methods

A novel method for the measurement of in vitro fatty acid 2-hydroxylase activity by gas chromatography-mass spectrometry

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Abstract Fatty acid 2-hydroxylase (FA2H), encoded by the *FA2H* gene, is an enzyme responsible for the de novo synthesis of sphingolipids containing 2-hydroxy fatty acids. 2-Hydroxy sphingolipids are highly abundant in the brain, as major myelin galactolipids (galactosylceramide and sulfatide) contain a uniquely high proportion (~50%) of 2-hydroxy fatty acids. Other tissues, such as epidermis, epithelia of the digestive tract, and certain cancers, also contain 2-hydroxy sphingolipids. The physiological significance of the 2-hydroxylation on *N*-acyl chains of subsets of sphingolipids is poorly understood. To study the roles of FA2H and 2-hydroxy sphingolipids in various tissues, we developed a highly sensitive in vitro FA2H assay. FA2H-dependent fatty acid 2-hydroxylation requires an electron transfer system, which was reconstituted in vitro with an NADPH regeneration system and purified NADPH:cytochrome P-450 reductase. A substrate [3,3,5,5-D_4]tetracosanoic acid was solubilized in α-cyclodextrin solution, and the 2-hydroxylated product was quantified by gas chromatography-mass spectrometry after conversion to a trimethylsilyl ether derivative. When the microsomes of FA2H-transfected COS7 cells were incubated with the electron transfer system and deuterated tetracosanoic acid, deuterated 2-hydroxy tetracosanoic acid was formed in a time- and protein-dependent manner. With this method, FA2H activities were reproducibly measured in murine brains and tissue culture cell lines.— Alderson, N. L., M. D. Walla, and H. Hama. **A novel method for the measurement of in vitro fatty acid 2-hydroxylase activity by gas chromatography-mass spectrometry. *J. Lipid Res.* 2005.** 46: **1569–1575.**

Supplementary key words FA2H • fatty acid alpha-hydroxylase • 2-hydroxy fatty acid

Sphingolipids are a large class of lipids ubiquitously present in eukaryotic cell membranes. The structural diversity of sphingolipids stems from >300 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 the de novo synthesis of ceramide and is catalyzed by fatty acid 2-hydroxylase (FA2H; also known as fatty acid α-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 esters of galactosylceramide) contain a high proportion (~50%) of 2-hydroxy fatty acids (1–3). The roles for galactosylceramide and sulfatide in myelination and axo-glial organization have been demonstrated in studies of knockout mice that lack these lipids (4–6), and the significance of the 2-hydroxylation of these myelin lipids has long been speculated (7). 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 (8, 9). A number of studies also showed 2-hydroxy sphingolipids in liver and kidney, epithelia of the digestive tract, and other tissues (10–13). Despite the prevalence of 2-hydroxy sphingolipids, FA2H has not been studied in extraneural tissues, and the physiological roles for extraneural 2-hydroxy sphingolipids are poorly understood.

Brain fatty acid 2-hydroxylation activity was first demonstrated by the conversion of [1-^{14}C]tetracosanoic acid to 2-hydroxy tetracosanoic acid when injected into rat brains (14). Subsequently, an in vitro FA2H assay was developed using [1-^{14}C]tetracosanoic acid as a substrate, brain homog-

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Abbreviations: FA2H, fatty acid 2-hydroxylase; TMS, trimethylsilyl.

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enate as an enzyme source, and assay products analyzed by thin-layer chromatography (15). With this method, biochemical properties of this enzyme have been studied in rat and mouse brains (7, 15–19). The rat brain FA2H requires molecular oxygen, Mg^{2+}, pyridine nucleotides (NADPH or NADH), and microsomal electron transport proteins (18, 19). The rat brain FA2H was insensitive to carbon monoxide, indicating that it was not a P-450 enzyme but another type of mixed function oxygenase (15). 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 (15). 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 FA2H that is highly expressed in the human brain (20). 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 (21). In the course of the study, we developed an improved in vitro FA2H 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 (20). Here, we report the details of the assay and an application of the method to a benchtop quadrupole mass spectrometer for convenient measurement of FA2H 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 [3,3,5,5-D_4]tetracosanoic acid (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 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 medium containing 50% Dulbecco's modified Eagle's medium (as above) and 50% RPMI 1640 supplemented with L-glutamine. Where indicated, COS7 cells were transfected with pcDNA3-hFA2H (20) using FuGene 6 transfection reagent (Roche Applied Science, Indianapolis, IN).

Preparation of microsomal fractions from tissue culture cells

Approximately 1×10^6 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 resuspended in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 1% glycerol, and 1 mM PMSF) and lysed by freeze-thawing, followed by sonication (Fisher Sonic Dismembrator model 500; amplitude, 29%; 5 s on, 0.1 s off for 12 cycles). Cell lysates were centrifuged at 1,000 *g* for 5 min to remove unbroken cells and large debris. The supernatants were centrifuged at 100,000 *g* for 2 h 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 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 the Pierce BCA Protein Assay Kit (Rockford, IL).

Preparation of postnuclear fractions from postnatal mouse brain

Whole brains were immediately excised from adult mice after 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. All samples were immediately frozen at −70°C until analyses. After thawing, homogenized tissue was sonicated (Fisher Sonic Dismembrator model 500; amplitude, 29%; 5 s on, 0.1 s off for 12 cycles), and cell lysates were centrifuged at 1,000 *g* for 5 min to remove unbroken cells and large debris. The supernatant was removed and placed on ice, and an aliquot was taken for immediate protein determination.

Fatty acid 2-hydroxylation activity measurement

In a 50 ml polypropylene tube, microsomal fractions (25–100 μg of protein) or crude brain homogenate (50 μg of 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 of human NADPH:cytochrome P-450 reductase, and a substrate, 1 μg (2.7 nmol) 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. After 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 pmol 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 of 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 × 100 mm screw-cap test tubes (Fisher Scientific; catalog number 14-959-25A). Fatty acids were derivatized and quantified as described below.

Measurement of FA2H assay product by GC-MS

The methyl esters of fatty acids were prepared as described previously (20). Briefly, anhydrous methanolic HCl was prepared by dropwise addition of acetyl chloride to methanol, and 1 ml was 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-OH groups were further derivatized to trimethylsilyl (TMS) ethers by the addition of 125 μl of Tri-Sil reagent and incubated at room temperature for 30 min tightly capped.

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Derivatized samples (1–2 μl) were analyzed by one of the two GC-MS systems. For activities in transfected COS7 cells (see Figs. 2–4), the samples were directly applied to a Hewlett-Packard 5890 gas chromatograph with the injector in splitless mode. The 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) (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 was increased to 300°C at 10°C/min. Mass spectra data were obtained on a VG-70S magnetic sector mass spectrometer after electron impact 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 benchtop GC-MS system. 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 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), 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 was increased to 300°C at 10°C/min. Mass spectra data were obtained on a Shimadzu GCMS-QP2010 mass spectrometer after electron impact ionization. Peaks of the target analytes and internal standard were processed using the GC-MS Lab Solutions software (Shimadzu Scientific).

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 C23 fatty acid methyl ester. The ions monitored for 2-hydroxy [3,3,5,5-D_4]tetracosanoic acid had masses of 415 and 459, corresponding to m-15 and m-59, respectively. The activities were calculated as picomoles of 2-hydroxy [3,3,5,5-D_4]tetracosanoic acid per milligram of protein per minute.

RESULTS AND DISCUSSION

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

FA2H activity was first demonstrated in vitro using a rat brain homogenate (15). The rat brain enzyme required molecular oxygen, Mg^{2+}, and pyridine nucleotides, which suggested that the enzyme was a mixed function oxidase (15). Cytochrome P-450 enzymes were not implicated in this reaction, because the activity was not inhibited by carbon monoxide (15). In modified assays with microsomal fractions, rat brain FA2H was shown to require a microsomal electron transfer system (18, 19). All of these characteristics are consistent with the predicted properties of the human *FA2H* gene product (20). The nucleotide sequence of the *FA2H* gene indicates that FA2H protein (372 amino acids) is a member of a membrane-bound desaturase/hydroxylase family with the conserved nonheme 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 (22). 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. Because NADPH is a more effective electron donor than NADH for the rat brain enzyme (18), 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 → cytochrome b_5 domain of FA2H → catalytic site of FA2H (**Fig. 1**). It is of interest that the rat brain enzyme was activated by a heat-stable, water-soluble cofactor (15), which was later shown to be glucose 6-phosphate (23). 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 FA2H 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 by-products by multiple chromatographic processes before quantification (15, 16). To simplify the assay, we used the deuterated fatty acid [3,3,5,5-

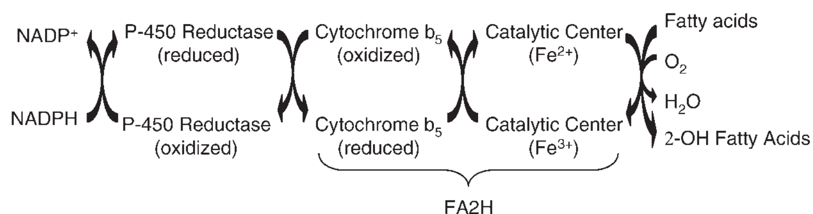

Fig. 1. An electron transport system reconstituted in the in vitro fatty acid 2-hydroxylase (FA2H) assay. The arrows indicate the flow of electrons. The intramolecular cytochrome b_5 domain is necessary for the activity of FA2H (20), which is believed to serve as a component of an electron transfer system 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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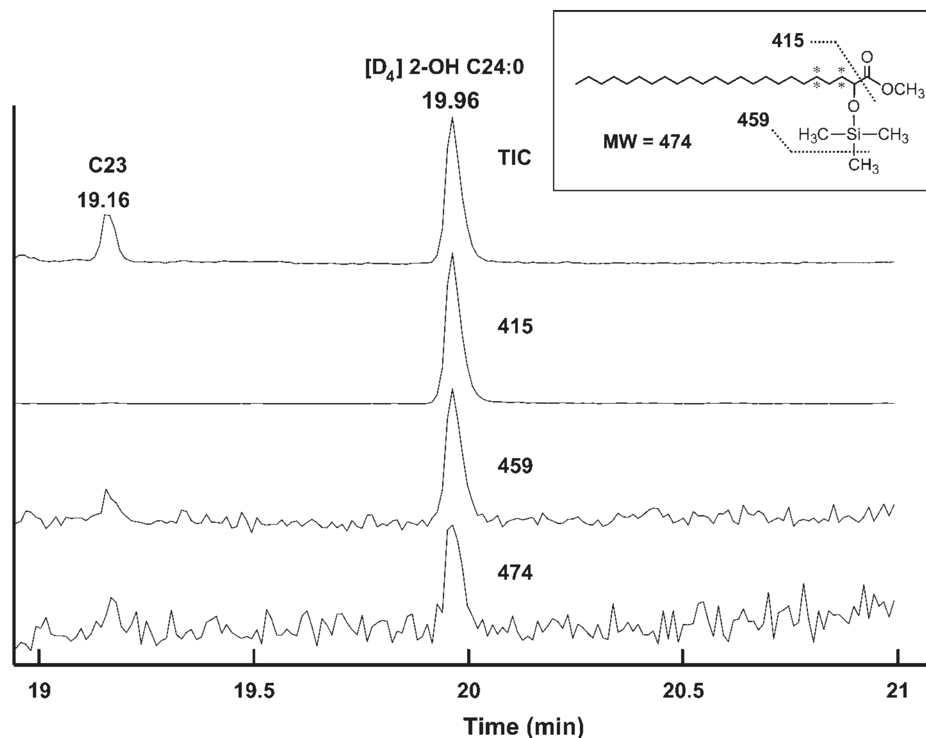

Fig. 2. Chromatograms of trimethylsilyl (TMS) ether of 2-hydroxy tetracosanoic acid methyl ester formed in an in vitro reaction. Total ion chromatogram (TIC) and selected ion chromatograms corresponding to MW = 474, m-15, and m-59 are shown. The inset shows the fragmentation of the TMS ether of [3,3,5,5-D_4]2-hydroxy tetracosanoic acid methyl ester. Asterisks indicate the positions of deuterium.

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 (15). A difficulty associated with this substrate was the delivery of this highly hydrophobic molecule to the enzyme. The rat brain FA2H was sensitive to detergents, and the substrate was coated on the surface of Celite (diatomaceous earth) in the previous assay system (15). 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 enzyme reactions (24). Microsomes of FA2H-transfected COS7 cells were a convenient source of the enzyme to establish the assay system.

One microgram of deuterated tetracosanoic acid (final concentration 1.8 μM) was incubated with the microsomes, an NADPH regeneration system, and NADPH:cytochrome P-450 reductase, and fatty acids were extracted after adding 1 pmol of an internal standard, tricosanoic

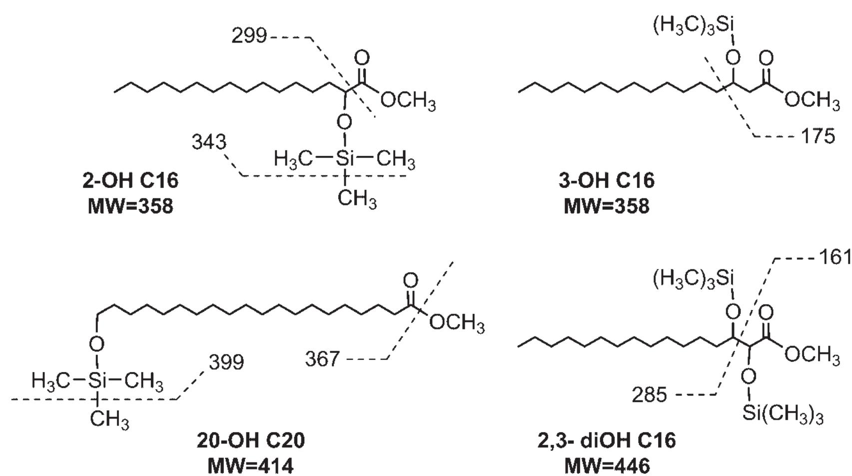

Fig. 3. Fragmentation patterns of hydroxy fatty acid derivatives. The molecular weights (MW) of unique fragments used for GC-MS quantification are shown.

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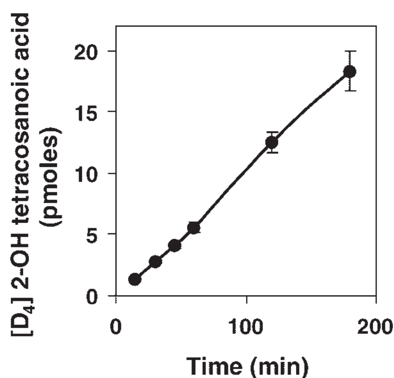

Fig. 4. Time course of fatty acid 2-hydroxylation. The microsomal fraction was prepared from COS7 cells transfected with pcDNA3-hFA2H. The assay mixtures contained 50 μg of microsomal proteins in the presence of an NADPH regeneration system and human NADPH:P-450 reductase. Data are shown as means ± SD of duplicate measurements.

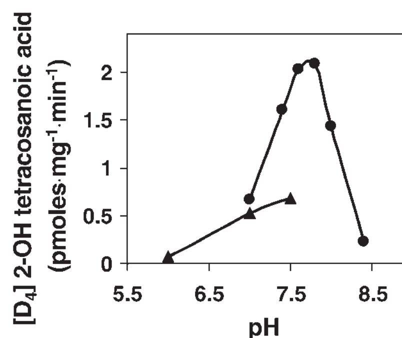

Fig. 6. pH profile of FA2H activity. Microsomal fractions were prepared from COS7 cells transfected with pcDNA3-hFA2H. The reaction mixtures contained 50 μg of microsomal proteins in the presence of an NADPH regeneration system, human NADPH:P-450 reductase, and 2.7 mM potassium phosphate (pH 6.0, 7.0, or 7.5) or 2.7 mM Tris-HCl (pH 7.0, 7.4, 7.6, 7.8, 8.0, or 8.4). Reaction mixtures were incubated for 120 min. The pH of the reaction mixture remained unchanged at the end of incubation.

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 coeluted with TMS ether of 2-hydroxy tetracosanoic acid methyl ester. The molecular ion of this compound had a mass of 474, which was 4 mass units larger than the TMS derivative of 2-hydroxy tetracosanoic acid. Fragmentation of this compound generated two distinct ions with masses of 459 and 415, which are also 4 mass units larger than the corresponding ions generated from the nondeuterated 2-hydroxy tetracosanoic acid derivative (**Fig. 2**). Other hydroxy fatty acids were distinguishable from 2-hydroxy fatty acids based on unique fragmentation patterns. As shown in **Fig. 3**, fragmentation of 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. Based on the retention time and the unique fragmentation pattern, we concluded that the lipid species shown in Fig. 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 subfemtomole level.

With this detection method, the FA2H activity assay was established using microsomes of COS7 cells transiently transfected with pcDNA3-hFA2H. These cells highly express the human FA2H and served as a convenient enzyme source to determine the assay condition. In the presence of 50 μg of microsomal proteins, the formation of deuterated 2-hydroxy tetracosanoic acid was linear up to 3 h at a substrate conversion rate of 0.25%/h (**Fig. 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 (15), and the direct substrate of the enzyme

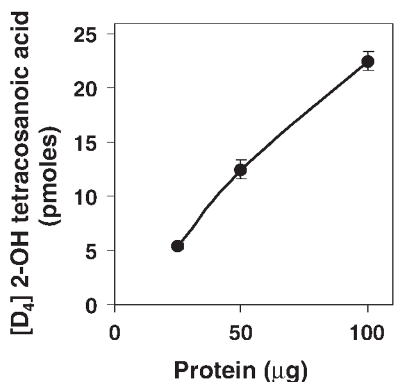

Fig. 5. Fatty acid 2-hydroxylation as a function of protein concentration. Microsomal fractions were prepared from COS7 cells transfected with pcDNA3-hFA2H. The assay mixtures contained the indicated amounts of microsomal proteins in the presence of an NADPH regeneration system and human NADPH:P-450 reductase. Reaction mixtures were incubated for 120 min at 37°C. Data are shown as means ± SD of duplicate measurements.

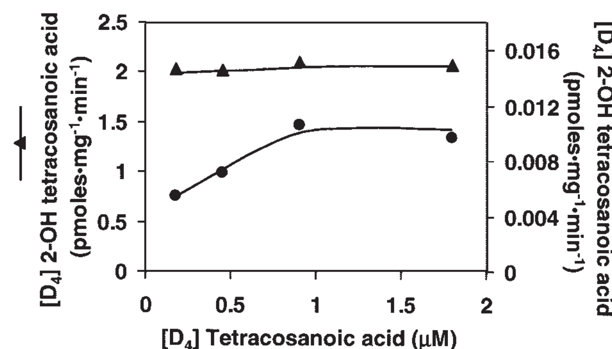

Fig. 7. Effect of substrate concentration on FA2H activities. The reaction mixtures contained 50 μg of microsomal proteins from FA2H-transfected COS7 cells (triangles) or murine brain homogenates (circles) and were incubated for 120 or 180 min, respectively. The substrate conversion was 4.5% (at 0.18 μM with FA2H-transfected COS7 microsomes) or less.

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remained unclear (7, 15, 19). Although we have not tested ceramide 2-hydroxylation by FA2H, the time course shown in Fig. 4 provides strong evidence that FA2H uses 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 used by ceramide synthases.

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

The pH profile shown in **Fig. 6** indicated that FA2H activity of FA2H-transfected COS7 microsomes was highest at pH 7.6–7.8, which is consistent with the optimum pH for the rat brain enzyme (15). It should be noted that this assay is a multicomponent system involving three enzymes (FA2H, NADPH:cytochrome P-450 reductase, and glucose 6-phosphate dehydrogenase). The linear correlation between protein concentration and activities (Fig. 5) shows that FA2H is the limiting component at pH 7.6. It is possible that the rate of electron transport could have become suboptimal at other pH conditions.

With crude biological samples, endogenous lipids could interfere with the FA2H 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 FA2H. When 0.18–1.8 μM deuterated tetracosanoic acid was added in the assay with 50 μg of microsomal proteins of FA2H-transfected COS7 cells, the 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. 7**). This result also indicates that the apparent K_m for tetracosanoic acid is <0.18 μM, which is significantly lower than the reported K_m for the rat brain enzyme (4.2 μM) (15). These values are not directly comparable because different methods of substrate delivery were used (Celite complexes vs. cyclodextrin inclusion complexes). Furthermore, the reaction product in the previous assay was detected as 2-hydroxy ceramide, which was generated by multiple enzyme reactions.

When crude murine brain homogenates (50 μg of protein) were used as enzyme sources, 2-hydroxylation was not saturated at substrate concentrations of <0.9 μM, presumably because of a relatively high concentration of lipids in the brain (Fig. 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.

TABLE 1. FA2H activities in tissue culture cells and mouse brain homogenates

Sample	FA2H Activity
	pmol/mg/min
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

FA2H, fatty acid 2-hydroxylase. For the measurement of activities in mouse brains, whole brain homogenates were used as enzyme sources. Assay mixtures were incubated with 50 μg of 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 of proteins for 120 min. Data are shown as means ± SD of triplicate measurements.

FA2H activity measurement by a benchtop GC-MS system

The assay described above was initially developed using a high-resolution magnetic sector mass spectrometer. Although this instrument provides unsurpassed sensitivity, the highly specialized instrument is not commonly used in research laboratories and is not practical for routine analyses. Therefore, we applied the same method to a benchtop system with a quadrupole mass spectrometer. Although the limit of detection on the benchtop instrument was ~100-fold higher (at the femtomole level) compared with the magnetic sector mass spectrometer, the activity in FA2H-transfected COS7 cells was readily detectable with this instrument (data not shown). To test whether this instrument was useful for various biological samples, we first measured FA2H activities in mouse brains. When whole brain homogenates (50 μg of protein) were incubated with the assay mixture as described above, deuterated 2-hydroxy tetracosanoic acid was reproducibly formed (**Table 1**). Subsequently, activities were measured in commonly used cell lines: COS7, HeLa, and A549. To our knowledge, FA2H activities have never been shown in tissue culture cells. As shown in Table 1, all three cell lines had relatively high FA2H activities. Consistent with this result, we observed free 2-hydroxy fatty acids in these cells (data not shown). It is likely that FA2H and 2-hydroxy sphingolipids have been overlooked in many tissues and cell lines because of their low abundance. The new assay will allow us to reevaluate the presence of FA2H and 2-hydroxy sphingolipids in various biological samples.

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