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Metabolism of Very Long-Chain Fatty Acids: Genes and Pathophysiology

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Abstract

Fatty acids (FAs) are highly diverse in terms of carbon (C) chain-length and number of double bonds. FAs with C>20 are called very long-chain fatty acids (VLCFAs). VLCFAs are found not only as constituents of cellular lipids such as sphingolipids and glycerophospholipids but also as precursors of lipid mediators. Our understanding on the function of VLCFAs is growing in parallel with the identification of enzymes involved in VLCFA synthesis or degradation. A variety of inherited diseases, such as ichthyosis, macular degeneration, myopathy, mental retardation, and demyelination, are caused by mutations in the genes encoding VLCFA metabolizing enzymes. In this review, we describe mammalian VLCFAs by highlighting their tissue distribution and metabolic pathways, and we discuss responsible genes and enzymes with reference to their roles in pathophysiology.

Key Words: Sphingolipids, Glycerophospholipids, ELOVL, Ceramide, Ichthyosis, Leukodystrophy

CELLULAR FATTY ACIDS (FAS): CHAIN-LENGTH AND NUMBER OF DOUBLE BONDS

FAs are classified according to their carbon (C) chain-length and the number of double bonds (Fig. 1). Long-chain FAs (LCFAs) have chain-lengths of C11-20, of which C16 and C18 LCFAs are the most abundant FA species in mammalian cells. FAs longer than C20 (C>20) are called very long-chain FAs (VLCFAs) and are less abundant than LCFAs. VLCFAs with C22 and C24 are found ubiquitously throughout the body. VLCFAs with C≥26 are often sub-classified into ultra long-chain FAs (ULCFAs) and are found in specific tissues, including the skin, retina, meibomian gland, testis, and brain.

Another classification of FAs is based on the number of double bonds. FAs are classified into saturated FAs (SFAs; no double bond), monounsaturated FAs (MUFAs; one double bond) and polyunsaturated FAs (PUFAs; two or more double bonds) (Fig. 1). PUFAs are further sub-classified into *n*-3 (or ω3) and *n*-6 (or ω6) series depending on the position of the terminal double bond, i.e. the double bond most distant from the carboxyl group. In the *n*-x series, x indicates the ordinal number of carbon atom with a double bond from the end of the carbon chain.

By combining these two classifications, arachidonic acid for example, an *n*-6 FA with chain-length C20 and four double bonds is denoted by C20:4*n*-6. Linoleic acid (C18:2*n*-6) and α-linolenic acid (C18:3*n*-3) are essential FAs (EFAs) that must be consumed through food, since humans are unable to synthesize them.

ENZYMES RESPONSIBLE FOR FA ELONGATION

FAs are elongated in the form of acyl-CoA, in which FAs are covalently linked to coenzyme A via thioester bonds. Elongation of ≥C16 FAs that are either synthesized *de novo* by the FA synthase in the cytoplasm or absorbed from food, occurs in the endoplasmic reticulum (ER). FA elongation proceeds through repetition of the FA elongation cycle whereby two carbons are added to the carboxyl end in each cycle (Fig. 2) (Jakobsson *et al*., 2006; Guillou *et al*., 2010). The FA elongation cycle consists of four sequential reactions: condensation, reduction, dehydration, and reduction. The first condensation reaction is the rate-limiting step, in which 3-ketoacyl-CoA is produced by condensation of acyl-CoA with malonyl-CoA. This reaction is catalyzed by FA elongase. FA elongases constitute the ELOVL family of proteins, and there are seven isozymes (ELOVL1-7) in mammals (Jakobsson *et al*., 2006; Guillou *et al*., 2010; Ohno *et al*., 2010). In the second reduction step, 3-ketoacyl-CoA is converted to 3-hydroxyacyl-CoA using nicotinamide adenine

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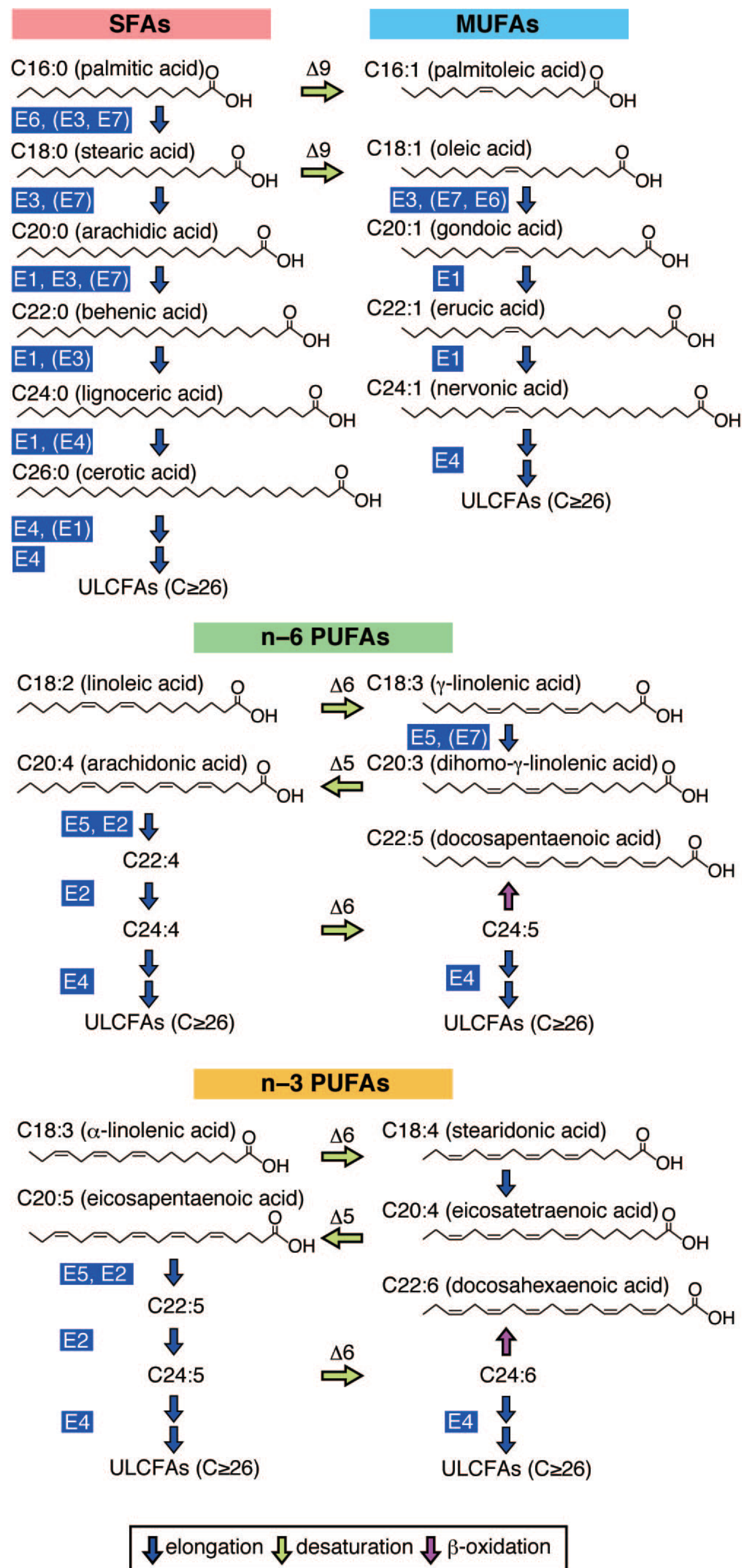

Fig. 1. Human FA elongation pathways. The FA elongation pathways of SFAs, MUFAs and PUFAs are illustrated. ELOVL isozymes (E1-E7) responsible for each elongation step are indicated. Parentheses denote ELOVLs that exhibit weak activity toward the indicated substrates. Δ5, Δ6 and Δ9 represent Δ5-, Δ6- and Δ9-desaturase, respectively. FA: fatty acid; SFA: saturated FA; MUFA: monounsaturated FA; PUFA: polyunsaturated FA.

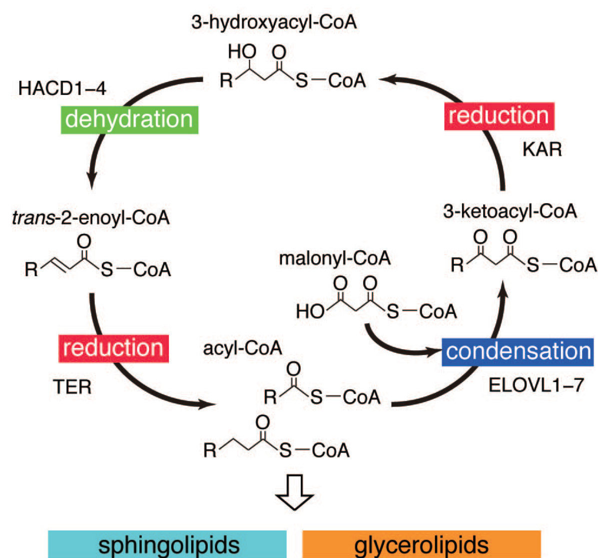

Fig. 2. Mammalian FA elongation cycle. The FA elongation cycle and enzymes involved in each step are illustrated. In each cycle, acyl-CoA incorporates two carbon units from malonyl-CoA.

dinucleotide phosphate (NADPH) as a cofactor. The 3-ketoacyl-CoA reductase responsible for this reaction is KAR (Moon and Horton, 2003). In the third dehydration step, 3-hydroxyacyl-CoA is converted to *trans*-2-enoyl-CoA, and this reaction is catalyzed by 3-hydroxyacyl-CoA dehydratase (HACD) (Ikeda *et al*., 2008). This third step appears to be another rate-limiting step, since some 3-hydroxyacyl-CoAs accumulate at low but significant levels in *in vitro* FA elongation assays (Abe *et al*., 2013). There are four mammalian HACD isozymes (HACD1-4) (Ikeda *et al*., 2008). In the last reduction step, *trans*-2-enoyl-CoA is converted to acyl-CoA, which is longer than the original acyl-CoA by two carbons. The *trans*-2-enoyl-CoA reductase responsible for this reaction is TER, and the reaction requires NADPH as a cofactor (Moon and Horton, 2003).

In vitro FA elongation assays and knockdown or knockout (KO) of *ELOVL1-7* genes uncovered that ELOVL1-7 exhibits substrate specificity; each isozyme prefers acyl-CoAs with specific chain-lengths and/or degree of saturation (Fig. 1) (Guillou *et al*., 2010; Ohno *et al*., 2010). ELOVL1 elongates saturated and monounsaturated C20-C26 acyl-CoAs (Ohno *et al*., 2010). ELOVL1 production of C24:0 and C24:1 acyl-CoAs is essential for the synthesis of C24 sphingolipids (discussed later); in *Elovl1* KO mice, C24 sphingolipids are severely reduced in various tissues (Sassa *et al*., 2013). ELOVL2 and ELOVL5 elongate PUFAs. ELOVL2 elongates C20-C22 polyunsaturated acyl-CoAs, while ELOVL5 elongates C18-C20 polyunsaturated acyl-CoAs (Ohno *et al*., 2010). In *Elovl2* KO mice, the levels of C22:4*n*-6 and C22:5*n*-3 FAs are increased, whereas the levels of derivatives thereof are severely reduced, indicating that Elovl2 is critical for the elongation of C22-CoAs of both the *n*-3 and *n*-6 series (Zadravec *et al*., 2011). Moreover, *Elovl2* deficiency causes the near absence of ultra long-chain (ULC)-PUFAs (C28:5*n*-6 and C30:5*n*-6) in the testis and male infertility with a complete arrest of spermatogenesis (Zadravec *et al*., 2011). Analysis of *Elovl5* KO mice demonstrated that Elovl5 is essential for the elongation of C18-CoAs of both *n*-3 and *n*-6 series in the liver (Moon *et al*., 2009).

Table 1. Substrate specificity and tissue distribution of mammalian CERS isozymes

Isozyme	Preferred substrates	mRNA expression
CERS1	C18	Brain, skeletal muscle
CERS2	C22–C24	Ubiquitous, high in liver, kidney, lung
CERS3	≥C26	Skin, testis
CERS4	C18–C20	Lung, heart
CERS5	C16	Ubiquitous, high in brain, kidney, testis
CERS6	C16	Ubiquitous, high in brain, liver, thymus

Elovl5 KO mice exhibit hepatic steatosis due to the activation of sterol regulatory element-binding protein (SREBP)-1c and its target genes involved in FA and triglyceride synthesis (Moon *et al*., 2009). ELOVL3 and ELOVL7 elongate both saturated and unsaturated C16-C22 acyl-CoAs, with the highest activity toward C18-CoAs (Ohno *et al*., 2010; Naganuma *et al*., 2011). *Elovl3* is expressed in the skin sebaceous glands and hair follicles as well as brown adipose tissue. *Elovl3* KO mice exhibit accumulation of C20:1 FAs in the skin that is associated with a sparse hair coat and defects in water repulsion (Westerberg *et al*., 2004). ELOVL4 also elongates both saturated and unsaturated acyl-CoAs, but it is specialized for the synthesis of ULCFAs (Ohno *et al*., 2010). In *Elovl4* KO mice and homozygous Stargardt disease 3 (STGD3) model mice that carry a pathogenic mutation in the *Elovl4* gene, ULCFAs in the epidermis were completely missing (Li *et al*., 2007; Vasireddy *et al*., 2007). ELOVL6 elongates shorter acyl-CoAs as compared to other ELOVLs, with activity toward C12:0-C16:0 acyl-CoAs (Moon *et al*., 2001). Consistent with this, the levels of C16:0 and C16:1 FAs are increased whereas the levels of C18:0 and C18:1 FAs are reduced in the liver of *Elovl6* KO mice (Matsuzaka *et al*., 2007). Interestingly, *Elovl6* KO mice are protected from obesity-induced insulin resistance despite becoming obese and developing hepatosteatosis (Matsuzaka *et al*., 2007).

C24 VLCFAS IN SPHINGOLIPIDS

Most saturated and monounsaturated VLCFAs are found as acyl moieties of sphingolipids. Sphingolipids contain ceramide (Cer) as a backbone, in which FAs are amide-linked to the sphingoid base. Cers are converted to sphingomyelin (SM) and glycosphingolipids by addition of a polar head group of phosphocholine and sugars, respectively.

Cer synthase (CERS) catalyzes an amide bond formation between acyl-CoA and the sphingoid base (Mizutani *et al*., 2009; Tidhar and Futerman, 2013). Six mammalian CERS isozymes (CERS1-6) have been identified, and each CERS isozyme exhibits characteristic substrate specificity toward acyl-CoAs with specific chain-lengths (Table 1) (Mizutani *et al*., 2009; Tidhar and Futerman, 2013). CERSs exhibit ubiquitous or tissue specific distribution patterns and may determine the tissue distribution patterns of sphingolipids with specific chain-lengths (Table 1) (Mizutani *et al*., 2009; Tidhar and Futerman, 2013).

In most tissues, chain-lengths of sphingolipids range from C16 to C24 with the major FAs being C16:0 palmitic acid,

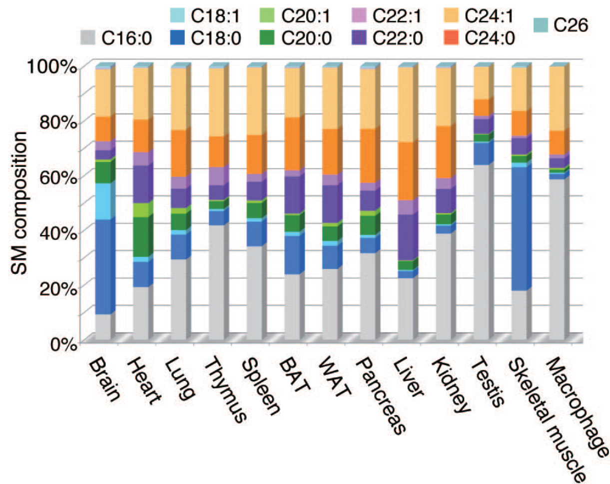

Fig. 3. FA compositions of SM. FA compositions of SM in indicated mouse tissues determined by liquid chromatography-mass spectrometry analysis are illustrated. SM: sphingomyelin; BAT: brown adipose tissue; WAT: white adipose tissue.

C24:0 lignoceric acid, and C24:1 nervonic acid. However, the proportion of each FA varies considerably among tissues. FA compositions of SM in various mouse tissues are shown in Fig. 3. C24 VLCFAs constitute almost 50% of SMs in the liver, whereas they constitute less than 20% in the testis. As an exception, C18 LCFAs (C18:0 stearic acid and C18:1 oleic acid) rather than C16 LCFAs or C24 VLCFAs are predominant in the brain and skeletal muscle. FA composition of sphingolipids varies significantly depending on the cell type. In the nervous system, for example, neurons synthesize mainly C18 sphingolipids, while oligodendrocytes and Schwann cells, which wind tightly around axons to form myelin sheaths, predominantly synthesize C24 sphingolipids (Becker *et al*., 2008; Imgrund *et al*., 2009; Ginkel *et al*., 2012).

CERS2 is primarily responsible for the synthesis of C24 sphingolipids. In *CerS2* KO mice, the levels of C24 sphingolipids are severely reduced in tissues that normally contain high levels of C24 sphingolipids, such as the liver, kidney and brain (Imgrund *et al*., 2009; Pewzner-Jung *et al*., 2010b). ELOVL1 provides C24-CoAs for C24 sphingolipid synthesis via CERS2. *Elovl1* KO mice also exhibit a severe reduction in C24 sphingolipid levels in various tissues (Sassa *et al*., 2013). *CerS2* KO mice exhibit defects in myelin sheath stability associated with the near absence of myelin C22-C24 galactosylCer (GalCer) and sulfatide, a sulfated form of GalCer (Imgrund *et al*., 2009). *CerS2* KO mice also develop hepatocellular carcinomas (Imgrund *et al*., 2009; Pewzner-Jung *et al*., 2010a).

At the cellular and molecular levels, C24 sphingolipids have unique biophysical properties not possessed by C16 sphingolipids such as effects on membrane fluidity, lipid microdomain formation, and signaling across the membrane (Simons and Ikonen, 1997; Kasahara and Sanai, 2000; Sonnino *et al*., 2009; Silva *et al*., 2012). Studies using artificial lipid bilayers have suggested that the C24 FA moiety in sphingolipids may interdigitate with the opposing leaflet and facilitate the formation of lipid microdomains (Morrow *et al*., 1995). In neutrophils, C24 lactosylCers (LacCers) but not C16 LacCers are associated with the Src family kinase LYN in the plasma membrane microdomain; this association is necessary for neutrophil functions such as superoxide generation and migration (Iwabuchi *et al*., 2008). In HeLa cells, knockdown of *ELOVL1* or *CERS2* causes a shift in sphingolipid composition from C24 to C16 and increases susceptibility to apoptosis induced by diverse stimuli such as anticancer drugs (i.e. cisplatin), ultraviolet radiation, or C6 Cers (Sassa *et al*., 2012).

Yeast sphingolipids consist exclusively of saturated C26 VLCFAs (Ejsing *et al*., 2009). VLCFA synthesis is essential for yeast viability, and viable mutant strains with limited VLCFA synthesis exhibit defects in vesicular transport mainly in the late endosome/multivesicular body (Obara *et al*., 2013). VLCFAs may have a conserved role in vesicular trafficking systems, where a highly curved membrane is constantly appearing and disappearing to support vesicle budding and fusion.

ULCFAS IN EPIDERMAL CERS

Saturated and monounsaturated ULCFAs are found in the skin. The stratum corneum (SC), the outermost layer of the skin epidermis, is essential for the epidermal permeability barrier, which protects terrestrial animals against desiccation by transepidermal water loss and invasion of pathogens and toxic chemicals (Proksch *et al*., 2008). In the SC, the intercellular spaces are filled with multilamellar lipid layers called extracellular lipid lamella (ELL) (Breiden and Sandhoff, 2014; Rabionet *et al*., 2014). Cers account for ~50% of lipids in the ELL, although Cers are less abundant in most tissues since they are utilized as precursors of complex sphingolipids such as SMs and glycosphingolipids. C26-C36 ULCFAs are found in Cers in the ELL (Masukawa *et al*., 2008; t'Kindt *et al*., 2012). Most C≥30 ULCFAs in the epidermal Cers are ω-hydroxylated and esterified with linoleic acid (C18:2*n*-6) to form ω-*O*-acylCers or covalently bound to proteins (mainly involucrin) of corneocytes, the latter form corneocyte lipid envelopes (CLEs) (Breiden and Sandhoff, 2014; Rabionet *et al*., 2014). EFA deficiency in human and other mammals causes ichthyosis-like cutaneous abnormalities including scaly skin and impaired permeability barriers (Prottey, 1977; Yamanaka *et al*., 1980). In EFA deficiency, linoleic acid (C18:2*n*-6) esterified with ω-OH ULCFAs in ω-*O*-acylCers is replaced by oleic acid (C18:1*n*-9) with a concomitant decrease in the epidermal permeability barrier, suggesting an essential function of linoleate-esterified ω-*O*-acylCers in constructing and maintaining epidermal permeability barrier integrity (Prottey, 1977; Yamanaka *et al*., 1980).

Our understanding on the pathway(s) and the enzymes involved in the synthesis of epidermal Cers is still incomplete. However, recent studies using KO mice identified the enzymes responsible for the synthesis of ULC-Cers. ULC-Cers are essential for the epidermal permeability barrier, thus a deficiency in ULC-Cers synthesis can be easily identified in KO mice by examining transepidermal water loss and dye penetration, and epidermal histology. Among the six CERS isozymes, CERS3 is exclusively required for ULC-Cer synthesis (Jennemann *et al*., 2012). Epidermal ULC-Cers are completely lost in *CerS3* KO mice, precluding the formation of the ELL and CLE, which leads to severe transepidermal water loss and early postnatal lethality (Jennemann *et al*., 2012). Consistent with the involvement of ELOVL4 in ULCFA synthesis (Fig. 1), *Elovl4* KO mice exhibit essentially identical skin phenotypes to that of *CerS3*

Table 2. VLCFA-related genes mutated in inherited diseases

Gene	Chromosome	Function	Disease
ELOVL4	6	FA elongase (condensation)	Stargardt-like macular dystrophy (STGD3) (dominant) Ichthyosis, nervous system abnormalities (recessive)
HACD1 (PTPLA)	10	3-Hydroxyacyl-CoA dehydratase	Myopathy
TER (TECR)	19	*Trans*-2-enoyl-CoA reductase	Non-syndromic mental retardation
CERS3	15	Cer synthase	Ichthyosis
ABCA12	2	Glucosylceramide transport into LB	Ichthyosis
FA2H	16	2-Hydroxylation of FA	Leukodystrophy with spastic paraparesis and dystonia
ABCD1	X	VLCFA-CoA transport into peroxisome	X-linked adrenoleukodystrophy (X-ALD)
ACOX1	17	VLCFA β-oxidation in peroxisome	Leukodystrophy, other nervous system abnormalities
HSD17β4	5	VLCFA β-oxidation in peroxisome	Leukodystrophy, other nervous system abnormalities

KO mice (Li *et al*., 2007; Vasireddy *et al*., 2007). CERS3 and ELOVL4 mRNA increases during keratinocyte differentiation (Mizutani *et al*., 2013). *Elovl1* KO mice also exhibit lethal epidermal permeability barrier deficiency (Sassa *et al*., 2013). ELOVL1 activity is regulated differently by CERS2 or CERS3 (Ohno *et al*., 2010; Mizutani *et al*., 2013; Sassa *et al*., 2013). In most tissues, ELOVL1 cooperates with CERS2 and elongates acyl-CoAs up to C24-CoAs, which are utilized for C24 sphingolipid synthesis by CERS2. However, in the upper epidermis, where ULC-Cers present in the ELL and CLE are synthesized, CERS3 is highly expressed and instructs ELOVL1 to elongate up to C26-CoAs, which are either utilized for the synthesis of C26-Cers by CERS3 or subject to further elongation up to C36-CoAs by ELOVL4 (Sassa *et al*., 2013).

Recently, recessive mutations in the *CERS3* gene on chromosome 15 and the *ELOVL4* gene on chromosome 6 have been identified in ichthyosis patients (Table 2) (Aldahmesh *et al*., 2011; Eckl *et al*., 2013; Radner *et al*., 2013). Lipid analysis of the CERS3 mutant keratinocytes derived from the patients revealed severe reductions in the levels of ULC-Cers including ω-*O*-acylCers and protein bound ω-OH Cers (Eckl *et al*., 2013; Radner *et al*., 2013).

Inherited ichthyoses form part of a large clinically and etiologically heterogeneous group of disorders of cornification, and most ichthyoses are associated with the impaired epidermal permeability barrier (Oji *et al*., 2010; Elias *et al*., 2012). Notably, along with *CERS3* and *ELOVL4*, some non-syndromic and syndromic forms of inherited ichthyosis include genes involved in lipid metabolism. Given the crucial role of ULC-Cers in epidermal permeability barrier, it is plausible that at least some of these genes may be involved in the synthesis or transport of ULC-Cers necessary for the formation of the ELL and CLE. Indeed, the *ABCA12* gene, which is mutated in harlequin ichthyosis, the most severe form of autosomal recessive congenital ichthyosis, encodes a member of the ATP-binding cassette (ABC) transporters likely involved in the transport of glucosylCers (GlcCers) into lamellar bodies (LB) (Table 2) (Akiyama *et al*., 2005; Akiyama, 2014). GlcCers in LBs are precursors of Cers including ω-O-acylCers and are converted to Cers upon secretion in the SC (Akiyama, 2014; Breiden and Sandhoff, 2014; Rabionet *et al*., 2014).

VLCFAS IN THE MEIBUM

The ocular surface is covered by a structure called the tear film (TF), which consists of aqueous tears and a mixture of diverse lipids (meibum) that are produced by lachrymal glands and meibomian glands, respectively (Butovich, 2013). The physiological functions of the TF are the maintenance of a smooth surface for light refraction, and, in analogy with the epidermal permeability barrier, the protection of underlying ocular structures including the cornea and conjunctiva from desiccation and infection. In humans, there are 30-40 and 20-30 meibomian glands in the upper and lower eyelids, respectively. In dry eye patients, the number of functional (yielding liquid secretion) meibomian glands is significantly decreased compared with asymptomatic controls (Korb and Blackie, 2008).

The major components of meibum are cholesteryl esters and wax esters (WEs), each accounting for ~30% of the total meibum lipids (Ohashi *et al*., 2006). The FA residues of human meibum cholesteryl esters are ~80% saturated, and their chain-lengths range from C18 up to C32, with saturated C24-C27 VLCFAs being the major species (Butovich, 2010). WEs are esters of FAs with fatty alcohols. The major WE species in human meibum have saturated C24-C26 VLC fatty alcohols that are esterified to oleic acid (C18:1) (Butovich *et al*., 2009). Meibum also contains ω-*O*-acylVLCFAs, which consist of ω-OH monounsaturated ULCFAs (C30-C34) esterified with monounsaturated C16 or C18 FA (Butovich *et al*., 2009). However, neither ω-*O*-acylCers nor regular Cers were found in the human meibum, and FAs (palmitoleic acid, C16:1*n*-7 and oleic acid, C18:1*n*-9) esterified to ω-OH ULCFAs are different from those (linoleic acid, C18:2*n*-6) esterified to epidermal ω-OH Cers (Butovich *et al*., 2009).

DOCOSAHEXAENOIC ACID (DHA) IN THE TESTIS, BRAIN, AND RETINA

VLC-PUFAs include DHA (C22:6*n*-3) (Fig. 1). DHA is abundant in glycerophospholipids in the testis, brain, and retina. In the spermatozoa, DHA is almost exclusively found in phosphatidylcholine (PC) or phosphatidylethanolamine (PE), whereas *n*-6 PUFAs, including docosapentaenoic acid (DPA; C22: 5*n*-6), are uniformly distributed in various glycerophospholipid species (Lin *et al*., 1993). In the brain gray matter, DHA is predominantly found in phosphatidylserine (PS) and PE (Sastry, 1985). About 50-60% of the PE in the brain is present in the form of plasmalogen, which contains a vinyl ether bond at the *sn*-1 position instead of an ester bond. DHA is enriched in both PE and PE plasmalogen (Sastry, 1985). In

the retina, DHA is the major FA (~30%) in the disc membrane of retinal photoreceptor outer segment, where photopigment rhodopsin processes phototransduction (Sangiovanni and Chew, 2005). DHA accounts for 20-30% of the FAs in PC, PE, and PS of outer segment disc membranes. Newborns with *n*-3 FA deficiency exhibit reduced light sensitivity of retinal rod photoreceptors, and DHA supplementation enhances visual resolution acuity (Uauy *et al*., 2001).

One of the functions of DHA is to generate lipid mediators, which actively turn off the inflammatory responses in tissues. The lipid mediators formed from DHA include D-series resolvin (resolvin D1-D4), protectin D1 (neuroprotectin D1), and maresin 1 (Bannenberg and Serhan, 2010). These molecules constitute novel families of lipid mediators that are structurally unrelated to authentic eicosanoids, such as prostaglandin or leuztriene, and that display potent anti-inflammatory and tissue-protective actions such as reduced neutrophil migration and activation of phagocytosis by macrophages (Bannenberg and Serhan, 2010).

In the retina, continuous light absorption by photoreceptors induces oxidative stress in the photoreceptor outer segments. To replace the damaged outer segments, photoreceptors shed the distal tips of the outer segments, which are phagocytosed by retinal pigment epithelial (RPE) cells. RPE cells respond to oxidative stress by synthesizing protectin D1 from DHA in the phagocytosed outer segment membranes (Bazan *et al*., 2010). Protectin D1 promotes the survival of RPE cells, and, as a consequence, photoreceptor cell integrity (Mukherjee *et al*., 2007).

ULC-PUFAS IN THE TESTIS, BRAIN, AND RETINA

ULC-PUFAs are found in the testis, brain, and retina (Agbaga *et al*., 2010). In the mammalian testis and spermatozoa, *n*-6 and *n*-3 ULC-PUFAs with chain-lengths of C26-C32 and 3-6 double bonds are present uniquely in sphingolipids including SMs, Cers, and fucosylated glycosphingolipids (FGSLs) (Sandhoff *et al*., 2005; Furland *et al*., 2007b; Rabionet *et al*., 2008). The level of ULC-PUFAs in these sphingolipids increase with the onset of spermatogenesis, and ULC-PUFAs account for up to 15% and 40% of rat testicular SMs and Cers, respectively (Furland *et al*., 2007b). *Galgt1* gene KO mice (encoding GM2 synthase) lack a subset of ULC-PUFAs-containing FGSLs and are infertile with an arrest of spermatogenesis at the stage of round spermatids (Sandhoff *et al*., 2005). Interestingly, ULC-PUFAs-containing SMs and Cers are enriched in the head of the spermatozoa, and ULC-PUFAs-containing SMs are converted to the corresponding Cers during sperm capacitation *in vitro*, suggesting a role for ULC-PUFAs-containing SMs and Cers in acquiring competency for fertilization (Furland *et al*., 2007a).

In the brain, *n*-6 and *n*-3 series of ULC-PUFAs with chain-lengths of C26-C38 and 4-6 double bonds are found in PCs at the sn-1 position, with *n*-6 series ULC-PUFAs being predominant (Poulos *et al*., 1988). In rats, the levels of PCs containing ULC-PUFAs in the brain are higher in the neonatal and early postnatal periods than in adults, suggesting a role in postnatal brain development (Robinson *et al*., 1990).

ELOVL4 is involved in the synthesis of ULC-PUFAs as well as saturated and monounsaturated ULCFAs (Fig. 1). The recessive mutations in the *ELOVL4* gene mentioned above are associated with not only ichthyosis but also seizures, intellectual abnormality, and spastic quadriplegia, suggesting the importance of ULC-PUFAs in brain development and physiology (Aldahmesh *et al*., 2011).

In the retina, similar to the brain, ULC-PUFAs are found at the *sn*-1 position of PCs. These ULC-PUFAs are predominantly *n*-3 series with chain-lengths of C26-C36 and 3-6 double bonds (Aveldano and Sprecher, 1987). These ULC-PUFA-containing PCs also have PUFAs (predominantly DHA) at the *sn*-2 position, thus as many as 12 double bonds are present in a single PC molecule (Aveldano and Sprecher, 1987). In retina-specific *Elovl4* KO mice, ULC-PUFA-containing PCs in the adult retina were severely decreased (Harkewicz *et al*., 2012).

STDG3 is an early onset, autosomal dominant form of macular degeneration that is characterized by decreased visual acuity, flecks in fundus flavimaculatus, and macular dystrophy (Donoso *et al*., 2001). STDG3 is caused by mutations in the *ELOVL4* gene on chromosome 6 (Table 2) (Zhang *et al*., 2001; Vasireddy *et al*., 2010). All three STDG3 causative mutations are located in the last exon (exon 6) of ELOVL4 and result in the production of C-terminally truncated proteins lacking the ER retention signal. These dominant mutations are located closer to the C-terminus of the ELOVL4 protein than the recessive mutations that result in ichthyosis and other nervous system abnormalities, and it may explain the differences in the symptoms and the mode of inheritance. A knock-in mouse model carrying one of the STGD3 mutations was generated and displayed characteristic features associated with the STGD3, such as the accumulation of lipofuscin in RPE and photoreceptor degeneration (Vasireddy *et al*., 2006). STGD3 mutant ELOVL4 proteins exhibited no activity *in vitro* and were misrouted to perinuclear aggresomes (Karan *et al*., 2005; Okuda *et al*., 2010). Moreover, STGD3 mutant ELOVL4 proteins are able to form hetero-oligomeric complexes with other components of the elongation machinery as well as homo-oligomeric complex with wild type ELOVL4 (Okuda *et al*., 2010).

2-HYDROXYLATED (2-OH) VLCFAS IN THE MYELIN

A subset of sphingolipids contains 2-OH VLCFAs. 2-OH VLCFAs are found almost exclusively in sphingolipids and are abundant in Cers in the epidermis and GalCers in the brain and kidney (Hama, 2010). In the brain, myelin contains abundant amounts of C24 GalCers and C24 sulfatides. The levels of 2-OH GalCers and 2-OH sulfatides increase during the course of myelination (Alderson *et al*., 2006).

2-Hydroxylation of FAs is catalyzed by the fatty acid 2-hydroxylase (FA2H) (Mizutani *et al*., 2008; Hama, 2010). Mutations in the *FA2H* gene on chromosome 16 are associated with leukodystrophy with spastic paraparesis and dystonia (Table 2) (Edvardson *et al*., 2008), indicating the importance of 2-OH GalCer/sulfatide for myelin formation and maintenance (Hama, 2010).

OTHER DISEASE-ASSOCIATED MUTATIONS IN THE VLCFA SYNTHESIS PATHWAY

A mutation in the *HACD1* gene (*PTPLA*) on chromosome 10 was identified in congenital myopathy characterized by

the severe hypotonicity and the absence of deep tendon reflexes (Table 2) (Muhammad *et al*., 2013). HACD1 is one of the four mammalian HACD isozymes (HACD1-4) and catalyzes the third step of the FA elongation cycle: dehydration of 3-hydroxyacyl-CoA to *trans*-2-enoyl-CoA (Fig. 2) (Ikeda *et al*., 2008). The expression of *HACD1* gene is highly specific to the heart and skeletal muscle (Li *et al*., 2000). The mutant HACD1 protein, which is C-terminally truncated by the introduction of nonsense codon at the Tyr residue 248 (Tyr248Stop), exhibits no activity toward 3-hydoroxy C16:0-CoA *in vitro* (Muhammad *et al*., 2013). A mutation of the dog *HACD1* gene caused by the insertion of a short interspersed element (SINE) in exon 2 also causes myopathy (Pelé *et al*., 2005). SINE insertion leads to multiple splicing defects and severely reduces the amount of wild type transcripts.

A mutation in the *TER* (*TECR*) gene on chromosome 19 encoding *trans*-2-enoyl-CoA reductase has been identified in an autosomal recessive form of non-syndromic mental retardation (Table 2) (Çalişkan *et al*., 2011). TER catalyzes the fourth step and last of the FA elongation cycle: NADPH-dependent reduction of *trans*-2-enoyl-CoA to acyl-CoA (Fig. 2) (Moon and Horton, 2003). The mutation substitutes the Pro residue at 182 to Leu (P182L) in the TER protein (Çalişkan *et al*., 2011). TER is an ER resident membrane protein with six predicted membrane-spanning domains, and the Pro-182 residue is likely to be located in the second luminal loop. The P182L mutation reduces both the activity and stability of the TER protein, thereby impairing the FA elongation cycle (Abe *et al*., 2013). Lipid analysis of B-lymphoblastoid cell lines derived from patients revealed a change in the sphingolipid profile and decreased levels of C24 Cers and C24 SMs (Abe *et al*., 2013).

MUTATIONS IN THE VLCFA DEGRADATION PATHWAY

Impairment in VLCFA degradation pathway leads to several diseases (Table 2). VLCFAs are transported, as VLCFA-CoAs, into peroxisomes, where they are subjected to β-oxidization into long-chain or shorter acyl-CoAs, followed by transport to the mitochondria and further cycles of β-oxidization (Wanders, 2014). Peroxisomal membrane protein ABCD1, a member of ABC transporters encoded by the *ABCD1* gene on X chromosome, transports VLCFA-CoAs into peroxisomes (van Roermund *et al*., 2008; Morita and Imanaka, 2012). Mutations in the *ABCD1* gene cause X-linked adrenoleukodystrophy (X-ALD), the most common peroxisomal disorder with more than 600 different mutations characterized by progressive demyelination and adrenal insufficiency (Table 2) (Mosser *et al*., 1993; Berger *et al*., 2010; Engelen *et al*., 2012). A defect in ABCD1 impairs the VLCFA degradation process, resulting in elevated levels of saturated C24-C26 VLCFAs in the plasma, brain, adrenal grand, and other tissues (Berger *et al*., 2010; Kemp *et al*., 2012). The accumulation of saturated VLCFAs is believed to play a crucial role in the pathogenesis of X-ALD such as demyelination with inflammation (Paintlia *et al*., 2003). As mentioned earlier, ELOVL1 is responsible for the synthesis of saturated and monounsaturated C24-C26 VLCFAs such as those accumulated in X-ALD. In fibroblasts derived from X-ALD patients, suppression of VLCFA synthesis by knockdown of *ELOVL1* partially restores C26:0 levels, suggesting ELOVL1 is a potential target for the treatment of X-ALD (Ofman *et al*., 2010). Lorenzo's oil, a 4:1 mixture of glyceryl trioleate and glyceryl trierucate, has been used to reduce the saturated VLCFA level in the plasma of X-ALD patients (Rizzo *et al*., 1989; Berger *et al*., 2010). Biochemical analysis demonstrated that oleic and erucic acids inhibit ELOVL1, suggesting that inhibition of ELOVL1 may be an underlying mechanism by which Lorenzo's oil exerts its action (Sassa *et al*., 2014)

Peroxisomal β-oxidation of VLCFAs consists of four sequential reactions: dehydrogenation, hydration, dehydrogenation, and thiolytic cleavage (Wanders, 2014). The first dehydrogenation step, which converts acyl-CoA to *trans*-2-enoyl-CoA, is catalyzed by acyl-CoA oxidase 1 (ACOX1). Mutations in the *ACOX1* gene on chromosome 17 result in autosomal recessive peroxisomal acyl-CoA deficiency (or pseudoneonatal adrenoleukodystrophy) with similar clinical features to those of X-ALD including the accumulation of VLCFAs and leukodystrophy (Table 2) (Poll-The *et al*., 1988). Both the second and third steps of VLCFA β-oxidation, hydration of *trans*-2-enoyl-CoA, and dehydrogenation of 3-hydroxyacyl-CoA, respectively, are catalyzed by single enzyme 17β-hydroxysteroid dehydrogenase 4 (HSD17β4; D-bifunctional protein). Homozygous mutations in the *HSD17β4* gene on chromosome 5 have been identified in the disorder called D-bifunctional protein deficiency with clinical features similar to those of peroxisomal acyl-CoA deficiency and X-ALD (Table 2) (Watkins *et al*., 1995). The fourth step, which converts 3-ketoacyl-CoA to acyl-CoA, is catalyzed by peroxisomal 3-oxoacyl-CoA thiolase encoded by the *acetyl-CoA acyltransferase 1* (*ACAA1*) gene on chromosome 3 (Wanders, 2014). No mutations in the *ACAA1* gene associated with disorders have been identified.

CONCLUSIONS

VLCFAs are FAs with a chain-length of C>20. VLCFAs with C≥26 are sub-classified as ULCFAs and found in limited tissues including the skin, retina, brain, testis, and meibomian gland. VLCFAs are variously present in sphingolipids, glycerophospholipids, and other forms of lipids including ω-*O*-acyl-ULCFAs. VLCFAs are synthesized by the FA elongation cycle in the ER. Identification of the enzymes that participate in FA elongation, modification, transport, and degradation enabled the study of specific VLCFA species. VLCFAs play multiple roles not substituted by LCFAs. KO mice for VLCFA-related genes exhibit various phenotypes, and mutations in VLCFA-related genes cause inherited disorders including ichthyosis, macular dystrophy, myopathy, mental retardation, and demyelination. VLCFAs may regulate cellular functions by affecting membrane properties including membrane fluidity, permeability, curvature, and lipid microdomain formation. Nevertheless, our present knowledge of VLCFAs is still the tip of the iceberg. Further identification of novel VLCFA species, VLCFA-related metabolic pathways, genes, and disorders will reveal the diverse and unique functions of VLCFAs, and may lead to intervention for lipid-associated diseases.

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