant role in cell cycle arrest, thus enabling continued cell migration and proliferation.

This body of work is a comprehensive study of FA2H dependent fatty acid 2-hydroxylation in the mammalian nervous systems. However, the role of 2-hydroxy fatty acid containing lipids in myelin cannot be adequately addressed until a FA2H knock-out mouse is available. Future studies utilizing the FA2H knockout mouse will address the structural role of 2-hydroxy lipids in the formation of myelin sheets, the role of FA2H in the maintenance of myelin lipids, the role of 2-hydroxy lipids in nerve conduction, and the effect of FA2H knock-out on oligodendrocyte and Schwann cell migration and differentiation.

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