Fatty Acid 2-Hydroxylation in Mammalian Sphingolipid Biology

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Abstract

2-Hydroxy fatty acids (hFA) are important components of a subset of mammalian sphingolipids. The presence of hFA in sphingolipids is best described in the nervous system, epidermis, and kidney. However, the literature also indicates that various hFA-sphingolipids are present in additional tissues and cell types, as well as in tumors. Biosynthesis of hFA-sphingolipids requires fatty acid 2-hydroyxlase, and degradation of hFA-sphingolipids depends, at least in part, on lysosomal acid ceramidase and the peroxisomal fatty acid α-oxidation pathway. Mutations in the fatty acid 2-hydroxylase gene, *FA2H*, have been associated with leukodystrophy and spastic paraparesis in humans, underscoring the importance of hFA-sphingolipids in the nervous system. In the epidermis, hFA-ceramides are essential for the permeability barrier function. Physiological function of hFA-sphingolipids in other organs remains largely unknown. Recent evidence indicates that hFA-sphingolipids have specific roles in cell signaling.

Keywords

Fatty acid 2-hydroxylase; fatty acid alpha-hydroxylase; FA2H; hydroxy fatty acid; hydroxy sphingolipids

1. INTRODUCTION

Sphingolipids are ubiquitous eukaryotic membrane lipids with highly diverse structures with hundreds of distinct headgroups attached to various ceramides. In mammalian sphingolipids, both the sphingoid base and the *N*-acyl chain of ceramide can vary with regard to alkyl chain length, hydroxylation, and desaturation [1]. The topic of this review is a subset of mammalian sphingolipids, hFA-sphingolipids, with 2-hydroxy fatty acid as the *N*-acyl chain of ceramide. Fatty acid 2-hydroxylase converts fatty acid to hFA, which is then incorporated into hFA-ceramide and complex hFA-sphingolipids (Fig. 1). However there is no evidence in the literature supporting the incorporation of hFA into glycerolipids in mammals.

The presence of hFA in mammalian sphingolipids has been known since the early 1900's (for review of early works, see reference [2]). Early investigations described high concentrations of hFA in brain galactosylceramide (GalCer), a major lipid component of the myelin sheath. Decades later, Kishimoto and Radin reported finding hFA-sphingolipids outside of the nervous

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system [3]. Subsequently, in a number of studies, researchers have shown that hFA-sphingolipids are present in various mammalian tissues and tumors. Despite their prevalence, physiological functions of hFA-sphingolipids remain largely unknown.

In parallel with tissue sphingolipid analyses, in vitro biochemical studies of rat brain fatty acid 2-hydroxylase were initiated by Kishimoto and colleagues [4], and their subsequent studies established key characteristics of this enzyme [5–8]. Molecular genetic approaches led to the identification of the *FA2H* gene, which encodes a fatty acid 2-hydroxylase with the characteristics of the rat brain enzyme [9,10].

Recent work by Edvardson et al. describes a leukodystrophy and spastic paraparesis in humans caused by mutations in the *FA2H* gene [11]. This discovery underscores the importance of hFA-sphingolipids in the nervous system. Also revealed in the study was additional enzyme (s) that can produce hFA outside of the nervous system in the absence of FA2H [11]. Thus, the story of hFA and hFA-sphingolipids is more complex than previously predicted.

Summarized below are the current knowledge about the biosynthesis and turnover of hFA-sphingolipids, tissue distribution of hFA-sphingolipids and their postulated functions, and brief description of recent developments in cell signaling, mediated specifically by hFA-sphingolipids.

2. BIOSYNTHESIS AND TURNOVER OF HFA-SPHINGOLIPIDS

2.1 Fatty acid 2-hydroxylation by FA2H

There are at least two types of fatty acid 2-hydroxylase in mammals; the NAD(P)H-dependent monooxygenase encoded by the *FA2H* gene, and the α-ketoglutarate-dependent monooxygenase encoded by the *PHYH* gene. FA2H is a 43-kDa (372-aa) integral membrane protein of the ER [9,10]. Mutations in the *FA2H* gene are associated with leukodystrophy and spastic paraparesis in humans [11], indicating that its primary function is to synthesize myelin hFA-GalCer (see §3.1). FA2H has two functional domains; an N-terminal cytochrome b_5 domain and the C-terminal catalytic domain consisting of four potential transmembrane domains (Fig. 2). The putative catalytic site contains the histidine motif conserved among membrane-bound monooxygenases [consensus: $HX_{(3–4)}H\ X_{(7–41)}\ HX_{(2–3)}HH\ X_{(61–189)}\ (H/Q)X_{(2–3)}HH$] [12]. The 8 histidine residues are thought to coordinate the non-heme di-iron cluster at the active site of the enzyme [12]. Studies by Kishimoto and coworkers in the 70's and 80's demonstrated that rat brain fatty acid 2-hydroxylase was a microsomal enzyme [5] that required molecular oxygen, Mg^{2+}, pyridine nucleotides (NADPH is preferred to NADH), heat-stable cofactors [4], and microsomal electron transfer proteins [7]. These characteristics indicate that the rat brain enzyme is a mixed-function monooxygenase, which is consistent with the features of FA2H predicted from the primary structure. Indeed, the in vitro fatty acid 2-hydroxylase activity of FA2H is dependent on cytochrome P450 reductase, NADPH, and an NADPH regeneration system [9]. The N-terminal cytochrome b_5 domain is also necessary for maximal activity, presumably providing electrons to the catalytic di-iron [9]. Thus, all available evidence suggests that FA2H is the same enzyme previously characterized in rat brain. FA2H converts free fatty acid to free 2-hydroxy fatty acid in vitro [9,13], which is also likely the case in vivo [14]. The possibility of direct hydroxylation of *N*-acyl chains of ceramide or other sphingolipids by FA2H has not been examined.

2.2 Acyl-CoA 2-hydroxylation by PHYH

The *PHYH* gene product, phytanoyl-CoA 2-hydroxylase, is an α-ketoglutarate-dependent acyl-CoA 2-hydroxylase in the peroxisomal α-oxidation pathway (for review, see [15]). Its primary function is degradation of branched-chain fatty acids. Deficiencies of this enzyme cause Refsum's disease characterized by accumulation of phytanic acid in various organs. In addition

to branched-chain acyl-CoA, PHYH can catalyze 2-hydroxylation of straight-chain acyl-CoA in vitro [16,17]. Whether PHYH is responsible for the FA2H-independent fatty acid 2-hydroxylation in dermal fibroblasts reported by Edvardson et al. [11] is an open question. If the FA2H-independent hFA formation occurs in peroxisomes, hFA would need to be transported from peroxisomes to the ER where hFA-ceramide is synthesized. Another possibility might be "alternative trafficking" of PHYH to the ER so that PHYH could contribute to the de novo hFA-ceramide synthesis within the ER.

2.3 Synthesis of hFA-ceramide and complex hFA-sphingolipids

Current evidence supports that hFA-sphingolipids are synthesized by the same set of enzymes as non-hydroxy sphingolipids, except for fatty acid 2-hydroxylase. After 2 hydroxylation, hFA is presumably activated to CoA ester by acyl-CoA synthetases (Fig. 1). In the next step, 2-hydroxy acyl group is transferred to the primary amine of dihydrosphingosine by one of the 6 isoforms of dihydroceramide synthase (CerS), which are all capable of synthesizing hFA-dihydroceramide [18]. Subsequent oxidation by one of the two dihydroceramide desaturases converts hFA-dihydroceramide to hFA-ceramide, which is the common precursor of all complex hFA-sphingolipids. The levels of hFA-ceramide range from 1–3% of total cellular ceramide in many established cell lines (our unpublished data) to over 50% in skin keratinocytes [19] and intestinal epithelial cells [20–22].

Ceramide is converted to sphingomyelin (SM), glucosylceramide (GlcCer), and GalCer, by the addition of phosphocholine, glucose, or galactose, respectively, at the C-1 hydroxyl group of ceramide. Various monosaccharides can be sequentially added to GlcCer to form complex glycosphingolipids, whereas SM is an end product of biosynthesis. GalCer can be converted to two known glycosphingolipids, one of which is another major myelin component sulfatide. Most of the enzymes involved in the synthesis of these sphingolipids appear to utilize hFA-ceramide because various hFA-sphingolipids are present in many different tissues and cell types (see §3). The best-characterized example is UDP-galactose:ceramide galactosyltransferase (CGT), encoded by the *UGT8* gene. CGT has strong preference for hFA-ceramide over non-hydroxy ceramide [23]. Both FA2H and CGT are highly upregulated in myelinating oligodendrocytes and Schwann cells, which enables these cells to produces vast quantities of hFA-GalCer in myelin.

2.4 Hydrolysis of hFA-ceramide by ceramidase and saposin D

Complex sphingolipids mostly reside in the extracellular leaflet of the plasma membrane. Once they are internalized by endocytosis and have reached the lysosomes, the cell surface sphingolipids are accessible to lysosomal sphingolipid hydrolases. Several lysosomal enzymes can remove specific sugar headgroups of complex sphingolipids, as well as acid sphingomyelinase and acid ceramidase. Degradation of sphingolipids has been extensively studied in association with the lysosomal storage disorders, which are caused by deficiencies in one of the lysosomal degradation enzymes (for review, see [24]).

Removal of the headgroup from hFA-sphingolipids generates hFA-ceramide in the lysosome, which can be hydrolyzed by lysosomal acid ceramidase into sphingosine and hFA (Fig. 3). Deficiencies in this enzyme cause the lysosomal storage disorder Farber's disease. Both ceramide and hFA-ceramide accumulate in the kidney, cerebellum, lung, and stomach of Farber's disease patients [25,26], suggesting that hFA-ceramide/sphingolipids are continuously synthesized and turned over in normal cells in these organs. Interestingly, mouse mutants with defective saposin D accumulate hFA-ceramide in kidney and cerebellum, resulting in renal tubular degeneration and a selective loss of cerebellar Purkinje cells [27]. Saposin D is one of five known sphingolipid activator proteins that bind distinct sphingolipids and "present" the sphingolipid substrates to lysosomal hydrolases [28]. Based on the findings

in human acid ceramidase deficiency and saposin D knockout mice, it appears that saposin D and acid ceramidase function together to keep hFA-ceramide from accumulating. The reason for the selective susceptibility of certain cell types to accumulated hFA-ceramide could be a "traffic jam" effect that interferes with lipid metabolism, or more specific cytotoxicity of hFA-ceramide. Ceramide can induce apoptosis in many cell types, and there is some evidence for enhanced apoptosis-inducing activity of hFA-ceramide compared to non-hydroxy ceramide [29]. This effect is likely cell type-specific, as hFA-ceramide had significantly lower activity than non-hydroxy ceramide in inducing DNA fragmentation in human promonocytic U937 cells [30].

2.5 Peroxisomal α-oxidation pathway and odd-chain fatty acids

Hydrolysis of hFA-ceramide by ceramidases yields hFA and sphingosine (Fig. 3). Metabolism of sphingosine has been extensively studied and will not be discussed here. The fate of hFA is not fully understood, and evidence for a salvage pathway to recycle hFA for the biosynthesis of hFA-ceramide is indirect. The only known pathway for degradation of hFA is the peroxisomal α-oxidation [31]. As mentioned in §2.2, this pathway has been studied in association with degradation of branched-chain fatty acids. The study by Foulon et al. demonstrates that the enzyme that catalyzes the one-carbon cleavage, 2-hydroxyacyl-CoA lyase, can utilize straight-chain fatty acyl CoA. When an even-chain hFA is oxidized in this process, an odd-chain fatty acid is produced, which could be further degraded by β-oxidation.

Odd-chain fatty acids can be synthesized de novo by priming of fatty acid synthesis with propionyl-CoA instead of acetyl-CoA, which occurs at a negligible rate in most cells. Interestingly, early studies showed a substantial amount of odd-chain fatty acids in rat brain GalCer (both hydroxy and non-hydroxy) [32,33], and their levels increased with age [32,33]. The presence of these odd-chain fatty acids cannot be fully explained by priming with propionyl-CoA. In fact, there is evidence for two pathways for generating odd-chain fatty acids in the brain; one is by the fatty acid synthase reaction primed with propionyl-CoA [34], and the other is shortening of even-chain fatty acid by one carbon via α-oxidation [35]. The study by Foulon et al. provides additional evidence that the peroxisomal α-oxidation pathway is an integral part of the metabolism of hFA in the brain (Fig. 3). Once activated to CoA esters, the hFA entering the peroxisomes would be ready for the cleavage by 2-hydroxyacyl-CoA lyase. A portion of the odd-chain fatty acids produced by α-oxidation may be degraded by β-oxidation, and the remaining may enter the synthesis pathway in the ER, resulting in GalCer with odd-chain fatty acids. Some of the odd-chain fatty acids can be 2-hydroxylated by FA2H in the ER, giving rise to odd-chain hFA-GalCer. This seems an inefficient way of recycling fatty acids, compared to direct recycling of hFA in a salvage pathway. This hypothetical pathway may serve the cells to maintain an optimal hydroxy/non-hydroxy fatty acid ratio, which can be achieved by modulating the balance between fatty acid 2-hydroxylation and α-oxidation of hFA.

3. TISSUE DISTRIBUTION OF HFA-SPHINGOLIPIDS AND THEIR BIOLOGICAL FUNCTIONS

3.1 hFA-sphingolipids in the nervous system

In the mammalian nervous system, nerve conduction is greatly facilitated by myelin, a lipid-rich membrane that wraps around the axon. The myelin sheath is a specialized structure with distinct lipid and protein constituents. Galactosylceramide (GalCer) and sulfatide make up approximately 30% of total myelin lipids, and more than half of these galactolipids contains hFA as their *N*-acyl chains [36]. No other mammalian tissues contain such high concentrations of hFA. The specific function of hFA-galactolipids in myelin is not fully understood, but there has been significant advance in our knowledge in the past few years.

The necessity of hFA-sphingolipids for the normal function of the nervous system became evident when mutations in the *FA2H* gene were discovered in patients with hereditary leukodystrophy with spastic paraparesis [11]. This study was conducted with 9 patients in three families who had normal early development but presented with gait disturbance due to spasticity in the lower limbs at 4–6 years old. In 2 patients from one family the disease was restricted to the lower limbs with no cognitive or speech impairment. In contrast, the disease rapidly progressed in 7 patients from the other two families. These 7 patients required walking aids at 7 years old and presented with spasticity extended to the upper limbs; dystonia in the trunk, limbs, and face; upper-motor neuron deficits; decline in cognitive abilities; and cerebellar dysfunction. Brain MRI indicated progressive white matter degeneration.

Homozygosity mapping and subsequent sequencing of candidate genes led to identification of two mutations in the *FA2H* gene. The 7 patients with the severe form of the disease carried a homozygous intronic point mutation that resulted in aberrant *FA2H* mRNA lacking exon 5 and exon 6. These exons encode 3 of the 4 putative transmembrane domains of FA2H that encompass the 8 conserved histidine residues (see Fig. 2). Targeted deletion of these exons in mice resulted in loss of hFA-galactolipids in both the central and peripheral nervous systems, providing additional evidence that the loss of hFA-galactolipids is the cause of this disorder (our unpublished data).

The two patients with the milder form of the disease carried a homozygous point mutation resulting in replacement of Asp 35 to Tyr in the N-terminal cytochrome b_5 domain of FA2H. The Asp residue is conserved in all known cytochrome b_5, including cytochrome b_5 domains of various enzymes, suggesting an essential function of this Asp in electron transport. The signs and symptoms of the milder form of the disease fall under the category of hereditary spastic paraplegia. Interestingly, one of the loci associated with hereditary spastic paraplegia, *SPG35*, has been mapped to chromosome region 16q21-q23 [37], the same region as the *FA2H* locus. It is possible that *SPG35* is an allelic form of *FA2H*.

Interestingly, fibroblasts isolated from patients with FA2H mutations showed fatty acid 2-hydroxylase activity in vitro, indicating that there is a second enzyme that is not present in the nervous system [11]. The identity of the hypothetical second enzyme is currently unknown.

The clinical findings of human FA2H deficiency indicate that hFA-sphingolipids are dispensable for the onset of myelination, but myelin is highly unstable in the absence of hFA-galactolipids and begins to disintegrate even before myelination is complete. Interestingly, targeted inactivation of the mouse *Fa2h* gene presented a different picture [38]. Despite the complete loss of hFA-sphingolipids, cellular, histological, and clinical phenotypes of *Fa2h* null mice are reportedly indistinguishable from the wild type mice for the most of their life. However, aged *Fa2h*-null mice had scattered axonal degeneration and loss of myelin in both the central and peripheral nervous systems. These observations indicate that hFA-sphingolipids are entirely dispensable for developmental myelination in mice, and are needed only for long-term stability of axons and myelin. Possibly, mice may have a mechanism that compensates for the deficits caused by the loss of hFA-sphingolipids. Such a mechanism could provide insight into the development of potential therapeutics to prevent and treat human FA2H deficiency.

3.2 hFA-Sphingolipids in the skin

The outermost layer of mammalian epidermis is the stratum corneum, which is made of flattened, enucleated keratinocytes and a unique extracellular lipid matrix produced by differentiating keratinocytes. The stratum corneum provides the permeability barrier against water and various environmental agents, such as chemicals and microorganisms. About half of the lipids in the stratum corneum are mixtures of ceramides, 40% of which contain hFA

[39]. These ceramides, together with cholesterol and free fatty acids (25% and 10% of the stratum corneum lipids, respectively) are essential for the permeability barrier function of the skin [40,41].

A study with cultured human skin keratinocytes demonstrated that FA2H provides the precursor hFA for the synthesis of the unique epidermal hFA-ceramides [19]. Interestingly, FA2H-silencing in cultured human keratinocytes greatly disturbed formation of the unique extracellular lipid matrix. During normal differentiation, these cells produce characteristic "lamellar bodies" filled with pieces of lipid lamellae, which are secreted outside the cell and assembled into extracellular lipid layers. With FA2H-silencing, formation and secretion of lamellar bodies were markedly diminished, and extracellular lipid layers were not formed. Thus, hFA-sphingolipids are not only the cargo in these lamellar bodies, but also play an essential role in controlling formation and transport of lamellar bodies. It should be noted that neither human FA2H deficiency, nor targeted inactivation of mouse *Fa2h* gene, causes obvious skin permeability barrier dysfunction. This is likely due to a second fatty acid 2-hydroxylase that allows the cells to proceed with differentiation and to form functional stratum corneum in the absence of FA2H. The second enzyme may be the same as the one observed in fibroblasts from patients with FA2H deficiency.

3.3 hFA-sphingolipids in other tissues

Kishimoto and Radin first reported the presence of sphingolipid-associated hFA in several extraneural tissues (spleen, kidney, lung, skin, plasma) in 1963 [3]. Subsequently, a number of reports described various hFA-sphingolipids in many tissues and organs. The most extensively documented example outside of the nervous system and epidermis is hFA-GalCer in kidney and other organs. First described by Makita in 1964, GalCer in human kidney is primarily hFA-GalCer [42]. The presence of hFA-GalCer in several organs was also shown in the twitcher mouse, a mutant mouse line that possesses a mutation in the gene encoding galactosylceramidase (*Galc*) and cannot hydrolyze the head group of GalCer. Consequently, GalCer (predominantly hFA form) accumulates in the kidney, liver, and lung of the twitcher mice [43]. It became evident that in many instances, the *N*-acyl chain of GalCer is predominantly hFA, which gave rise to the misconception that hFA is only associated with GalCer and not with other sphingolipids. Apparently, hFA is readily incorporated into GalCer because of the subcellular localization and substrate preference of CGT. Among the biosynthetic enzymes that transfer a head group to ceramide, CGT is the only one localized in the ER [44]. Consequently, CGT comes in contact with ceramide/hFA-ceramide before other enzymes do in other organelles (mostly Golgi). With CGT's strong preference for hFA-ceramide over non-hydroxy ceramide [23], most hFA-ceramide would be converted to GalCer in the ER if sufficient CGT was present, leaving little or no hFA-ceramide for SM or GlcCer synthesis. However, this does not mean that SM synthases and GlcCer synthase (GCS) are incapable of utilizing hFA-ceramide. In fact, a number of reports describe various hFA-sphingolipids other than hFA-GalCer in various organs. Representative studies are listed in Table 1. Based on the literature, it is safe to say that hFA can be incorporated into most, if not all, sphingolipids.

In most cases shown in Table 1, physiological functions of hFA-sphingolipids are unknown, but a couple of interesting facts are noteworthy. When developing animals were studied, a dramatic increase in hFA content was found in different tissues. For instance, myelin GalCer is mostly the non-hydroxy form, and the hFA content increased during developmental myelination [6]. A similar developmental change occurs in intestinal GlcCer. In neonatal rodents, intestinal GlcCer contains mostly non-hydroxy FA, and shifts to predominantly hFA during development [45–47]. A number of other examples are shown in Table 1. Another interesting transition is the ganglioside GM_3 in human liver. Riboni et al. found that hFA-

GM_3 was relatively minor in the liver of young adults, but that it continued to increase with age [48]. Although the physiological significance of such a transition is not fully understood, it may relate to the developmental and/or environmental requirement for higher stability/rigidity of the membrane in which these lipids reside.

An important physical characteristic of sphingolipids is the hydrogen-bonding capacity of ceramide, which allows lateral intermolecular interactions and influences the stability and permeability of the membrane [49]. Pascher speculated that the renal tubular cells and intestinal epithelial cells stand the physical stress in part due to the high hFA-sphingolipid contents [49]. Several biophysical studies support this hypothesis. The crystal structure of synthetic GalCer with 2-hydroxyoctadecanoic acid indicated that the 2-hydroxyl group participated in an extensive lateral network of hydrogen bonds between the galactose head group and the polar part of the ceramide backbone [50]. The monolayer behavior of synthetic ceramides revealed that the 2-hydroxyl group promoted the condensation to a close-packed arrangement [51]. Boggs and coworkers demonstrated that the 2-hydroxyl group in sphingolipids increased their phase transition temperature, suggesting that the 2-hydroxyl group contributes to the hydrogen-bonding network and stabilizes the gel phase of the lipids [52]. To confirm these effects in vivo, genetically modified cells and animals with or without hFA-sphingolipids would be needed. One such study using *Fa2h* knockout mice showed that detergent resistance of myelin lipid microdomains was indistinguishable between control and mutant mice, whereas long-term stability of myelin was clearly compromised in the mutant mice [38]. Human patients with FA2H deficiency underwent normal early development, followed by progressive white matter degeneration [11]. These studies foretell that efforts to determine physiological roles of hFA-sphingolipids, especially the long-term effect, will likely face significant challenges.

3.4 hFA-sphingolipids in cancer

Cancer cells often express aberrant cell surface antigens. Glycosphingolipids (GSL) are the first demonstrated tumor-associated cell surface antigens [53], many of which facilitate uncontrolled cell growth and metastasis [54,55]. Interestingly, some human tumors express aberrant GSL containing relatively high levels of hFA. These tumors include ovarian tumors [56], neuroblastomas [57], small cell lung carcinomas [58], and colon and liver adenocarcinomas [59,60]. Based on these observations, Ladisch proposed that elevated fatty acid 2-hydroxylation is a characteristic metabolic change in some human tumors [57]. In a study of transformed human urothelial cell lines, hFA-GSL were found in highly tumorigenic and invasive cell lines, but not in non-tumorigenic, non-invasive cell lines [61]. Whether hFA-GSL has specific roles in facilitating tumorigenesis and metastasis is a fascinating question, and could be addressed by targeted silencing/deletion of FA2H or another fatty acid 2-hydroxylase, whichever present in the cancer cells of interest.

Recently, Iwamori and colleagues reported a striking increase in hFA-GSL in drug-resistant human ovarian carcinoma cell lines [62,63]. The drug-resistance in these cells correlates with the amount of drug efflux pumps, suggesting an intriguing possibility that hFA-GSL may modulate the expression, stability, and/or activities of these pumps. It is also conceivable that elevated hFA-GSL is a byproduct of metabolic activities associated with drug resistance, such as upregulation of cytochrome P450 enzymes, many of which are hydroxylases. Some of them may catalyze fatty acid 2-hydroxylation, resulting in elevated hFA.

In a recent study of the antitumor drug PM02734, Herrero et al. reported that overexpression of *FA2H* increased the sensitivity of human colorectal and cervical carcinoma cells to this drug, while silencing *FA2H* rendered the cells resistant to the drug [64]. It is unlikely that the observed effect was non-specific permeabilization of the plasma membrane because the sensitivity to methyl methanesulfonate was not affected. It appears that hFA-sphingolipids interact with this drug and facilitate its uptake by the cell.

4. HFA-SPHINGOLIPIDS IN CELL SIGNALING

The remarkable expansion of the research on sphingolipid-mediated cell signaling was triggered by the discovery of ceramide as potential lipid second messenger [65]. A key discovery was the activation of hydrolysis of SM by vitamin D, leading to a transient increase of ceramide, which in turn stimulated cell differentiation [66]. In subsequent studies, enhanced de novo ceramide synthesis and reduced conversion to GlcCer were also found to be part of the stress response that increase cellular ceramide concentrations (for review, see [67]). In most ceramide-mediated signaling studies, no attention was paid to hFA-ceramide. As mentioned in §2.4, one study reported inactivity of hFA-ceramide in inducing DNA fragmentation in human promonocytic U937 cells [30].

In a recent study, we provided evidence that hFA-sphingolipids regulate cAMP-signaling in rat D6P2T Schwannoma cells [68]. Similar to Schwann cells, D6P2T cells respond to cAMP and show differentiation phenotypes, including withdrawal from the cell cycle and upregulation of myelin basic protein [69,70]. The cAMP-induced exit from the cell cycle is, in part, mediated by upregulation of the cyclin-dependent kinase inhibitors p21 and p27 [70, 71]. We found that cAMP-induced upregulation of p21 and p27 is greatly diminished by silencing *Fa2h*. This finding suggests that hFA or hFA-sphingolipids contribute to cAMP-dependent signaling pathways that regulate the cell cycle, thereby facilitating Schwann cell differentiation. Though the exact hFA-lipid species involved in this regulation has not been defined, altered *Fa2h* expression appears to influence phosphorylation/dephosphorylation status of a subset of proteins in response to cAMP (our unpublished observation). Identification of these proteins will provide clues to the pathways regulated by hFA-lipids.

For hFA-sphingolipids to participate in specific signaling pathways, there must be specific targets that recognize hFA-sphingolipids. A question arises as to whether proteins and other molecules can distinguish hFA-sphingolipids from non-hydroxy counterparts, and compelling evidence suggests that this is true for hFA-GSL. Some anti-GSL monoclonal antibodies and bacterial toxins have higher affinity for hFA-GSL than for non-hydroxy equivalents [72–74]. Cell surface GSLs often serve as specific binding sites for bacteria, some of which bind only to hFA-GSL and not to the non-hydroxy form, and vice versa [75–79]. The effects of hFA in these examples could be attributed to the hydrogen bonding capability mentioned in §3.3. The 2-hydroxyl group can influence the conformation of the carbohydrate head groups via hydrogen bonds [50], which could affect affinity for specific binding proteins. The evidence for molecular recognition between hFA-ceramide and non-hydroxy ceramide is scarcer. As mentioned in §2.4, there are indications for higher apoptosis-inducing activity of hFA-ceramide in some cell types [29].

In a non-sphingolipid area of lipid research, studies have uncovered potent biological activities of 2-hydroxyoleic acid (HOA). HOA was initially developed as a synthetic oleic acid analog and found to have potent anti-cancer activities in vitro and in animal models [80–82]. The anti-cancer activity of HOA is mediated, at least in part, by downregulation of dihydrofolate reductase [83]. HOA also has anti-hypertensive activity in rats [84] and modulates contractility of cardiomyocytes [85]. The mechanism of HOA action is not fully understood, and it has been attributed to its structural effects on cell membranes, rather than specific interactions with target proteins. However, specific fatty acid-protein interactions are well-documented, and there is an example of a G protein-coupled receptor GPR109B that binds with hFA (both 2- and 3-hydroxyoctanoic acid) [86]. Hence, bioactivities of HOA could well be mediated by specific interactions with target proteins.

What is not mentioned in the literature of HOA is that endogenous HOA is present as a component of hFA-sphingolipids in rat kidney, lung, plasma, and skin [3], and in intestinal

epithelial cells of monkey [22] and sheep [20,21]. Thus, endogenous and exogenous HOA appear to show distinct biological activities. One possible explanation for this might be that endogenous free hFA is very rapidly converted to hFA-ceramide in the ER or degraded in the peroxisomes, which would keep cellular free hFA levels extremely low. If this is the case, modulating the rate of hFA-ceramide synthesis or hFA degradation could be an effective mechanism to regulate cell signaling mediated by HOA and possibly by other hFA. This is highly speculative, and further investigation is warranted to determine the mechanism of action of HOA and to determine whether specific binding proteins for hFA and hFA-sphingolipids exist.

5. FUTURE PERSPECTIVES

This review illustrates a few issues that were often overlooked in mammalian sphingolipid studies. First, hFA can be incorporated into many, if not all, complex sphingolipids. Second, hFA-sphingolipids are present in many mammalian tissues and cell types. It may even be true that all cells contain hFA-sphingolipids, ranging from barely detectable levels to relatively high levels. As evident from this review, the current knowledge about the metabolism and physiological function of hFA-sphingolipids is very limited. At the cellular level, many questions remain to be addressed, such as trafficking and subcellular localization, potential targets, and selective stability/instability of hFA-sphingolipids.

A recent and emerging theme in sphingolipid biology is that the bioactivity of sphingolipids can be greatly influenced by the structure of the sphingoid base and the *N*-acyl chain of ceramide [67]. The physiological significance and the underlying mechanism for such diversity is only beginning to be understood. The precise composition of various ceramide species is determined by the relative activities of enzymes responsible for synthesis and degradation of various sphingoid bases, fatty acids, and ceramides. In the case of hFA-sphingolipids, the expression of *FA2H* is highly variable among cell types, and is inducible during cellular differentiation and by certain stimuli. In addition, there is a second enzyme that can produce hFA. With combinatorial up- and down-regulation of enzymes, the cell can adjust the portfolio of various ceramides in response to the ever-changing cellular environment, thereby fine-tuning the overall metabolic outcomes regulated by bioactive sphingolipids. In order to draw a complete picture of biology of not only hFA-sphingolipids but of all sphingolipids, the "ceramide code" needs to be deciphered. Fortunately, the field of sphingolipid biology is advancing rapidly with ever-evolving analytical technologies, molecular genetic tools and animal models. The study of hFA-sphingolipids will no doubt benefit from these advances, and many unanswered questions will be addressed in the near future. The complete translation of the ceramide code may become available soon.

Acknowledgments

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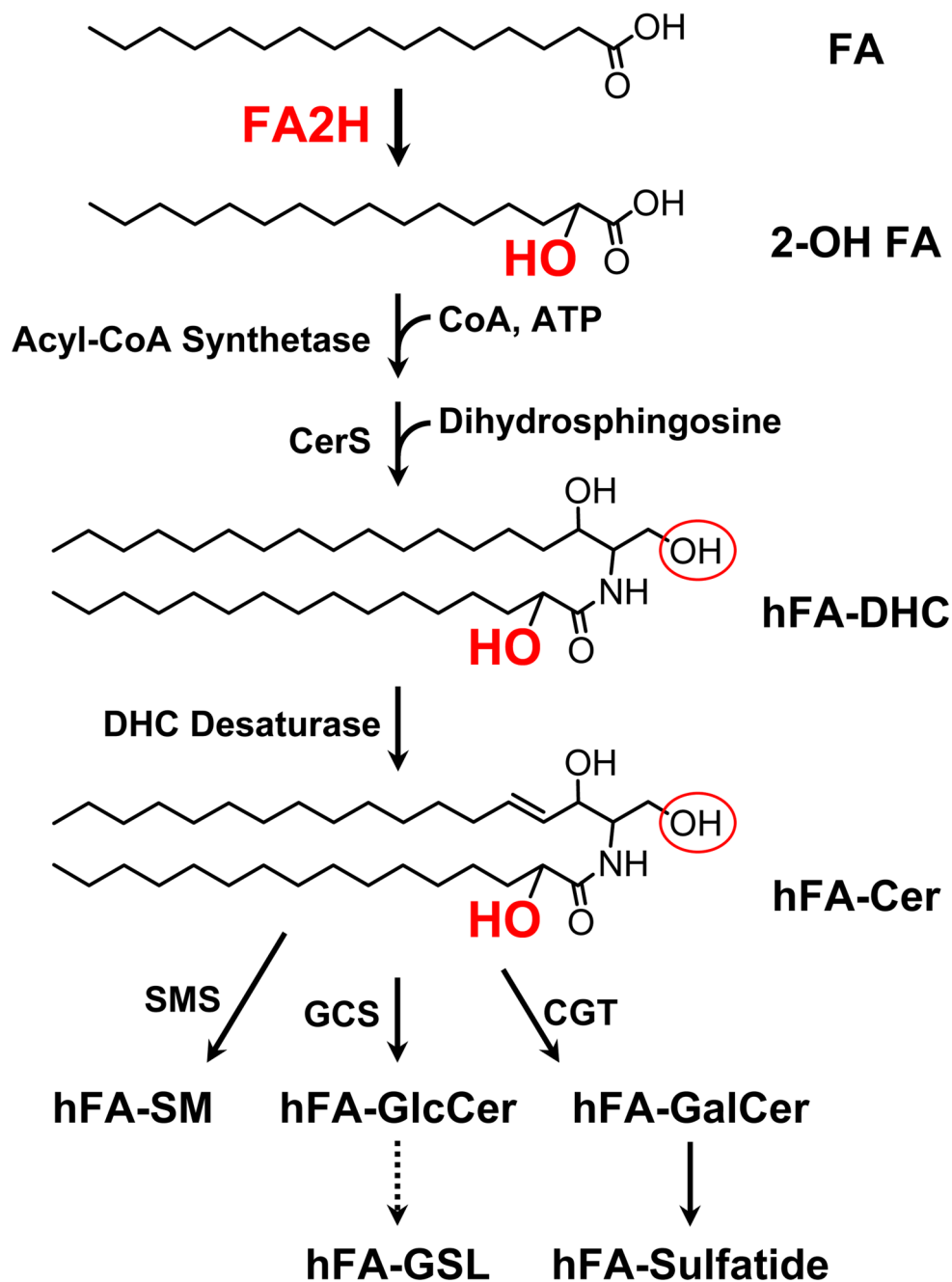
Fig. 1. Biosynthesis of hFA-sphingolipids
The pathway is identical to biosynthesis of non-hydroxy sphingolipids, except for the fatty acid 2-hydroxylation step. Note that various fatty acids (mostly C_{16}-C_{24}) can be 2-hydroxylated and incorporated into hFA-ceramide. The 2-hydroxyl group is shown in red, and the hydroxyl group at the C-1 position of ceramide to which a head group is transferred is indicated by a red circle. DHC, dihydroceramide; CerS, dihydroceramide synthase; Cer, ceramide; SMS, sphingomyelin synthase; CGT, UDP-galactose: ceramide galactosyltransferase; GCS, UDP-glucose:ceramide glucosyltransferase; GSL, glycosphingolipids.
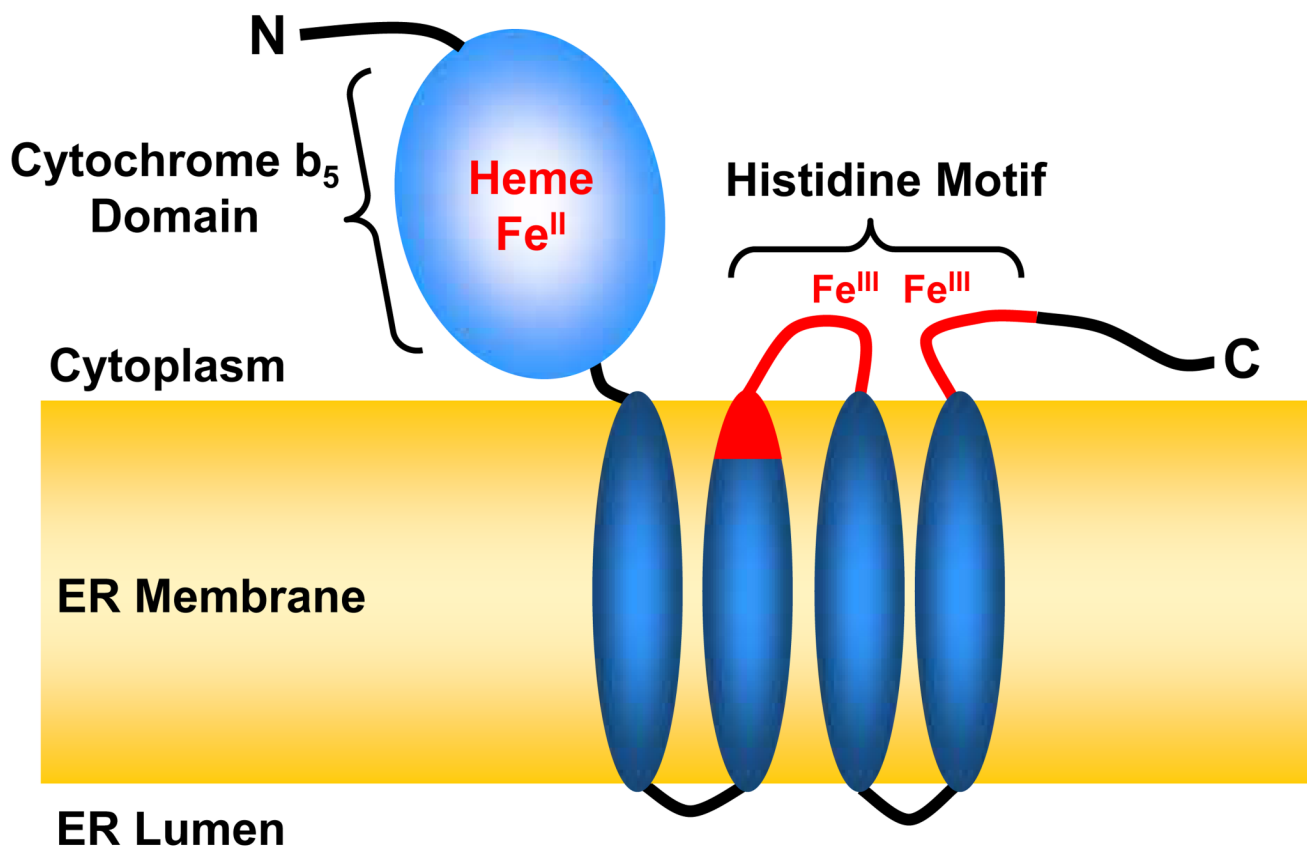
Fig. 2. FA2H is a membrane-bound monooxygenase

FA2H (372 aa) consists of two functional domains; the N-terminal cytochrome b_5 domain (1–92 aa) and the C-terminal catalytic domain. The topology of FA2H is postulated based on the topology of microsomal cytochrome b_5 [111] and the yeast FA2H homologue Scs7p [112, 113]. The 8 conserved histidines (H_{234}, H_{239}, H_{257}, H_{260}, H_{261}, H_{336}, H_{339}, H_{340}) are clustered in the two short segments facing the cytoplasm (shown in red). These histidines are thought to coordinate the non-heme di-iron. The 7 patients with an intronic mutation in the *FA2H* gene produce aberrant mRNA encoding a protein without the last three transmembrane domains that encompass the putative catalytic site.
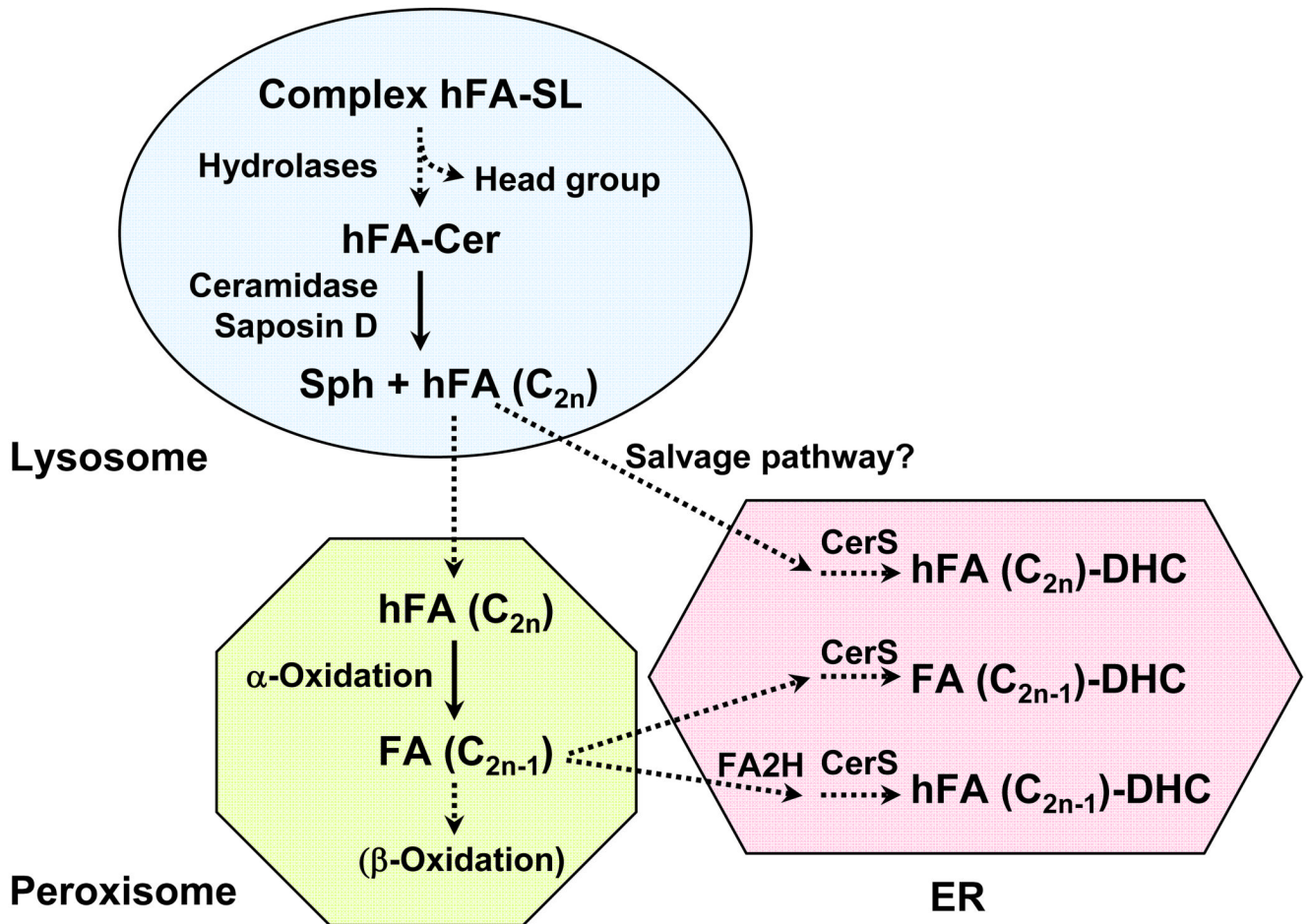
Fig. 3. Degradation of hFA-sphingolipids
Endocytosed sphingolipids are first hydrolyzed by lysosomal hydrolases to ceramide. hFA-ceramide is hydrolyzed by acid ceramidase with the assistance of saposin D. Currently, there is no direct evidence for the recycling of hFA via a salvage pathway. The only known degradation pathway of hFA is the peroxisomal α-oxidation pathway, in which one-carbon cleavage of even-chain fatty acids (C_{2n}) generates odd-chain fatty acids (C_{2n-1}). How hFA is transported from the lysosome to peroxisome is not known. SL, sphingolipids; Sph, sphingosine; DHC, dihydroceramide; CerS, dihydroceramide synthase.

TABLE 1

HFA-SPHINGOLIPIDS IN MAMMALS[a]

hFA-SL species and source	hFA content	Major hFA[b]	Note	Ref
hFA-Ceramide				
Human erythrocyte	0.9%	24:0 (58.7%) 16:0 (18%) 22:0 (10.5%)	Cer content was 5.6 µmol/100 ml packed cells.	[87]
Monkey intestinal mucosa	63.1% in small intestine		Cer was 22.4% of neutral SL.	[22]
	72.9% in colon		Cer was 35.1% of neutral SL.	
Horse kidney	11.6%	24:0 (29%) 23:0 (26%) 22:0 (22%) 20:0 (10%)		[88]
Sheep small intestinal mucosa	31.6% in fetal tissue	28:0 (34.8%) 26:0 (13.0%) 18:1 (9.8%) 30:0 (8.9%) 18:0 (6.6%)	Cer was 40.2% of neutral SL.	[21]
	70.2% in adult tissue	28:0 (35.5%) 30:0 (8.5%) 20:2 (6.8%) 18:1 (6.1%)	Cer was 65.0% of neutral SL.	
Sheep colonic mucosa	29.7% in fetal tissue		Cer was 62.7% of neutral SL.	[20]
	67.1% in adult tissue		Cer was 62.5% of neutral SL.	
Rat small intestine	11%	C20–C24	Cer content was 0.16 µmol/ml packed cells.	[89]
Rat liver	0.5% at P10 1.4% in adult		Cer content was 320 µg/g dry wt at P6, 700 µg/g dry wt at P14.	[90]
Rat kidney	0.6% at P10 3.2% in adult		Cer content was 180 µg/g dry wt at P6, 500 µg/g dry wt at P14.	
Guinea pig Harderian gland	14%	18:0 (19.9%) 23:0 (18.5%) 19:0 (12.3%) 22:0 (11.7%) 20:0 (10.9%)	Also contained branched-chain hFA (5.8% of hFA).	[91]
hFA-SM				
Bovine Rennet stomach	25%	16:0 (28%) 24:0 (22%) 23:0 (20%) 22:0 (15%)		[92]
Bovine rennet stomach	25%	16:0 (38%) 23:0 (16%) 22:0 (12%) 24:0 (12%)		[93]
Bovine kidney, intestinal mucosa		22:0, 23:0, 24:0		[94]
Bovine milk	<1%	23:0 (31.5%) 24:0 (21.8%) 22:0 (17.2%) 16:0 (9.2%)		[95]
Guinea pig Harderian gland	30.5%	18:0 (30.2%) 19:0 (20.0%) 20:0 (16.4%)	Also contained 1.4% branched-chain hFA	[96]

hFA-SL species and source	hFA content	Major hFA[b]	Note	Ref
			(16-methyl 19:0).	
Ram, bull, rat, boar testes and spermatozoa; human spermatozoa	In rat testis, undetectable at P20; 49% at P55	In rat testis, 28:4, 30:5, 31:5, 32:5		[97]
hFA-GlcCer/MHC				
Human liver GlcCer	20%	24:0 (46%) 22:0 (17%) 23:0 (17%) 24:1 (11%)	GlcCer was 19.1% of neutral GSL.	[98]
Human kidney GlcCer	49%	24:0 (32.1%) 22:0 (23.2%) 23:0 (19.2%) 24:1 (12.5%)	MHC (6–7% of neutral GSL) was a mixture of GlcCer and GalCer (1:1).	[99]
Human ureteral epithelial cells GlcCer	90%	24:0, 20:0, 21:0, 22:0, 23:0, 25:0	GlcCer was 75% of GSL	[100]
Human uterine endometrium GlcCer	30.9% in the secretory phase 3.8% in the proliferative phase	In the secretory phase, 24:0 (54.7%) 24:1 (14.9 %) 16:0 (11.7%) 22:0 (11.3%)		[101]
Monkey intestinal mucosa MHC	88.4% in small intestine		MHC (21.8% of neutral SL) was a mixture of GlcCer (90%) and GalCer (10%).	[22]
	93.4% in colon		MHC (14.8% of neutral SL) was a mixture of GlcCer (82%) and GalCer (18%).	
Bovine kidney MHC	>95%	22:0 16:0	MHC was a mixture of GlcCer (75%) and GalCer (25%).	[102]
Bovine milk GlcCer	<1%	23:0 (31.0%) 24:0 (19.7%) 22:0 (16.7%) 16:0 (12.6%)		[95]
Horse kidney MHC	78.9%	23:0 (25%) 24:0 (23%) 22:0 (20%)	MHC was a mixture of GlcCer (70.5%) and GalCer (29.5%).	[88]
Sheep small intestinal mucosa MHC	36.3% in fetal tissue	18:0 (23.1%) 18:2 (15.2%) 16:1 (12.9%) 22:0 (12.7%) 16:0 (12.1%) 20:0 (5.8%)	MHC (25.5% of neutral SL) was a mixture of GlcCer (80%) and GalCer (20%).	[21]
	69.1% in adult tissue	18:0 (41.5%) 16:0 (21.4%) 18:1 (4.1%)	MHC (13.6% of neutral SL) was a mixture of GlcCer (90%) and GalCer (10%).	
Sheep colonic mucosa MHC	32.6% in fetal tissue	18:1 (38.7%) 18:0 (36.8%) 18:2 (4.3%)	MHC (10.8% of neutral SL) was a mixture of GlcCer (80%) and GalCer (20%).	[20]
	64.2% in adult tissue	26:0 (45.2%) 16:0 (14.2%) 30:0 (12.2%) 28:0 (8.5%) 20:2 (6.8%)	MHC (12.6% of neutral SL) was a mixture of GlcCer (80%) and GalCer (20%).	

hFA-SL species and source	hFA content	Major hFA[b]	Note	Ref
Rat stomach MHC	55.5% in 20-day embryo 82.4% at P60	In 20-day embryo, 24:0 (57.8%) 22:0 (14.2%) 20:0 (7.0%) In P60, 24:0 (54%) 23:0 (21.7%) 22:0 (17.6%)	MHC was a mixture of GlcCer (95%) and GalCer (5%).	[103]
Rat intestinal villus and crypt cells GlcCer	63–68%	C24 (28–29%) C22 (20–24%) C20 (10–16%) C24:1 (10–12%)		[104]
Rat small intestinal epithelial cells	66% in villus tip	24:0 (34.7%) 16:0 (19.7%) 22:0 (15.9%) 24:1 (14.0%) 20:0 (9.2%) 23:0 (5.4%)		[105]
	32% in crypt cells	24:0 22:0 16:0 20:0 24:1		
Rat colon MHC	40% at birth 75% in adult		MHC (45–50% of neutral GSL) was mostly GlcCer (>90%) at birth and in adult	[46]
Mouse intestinal mucosa GlcCer	90.5%	24:1 (27.8%) 22:0 (26.0%) 24:0 (13.5%) 23:0 (12.0%) 16:0 (11.5%) 20:0 (6.4%)	Asialo GM$_1$ and GlcCer were the two major neutral GSL.	[106]
hFA-LacCer				
Human liver	10%	C24 (33%) C24:1 (18%) C23 (15%) C22 (13%)	LacCer was 30.3% of neutral GSL.	[98]
Human Uterine endometrium	26.2% in the secretory phase 4.7% in the proliferative phase	In the secretory phase, C24:0 (50.4%) C24:1 (21.8 %) 16:0 (8.0%)		[101]
Monkey intestinal mucosa	91.2% in small intestine	28:0 (42.5%) 26:0 (11.4%) 18:0 (10.2%)	LacCer was 27.6% of neutral SL.	[22]
	93.6% in colon	28:0 (43.9%) 18:2 (14.2%) 18:1 (13.6%) 26:0 (7.4%)	LacCer was 26.6% of neutral SL.	
Bovine milk	<1%	24:0 (29.5%) 23:0 (26.9%) 22:0 (15.4%) 16:0 (10.5%)		[95]
hFA-GM$_3$				
Human liver	43.1%	22:0 (28.8%) 18:0 (17.7%) 16:0 (17.4%)	(84 yr old female)	[107]
Human liver	30%	C24 (37%) C22 (20%) C23 (17%) C24:1 (15%)	GM$_3$ was 91.6% of liver gangliosides (11 yr old male).	[98]
Human liver	10% at 20 yr old 50% at 80 yr old	22:0 (32.3%) 24:0 (25.0%) 23:0 (16.1%) 24:1 (9.8%)	hFA composition was determined with a pool of 31 samples, 19–85 yr old.	[48]
Monkey intestinal mucosa	70.3% in small intestine	28:0 (42.2%) 30:0 (31.2%) 16:1 (7.2%)	GM$_3$ was 60.7% of gangliosides.	[22]
	76.4% in colon	30:0 (50.9%) 28:0 (20.8%) 16:1 (7.9%) 26:0	GM$_3$ was 61.6% of gangliosides.	

hFA-SL species and source	hFA content	Major hFA[b]	Note	Ref
		(7.3%)		
Sheep small intestinal mucosa	41.1% in fetal tissue		GM_3 was 41.5% of gangliosides.	[21]
	72.3% in adult tissue		GM_3 was 25.2% of gangliosides.	
Sheep colonic mucosa	36.3% in fetal tissue	26:0 (81.8%) 28:0 (5.5%) 24:0 (3.4%) 18:0 (2.4%)	GM_3 was 41.2% of gangliosides.	[20]
	72.1% in adult tissue	30:0 (44.7%) 24:0 (16.1%) 28:0 (15.6%) 18:0 (8.8%) 26:0 (9.9%) 18.1 (2.7%)	GM_3 was 22.3% of gangliosides.	
Rat intestinal villus and crypt cells	71–72%	C24 (23–25%) C22 (21–22%) C16 (14–15%) C24:1 (10–15%) C20 (9–14%)		[104]
Rat small intestine	69%	16:0 20:0 22:0 24:0 24:1	hFA-GM_3 was concentrated in the epithelial tissue. GM_3 in non-epithelial tissue contained no hFA.	[108]
Rat small intestine	no hFA at P0; 26.0% at P21; 41.5% at P38		Majority of hFA-GM_3 was in epithelium.	[45]
Adult rat small intestinal epithelium	49.4–82% among different strains			[109]
Other hFA-SLs				
Gangliosides in human kidney	11.7–28.9%	24:0 23:0 24:1 22:0	Values are for 2 main gangliosides and 2 minor gangliosides.	[110]
Globotriaosyl ceramide in human uterine endometrium	22.2% in the secretory phase 4.8% in the proliferative phase	In the secretory phase, C24:0 (51.8%) C24:1 (29.7 %) 16:0 (7.7%)		[101]
THC in sheep small intestinal mucosa	42.2% in fetal tissue	28:0 (29.4%) 26:0 (16.1%) 24:0 (11.8%) 16:0 (5.7)	THC was 17.3% of neutral SL.	[21]
	68.3% in adult tissue	18:0 (64.1%) 16:0 (20.8%) 18:1 (10.4%) 18:2 (4.2%)	THC was 18.9% of neutral SL.	
GD_{1a} in sheep small intestinal mucosa	34.3% in fetal tissue	24:0 (28.0%) 26:0 (25.1%) 18:0 (24.2%) 16:0 (7.3%) 20:0 (6.4%) 20:1 (4.4%)	GD_{1a} was 47.2% of gangliosides.	
	68.3% in adult tissue	26:0 (55.9%) 18:0 (16.7%) 24:0 (14.6%) 22:1 (4.5%)	GD_{1a} was 48.3% of gangliosides.	
THC in sheep colonic mucosa	40.1% in fetal tissue		THC was 25.5% of neutral SL.	[20]

hFA-SL species and source	hFA content	Major hFA[b]	Note	Ref
	69.4% in adult tissue		THC was 14.7% of neutral SL.	
GD$_{1a}$ in sheep colonic mucosa	31.2% in fetal tissue		GD$_{1a}$ was 47.5% of gangliosides.	
	67.4% in adult tissue		GD$_{1a}$ was 50.7% of gangliosides.	
Asialo GM$_1$ in mouse intestinal mucosa	79.8%	22:0 (27.2%) 24:1 (23.6%) 16:0 (21.4%) 23:0 (10.3%) 20:0 (10.2%) 24:0 (7.0%)	Asialo GM$_1$ and GlcCer were the two major neutral GSL.	[106]

[a]This table does not include hFA-sphingolipids that have been extensively documented (GalCer and sulfatide in the nervous system, kidney, and other organs; epidermal sphingolipids).

[b]Percent composition was calculated among hFA and does not include non-hydroxy FA.

P, postnatal day; MHC, monohexosylceramide; DHC, dihexosylcermaide; THC, tetrahexosylceramide. Neutral SL includes ceramide.