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FORUM REVIEW ARTICLE

Sphingolipid Metabolism, Oxidant Signaling, and Contractile Function of Skeletal Muscle

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Abstract

Significance: Sphingolipids are a class of bioactive lipids that regulate diverse cell functions. Ceramide, sphingosine, and sphingosine-1-phosphate accumulate in tissues such as liver, brain, and lung under conditions of cellular stress, including oxidative stress. The activity of some sphingolipid metabolizing enzymes, chiefly the sphingomyelinases, is stimulated during inflammation and in response to oxidative stress. Ceramide, the sphingomyelinase product, as well as the ceramide metabolite, sphingosine-1-phosphate, can induce the generation of more reactive oxygen species, propagating further inflammation. ***Recent Advances:*** This review article summarizes information on sphingolipid biochemistry and signaling pertinent to skeletal muscle and describes the potential influence of sphingolipids on contractile function. ***Critical Issues:*** It encompasses topics related to (1) the pathways for complex sphingolipid biosynthesis and degradation, emphasizing sphingolipid regulation in various muscle fiber types and subcellular compartments; (2) the emerging evidence that implicates ceramide, sphingosine, and sphingosine-1-phosphate as regulators of muscle oxidant activity, and (3) sphingolipid effects on contractile function and fatigue. ***Future Directions:*** We propose that prolonged inflammatory conditions alter ceramide, sphingosine, and sphingosine-1-phosphate levels in skeletal muscle and that these changes promote the weakness, premature fatigue, and cachexia that plague individuals with heart failure, cancer, diabetes, and other chronic inflammatory diseases. *Antioxid. Redox Signal*. 15, 2501–2517.

Introduction

Sphingolipid metabolism is emerging as a novel and important aspect of skeletal muscle biology. It is a challenging field of research. Lipid mediators are elusive and difficult to measure. Proteins that regulate sphingolipid signaling are not fully characterized, and it is likely that many remain undiscovered. As a result, data on this topic have accumulated slowly over the past two decades. Our current knowledge comes from disparate sources. A few labs have labored in this field systematically, but many observations are isolated events that emerged tangentially, products of research in related areas (14, 22, 118).

Despite these limitations, the data have now reached a critical mass. It is clear that sphingolipids are ubiquitous in skeletal muscle and have broad potential to influence muscle function (89). Sphingolipid metabolites alter the biochemical properties of key regulatory proteins and modulate cellular processes that range from satellite cell proliferation (94) to mitochondrial respiration (117), from apoptosis (16) to voltage-dependent calcium release (109). Muscle-derived oxidants—reactive oxygen species (ROS) and nitric oxide (NO) derivatives—play a complex role in these responses. Oxidants can influence regulatory enzymes to modulate sphingolipid metabolism (17) and also appear to serve as downstream messengers for sphingolipid signaling (103).

The current article explores the conceptual model that sphingolipids act *via* muscle-derived oxidants to modulate contractile function of skeletal muscle. The text reviews basic concepts of sphingolipid biology, integrating information on skeletal muscle where available, and outlines the links with redox homeostasis and contractile regulation. Our story is told in three sections, each reflecting a major element of the proposed model.

Sphingolipid Biochemistry, Cell Biology, and Signaling

Ceramides (Fig. 1) form the hydrophobic backbone of all complex sphingolipids (47) and consist of a long-chain sphingoid base and amid linked fatty acid that is either saturated or unsaturated and vary in length from 2 to 28 carbon atoms. In mammalian cells, the most commonly found ceramides have D-*erythro*-sphingosine and a saturated fatty acyl chain of 16 carbon atoms and are among the most hydrophobic lipids in the membrane. Dihydroceramide differs from ceramide inasmuch as the latter contains a trans 4, 5 double bond, which is essential for some of the bioactive roles of ceramide.

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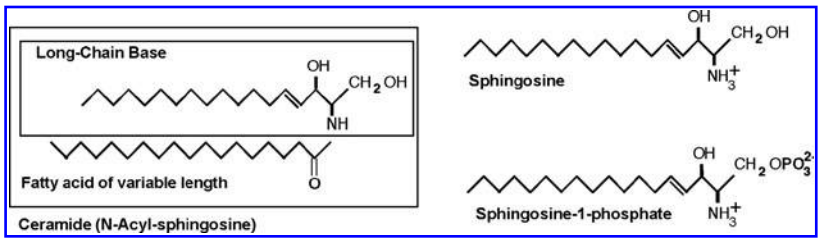

FIG. 1. Chemical structures of major sphingolipid metabolites. Diagrams illustrate structures of three commonly studied metabolites.

Sphingolipids are a diverse group of lipids (Fig. 2) with a critical role in cell membrane structure and organization of lipid signaling platforms. In addition, ceramide, sphingosine, and sphingosine-1-phosphate are bioactive signaling molecules that regulate cell survival, stress response, and complex physiological processes such as organogenesis, angiogenesis, and the innate immune response (Fig. 3). Ceramide has been extensively studied for its role as a cellular sensor to stress. The current paradigm states that the levels of ceramide are low in normal healthy cells, but increase during various stress conditions. Many extracellular agents, such as tumor necrosis factor-α (TNF), interleukin-1β (IL-1β), Fas ligand, ionizing radiation, and chemotherapeutic drugs, have been shown to trigger generation of ceramide through distinct metabolic pathways. The resulting transient elevation in ceramide content is found to initiate or modulate cellular responses by (1) inhibiting growth pathways, such as those mediated by Akt; (2) inducing apoptotic molecules, such as the caspases; and (3) activating proinflammatory second messengers, including c-Jun *N*-terminal kinase (JNK) and nuclear factor-kappa B (NF-κB). Ceramide directly interacts with and regulates phosphatases from the protein phosphatase 2 (PP2A) family (20), protein kinase C zeta (PKCζ), and Ceramide-activated Protein Kinase. Ceramide mediates the activation of JNK (21, 79, 101) and is required for activation of NF-κB (5, 127) in response to various cytokines.

Metabolic products of ceramide turnover, notably sphingosine-1-phosphate, have been similarly linked to the regulation of various cellular responses such as inflammation, proliferation, and apoptosis. In contrast to ceramide, however, sphingosine-1-phosphate has emerged as a soluble bioactive lipid that regulates cellular response by binding and activating a family of G-protein coupled receptors, termed sphingosine-1-phosphate receptors 1–5 ($S1P_{1-5}$).

Investigators have become increasingly aware that many aspects of sphingolipid metabolism are linked to the state of oxidative stress of the cells. Some have proposed that increases in ceramide levels and the onset of oxidative stress are mechanistically coupled and represent a point of integration of cellular stress response. These correlations have been studied in detail with regard to apoptotic cell death and have been reviewed elsewhere (7). Skeletal muscle is often exposed to oxidative stress, for example, during strenuous exercise, heat stress, chronic inflammatory diseases, and infection. Nevertheless, sphingolipid metabolism, signaling, or connections to oxidative stress have been rarely explored in this tissue. The goal of this review is to summarize and evaluate the existing literature as it relates to skeletal muscle.

De novo *synthesis of ceramide*

Sphingolipid biosynthesis starts in the endoplasmic reticulum (ER) with the condensation of L-serine and palmitoyl CoA to generate free sphingoid base, a reaction catalyzed by serine palmitoyltransferase (SPT) (see Table 1 for nomenclature). The product, 3-ketosphinganine, is first reduced to sphinganine and then acylated at the amide group by dihydroceramide synthase (CerS) forming dihydroceramide (90). CerS exhibit strict specificity in terms of the length of the fatty acid added to the sphingoid base and determine the fatty acid composition of sphingolipids in the cell. This is important, because emerging data suggest that ceramides with different fatty acids have different biological properties. Most dihydroceramides are immediately converted to ceramides by the introduction of a characteristic 4,5-double bond in the sphingoid base of the molecule.

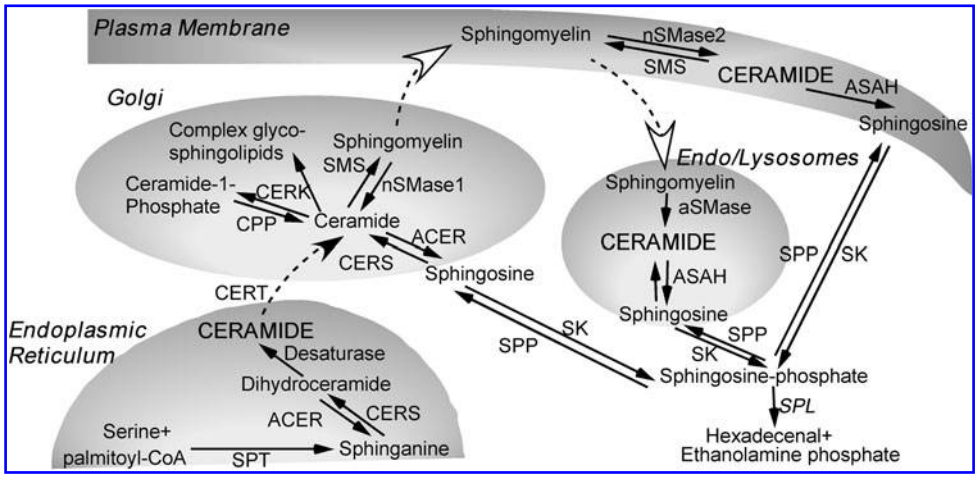

FIG. 2. Metabolism of sphingolipids in mammalian cells. SPT, Serine palmitoyltransferase; CERS, (dihydro) ceramide synthase; dihydroceramide desaturase; CERT, ceramide transfer protein; SMS, sphingomyelin synthase; CERK, ceramide kinase; CPP, ceramide-1-phosphate phosphatase; nSMase1, nSMase2, aSMase, sphingomyelinase; ASAH1,2, ACER, ceramidase; SK, sphingosine kinase; SPL, sphingosine phosphate lyase; SPP, sphingosine phosphate phosphatase.

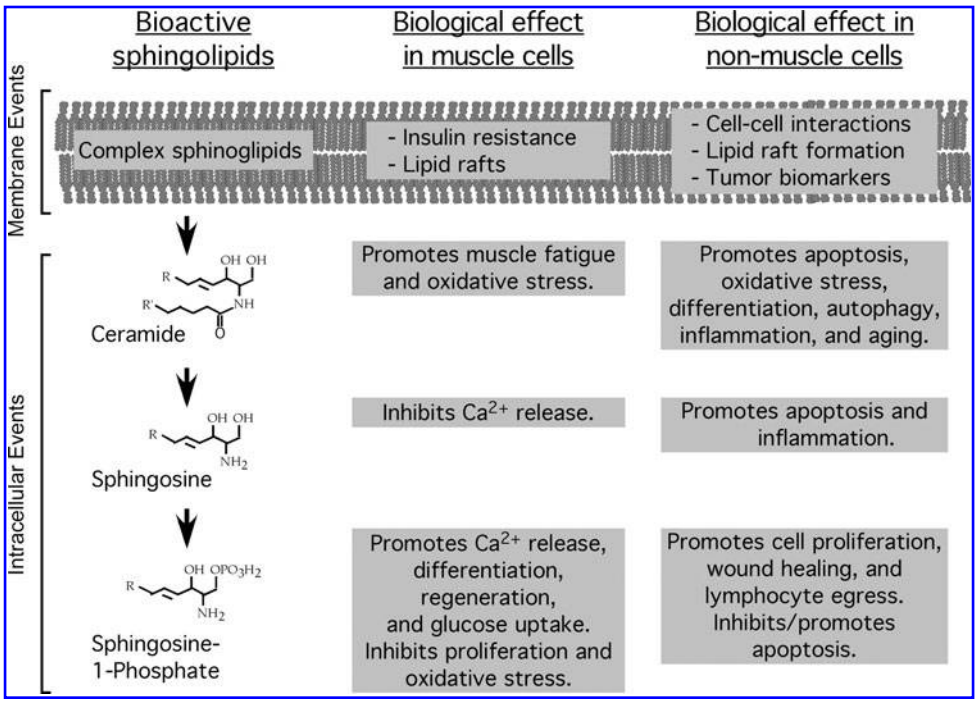

FIG. 3. Cellular responses to sphingolipid metabolites. Summary of biological activities reported for major sphingolipid mediators in muscle *versus* nonmuscle cell types.

Comparative analyses of the specific SPT activity in microsomes isolated from various tissues of the rat (89) show that microsomes from skeletal muscle exhibit very high SPT activity. Skeletal muscle is second only to lung in SPT activity and is well above the activities measured in kidney, spleen, liver, stomach, intestine, brain, ovary, heart, and pancreas. This suggests that skeletal muscle has very active *de novo* synthesis of sphingolipids. However, muscle was also the single tissue exhibiting very large animal-to-animal deviations in the measured SPT values, suggesting that SPT activity may be influenced by various factors, both external (diet, exercise status, *etc*.) and intrinsic (muscle fiber type). Indeed, many studies provide evidence to that extent: The activity and expression of SPT is upregulated after the consumption of diet rich in palmitic acid and in various animal model of obesity. The resulting accumulation of ceramide (and more complex metabolites, such as glucosylceramide, introduced below) is linked to the onset of insulin resistance in muscle. These studies have been extensively reviewed elsewhere (117, 118) and will not be discussed here. In turn, physical activity also has been found to affect skeletal muscle SPT mRNA level. The latter is significantly increased in men after 6 mo of inactivity, whereas endurance exercise completely prevents this increase (112). In the heart, inhibition of SPT was shown to decrease fatty acid oxidation, increase glucose oxidation, improve systolic function, and decrease apoptosis (98), suggesting a critical role for SPT in the pathogenesis of lipotoxic cardiomyopathy.

The next step in the *de novo* pathway of sphingolipid synthesis, ceramide synthase (CerS), is poorly characterized in muscle. Studies using the mycotoxin Fumonisin B1, which inhibits CerS, but does not affect SPT, provide evidence that CerS might be required for some muscle functions. Fumonisin B1 was shown to suppress palmitate-induced apoptosis in cardiomyocytes (34) and to delay myogenic differentiation (87); however, the specific forms of CerS involved in these processes were not identified. CerS2 is the major CerS expressed by most tissues, but this isoform is undetectable in skeletal muscle. Instead, skeletal muscle expresses high levels of CerS 1, 4, and 5 (72). The CerS2 enzyme exhibits high specificity toward long-chain fatty acids, such as C20, C22, and C26. CerS 1, 4 and 5 prefer shorter fatty acids: C18, C18/C20, and C16, respectively. The distinct expression profile of CerS isoforms indicates that skeletal muscles may have a unique make-up with regard to the fatty acid content of sphingolipids. Indeed, sphingolipids with C26:0 and C26:1 ceramides are the major species found in liver, based on mass spectrometry analyses, whereas the ceramide in vastus lateralis muscle (54) and C2C12 myotubes (37) seemed to contain mostly palmitic (C16) and stearic acid (C18).

Synthesis of complex sphingolipids and glycosphingolipids

Once ceramide/dihydroceramide is synthesized in the ER, it is carried to the Golgi apparatus and converted to sphingomyelin (SM) or glycosphingolipids (glucocerebrosides and gangliosides) by the addition of a distinct head group to its primary hydroxyl. These lipids are components of the outer leaflet of the plasma membrane and although are not considered "bioactive" molecules, complex sphingolipids are important modulators of lipid microdomains (lipid rafts) and participate in cell–cell and cell–matrix interactions. The ceramide substrate is transferred from the ER to the Golgi in a nonvesicular manner by ceramide transfer protein CerT1 (52). Mice carrying a deletion of CerT exhibit severely compromised cardiac function and die at embryonic day 11.5, suggesting a critical role for this novel protein in cardiac development.

SM synthase 1 (SMS1) and 2 (SMS2) catalyze SM synthesis by transferring a phosphorylcholine head group from phosphatidylcholine to the primary hydroxy group of ceramide

Table 1. Nomenclature of Proteins and Genes Involved in Sphingomyelin Synthesis and Degradation

Protein Name	*Protein abbreviation*	*Entry in Uniprot*	*Gene symbol*
Serine palmitoyltransferase 1	SPT1	LCB1_MOUSE	Sptlc1
Serine palmitoyltransferase 2	SPT2	LCB2_MOUSE	Sptlc2
Ceramide synthase 1	CERS1	LASS1_MOUSE	Lass1
LAG1 longevity assurance homolog 1			(Uog-1)
Ceramide synthase 2	CERS2	LASS2_MOUSE	Lass2
LAG1 longevity assurance homolog 2			
Ceramide synthase 3	CERS3	Q195J4_MOUSE	Lass3
Longevity assurance 3-like protein var. 2			
Ceramide synthase 4	CERS4	LASS4_MOUSE	Lass4
LAG1 longevity assurance homolog 4			
Ceramide synthase 5	CERS5	LASS5_MOUSE	Lass5
LAG1 longevity assurance homolog 5			
Ceramide synthase 6	CERS6	LASS6_MOUSE	Lass6
LAG1 longevity assurance homolog 6			
Dihydroceramide desaturase	DES2	Q8R2F2_MOUSE	Degs2
(Sphingolipid delta 4 desaturase)			
Ceramide kinase	CERK	Q52KP2_MOUSE	Cerk
Ceramide-phosphate phosphatase	CPP	LPP3_MOUSE	Ppap2B
(Lipid phosphate phosphohydrolase 3)			
Sphingomyelin synthase 1	SMS1	SMS1_MOUSE	Sgms1
(Phosphatidylcholine:ceramide cholinephosphotransferase 1)			
Sphingomyelin synthase 2	SMS2	SMS2_MOUSE	Sgms2
(Phosphatidylcholine:ceramide cholinephosphotransferase 2)			
Acid sphingomyelinase	ASMase	ASM_MOUSE	Smpd1
(Sphingomyelin phosphodiesterase 1)			
Neutral sphingomyelinase 1	NSMase1	NSMA_MOUSE	Smpd2
(Sphingomyelin phosphodiesterase 2)			
Neutral sphingomyelinase 2	NSMase2	NSMA2_MOUSE	Smpd3
(Sphingomyelin phosphodiesterase 2)			
Neutral sphingomyelinase 3	NSMase3	NSMA3_MOUSE	Smpd4
(Sphingomyelin phosphodiesterase 4)			(Kiaa1418)
Neutral sphingomyelinase 4	NSMase4	N/A	Smpd5
(Sphingomyelin phosphodiesterase 5)			
Acid ceramidase	ASAH1	ASAH1_MOUSE	Asah1
Neutral ceramidase	ASAH2	ASAH2_MOUSE	Asah2
Alkaline ceramidase 1	ACER1	ACER1_MOUSE	Asah3(Acer1)
Alkaline Ceramidase 2	ACER2	ACER2_MOUSE	Acer2 (Asah3l)
(Isoform 1 of alkaline ceramidase 2)			
Alkaline ceramidase 3	ACER3	ACER3_MOUSE	Acer3
(Alkaline phytoceramidase)		(APHC_MOUSE)	(Aphc)
Sphingosine kinase 1	SK1	O88885_MOUSE	Sphk1
Sphingosine kinase 2	SK2	SPHK2_MOUSE	Sphk2
Sphingosine-1-phosphate phosphatase 1	SPP1	SGPP1_MOUSE	Sgpp1
Sphingosine-1-phosphate phosphatase 2	SPP2	SGPP2_MOUSE	Sgpp2

Alternative names are given in parentheses.

(119). In turn, glucosylceramide synthase is a Golgi-localized, membrane-bound glycosyl transferase that catalyzes the glycosylation of ceramide to produce glycosylceramide. Glucosylceramide is a key player in the biosynthesis of more complex sphingolipids that are generated through stepwise addition of galactose, glucose, *N*-acetylglucosamine, or *N*-galactosamine (123). Golgi-synthesized complex glucosphingolipids and SM undergo vesicular trafficking to the plasma membrane, where they are localized mainly at the outer leaflet.

SM accounts for 7% of all phospholipids in skeletal muscle. This is relatively low as compared with other tissues such as liver (12%), kidney (17%), lung (20%), and brain (23%). The two SMSs, SMS1 and 2, are ubiquitously expressed in most tissues, including muscle and heart (59). These isoforms differ in subcellular localization; SMS1 is found in the Golgi apparatus, whereas SMS2 is localized to the plasma membrane. The functions of SMS1/2 in muscle tissue have not been explored, despite studies in the early 1980s that show significant differences in SM content of the sarcoplasmic reticulum (SR) among muscle types. Slow-twitch muscle fibers in soleus were found to contain thrice more SM than caudofemoralis (fast-twitch) fibers. These differences were proposed to have functional significance in regulating Ca^{2+}-ATPase activity (13).

Biochemical analyses of rabbit skeletal muscle have found that the major neutral cerebroside is glucosylceramide with only traces of lactosylceramide detectable in whole-muscle extracts. Glucosylceramide is found mostly in the sarcolemmal and T-tubular vesicles, but not in SR-2 or triad/mito-

chondrial fractions. Lactosylceramide has been selectively recovered from the less dense SR-1 fraction. Rabbit skeletal muscle is also characterized by the presence of ganglioside GM3 with approximately two-thirds associated with the sarcolemma.

Very little is known about the synthesis of these lipids in mammalian skeletal muscle. Differential enrichment of glycosylceramide and GM3 suggest compartmentalization of glucosyltransferase and galactosyltransferase, regulated membrane trafficking, or a specific sorting mechanism in skeletal muscle. These data also indicate different subcellular localizations for glucosyl-, galactosyl-, and sialyl-transferase with GM3 biosynthesis taking place in the sarcolemma of myotubes (93). Studies of glycoconjugate metabolism in developing rabbit skeletal muscle revealed a strong decrease in sialyltransferase activity of SR, compared with sarcolemma, during development from neonatal to adult stage. *In vitro* liposome-reconstitution studies suggest a different functional role for gangliosides as modulators of cytosolic calcium levels in muscle (93). Glucosphingolipid biosynthesis is tightly regulated during myogenesis *in vitro*, suggesting a role for membrane gangliosides in muscle cell differentiation (6, 75). One of the better-understood functions of glycosphingolipids is in regulation of insulin response of muscle. Inhibitor studies of glucosphingolipid synthesis strongly suggest that glucosylceramides promote insulin resistance and diabetes. Patients with Gaucher's disease, a lysosomal storage disease caused by the failure to degrade glucosylated ceramides, are insulin resistant (71). Conversely, knockout mice that lack GM3 synthase, which produces higher-order glycosphingolipids, have improved insulin sensitivity (117); and pharmacologic blockade of glycosphingolipid synthesis improves glycemic control and insulin sensitivity in animal models of type 2 diabetes (126).

Turnover of SM to ceramide

The sphingomyelinase (SMase) family is a group of biochemically and genetically different enzymes, all of which hydrolyze SM to ceramide and phosphorylcholine. SMase activities with neutral and acidic pH optima are found in most mammalian cells, and an SMase active in alkaline pH is localized in the intestinal wall. Currently, research is focused on four genes encoding different mammalian SMases: *smpd1* encodes two forms of acidic SMase. One is associated with the endosomal/lysosomal compartment. A second, secretory form is found in plasma and in conditioned medium of stimulated cells. *smpd2* and *smpd3* encode neutral SMase 1 (nSMase1) and 2 (nSMase2). Both are Mg^{2+}-dependent but differ in their subcellular localization and role in signaling. Data from several labs have shown that nSMase1 is a housekeeping enzyme with no particular function in signaling. nSMase2 is regulated by cytokines such as IL-1β and TNF and mediates some cytokine effects (28, 64, 81). The recently cloned *smpd4* encodes a novel nSMase3 isoform that is found predominantly in skeletal muscle and heart. A mitochondria-specific *smpd5* was also recently identified; its expression is highest in testis, pancreas, and fat but is low in heart. Finally, two novel SMase isoforms with MW of 92 and 53 kDa were purified from rabbit muscle over two decades ago (44). These isoforms are not linked to any of the known genes for SMase, and their role in muscle remains undefined.

Turnover of ceramide to sphingosine

The metabolic fate of SMase-generated ceramide is complex and depends on the site of its production. The principle metabolic pathway for ceramide is *via* ceramidase-mediated turnover to free fatty acid and a sphingoid base. Ceramide may also be recycled back to SM in the plasma membrane, or phosphorylated to ceramide-1-phosphate by a ceramide kinase. Two types of ceramide-hydrolyzing activities, with either an acidic or an alkaline pH optimum, have been described in muscle tissues (114). However, the basal ceramidase activity in muscle is low compared with other tissues.

Ceramidases are a family of five enzymes that differ in their biochemical characterization, subcellular localization, and pH optimum (80). Acid ceramidase (*N*-acylsphingosine amidohydrolase 1, ASAH1) is a heterodimer synthesized from a single protein precursor and has an acidic pH optimum. The enzyme localizes to the lysosomal/endosomal compartment and also can be secreted. ASAH1 mRNA is ubiquitously and highly expressed in kidney, lung, heart, and brain but is found at relatively low levels in spleen, skeletal muscle, and testes (76). A ceramidase with neutral pH optimum termed neutral ceramidase (*N*-acylsphingosine amidohydrolase 2, ASAH2) also has been cloned and characterized. ASAH2 is a heavily glycosylated protein that localizes to the plasma membrane and is ubiquitously expressed (80). Expression is particularly high in small intestines (68), suggesting a role in digestion of dietary sphingolipids. A form of ASAH2, ASAH2C, is proposed to function as a mitochondria-specific ceramidase (35) and is highly expressed in skeletal muscle and heart. This form, however, remains poorly characterized. Three alkaline ceramidases (ACER), known as ACER1, 2 and 3, have also been characterized. ACER1 and 3 are localized at the ER/Golgi, whereas the subcellular distribution of ACER2 is unknown. The tissue distribution of these ceramidases is not well characterized.

In skeletal muscle, sphingosine has been studied for its capacity to modulate Ca^{2+} release from the SR [for a review see Sabbadini *et al.* (109)], potentially influencing muscle contraction and force. The junctional T-tubule membranes have the highest sphingosine content, based on HPLC analyses of various subcellular membranes. T-tubules also contain both enzymes required to synthesize sphingosine, nSMase and ceramidase, although the genes that code for these enzymes are unknown. Sphingosine has been shown to block calcium release through the SR ryanodine receptor (RyR). Sphingosine-blocked calcium release is coincident with the inhibitory effects of sphingosine on [^{3}H]ryanodine binding to the RyR (109). In contrast to the ability of sphingosine to block RyR, another sphingolipid metabolite, lysosphingomyelin, can be a potent activator of calcium release. Lysosphingomyelin is produced from SM *via* a ceramidase-like activity (sphingolipid *N*-deacylase) and seems to act by binding to and activating SCaMPER (18), a double-pass membrane protein that was first characterized in cardiomyocytes. SCaMPER is localized to the sarcotubular junction together with dihydropyridine and RyRs. Antisense knockdown of SCaMPER mRNA substantially reduces sphingolipid-induced calcium release, suggesting that SCaMPER is a functional calcium channel.

Acute exercise increases ceramide turnover to sphingosine in soleus and gastrocnemius muscles (30). Strangely, this

increased turnover is paralleled by an increase in the rate of *de novo* synthesis of ceramide. The functional importance of this enhanced "recycling" of ceramide is not understood.

Sphingosine-1-phosphate and its receptor

Sphingosine is the major free sphingoid base in mammalian cells and is produced solely through ceramidases. Once generated by the ceramidase, sphingosine can be reacylated to reform ceramide as a part of the "salvage" pathway. Or, sphingosine can be phosphorylated by sphingosine kinase to form sphingosine-1-phosphate. Free sphinganine is the less abundant, saturated counterpart of sphingosine. Sphinganine is produced either through the *de novo* pathway or by the action of SMases and ceramidases on sphinganine-containing substrates and is an alternative substrate for the sphingosine kinases.

Two sphingosine kinase isoforms, SK1 and SK2, have been cloned and characterized in mammalian cells. SK1 and SK2 are products of separate genes and are highly homologous. SK1 is a cytosolic protein but can translocate to the plasma membrane in response to cellular stimuli. SK2 is localized to the ER and the nucleus [for recent in-depth reviews see Snider *et al.* (113) and Kim *et al.* (66)]. The SK1/SK2 double knockout is embryonic lethal due to inadequate blood vessel formation. In contrast, the single knockouts have no phenotypes due to apparent redundancies in function of the two isoforms (92). Sphingosine kinase activity is stimulated by growth factors, including insulin-like growth factor-1, platelet-derived growth factor, vascular endothelial growth factor and nerve growth factor, and by cytokines that include TNF and IL-1β. The product of this enzyme, sphingosine-1-phosphate, can act intracellularly to regulate cell function and gene expression. Sphingosine-1-phosphate can also be secreted, exerting extracellular effects as an autocrine/paracrine mediator. Evidence from C2C12 myotubes also suggests that sphingosine-1-phosphate can be generated at the extracellular leaflet of the plasma membrane by SK1 and can act in an autocrine manner (83). Five G protein-coupled surface receptors, termed $S1P_{1-5}$ (formerly known as Edg1, Edg5, Edg3, Edg6, and Edg8, respectively), are activated by sphingosine-1-phosphate, diversifying the biological effects of this lipid.

Sphingosine-1-phosphate can be de-phosphorylated back to sphingosine and, thus, recycled into the sphingolipid metabolism by two ER-located sphingosine-1-phosphate phosphohydrolases (74). Alternatively, it can be terminally degraded to hexadecenal and ethanolamine phosphate by sphingosine-1-phosphate lyase. Cloning of the Drosophila sphingosine-1-phosphate lyase gene (Sply) demonstrated the importance of sphingosine-1 phosphate for adult muscle development and integrity (56). Sply null mutants accumulate phosphorylated and unphosphorylated sphingoid bases and exhibit gross abnormalities in dorsal longitudinal flight muscles.

Sphingosine-1-phosphate signaling *via* sarcolemmal receptors promotes proliferation of adult stem cells and muscle regeneration after injury (94). Two sphingosine-1-phosphate receptors, $S1P_1$ and $S1P_3$, are expressed by quiescent satellite cells and are localized to the sarcolemma and T-tubules of skeletal muscle fibers; $S1P_1$ is found at the neuromuscular junction (125). Regeneration of soleus muscle after injury stimulates the expression of these receptors, whereas administration of sphingosine-1-phosphate at the time of injury increases the cross-sectional area of regenerating fibers; conversely, administration of anti-sphingosine-1-phosphate antibody has the opposite effect, attenuating fiber growth (27). Sarcolemmal receptors are likely to mediate these trophic effects, suggesting that sphingosine-1-phosphate can function as a growth factor for muscle fibers.

Sphingosine-1-phosphate can also act as an autocrine/paracrine mediator of profibrotic changes stimulated by transforming growth factor β1 (TGFβ1) (19). In undifferentiated C2C12 myoblasts, TGFβ1 upregulates SK1 in a Smad-dependent manner and alters the expression of sphingosine-1-phosphate receptors. These changes precede the induction of fibrotic markers by TGFβ1. Profibrotic changes can be prevented by inhibiting SK1 activity, using pharmacological probes or short interfering RNA, or by inhibiting $S1P_3$ expression.

Further evidence with regard to the functions of sphingosine-1-phosphate in skeletal muscle emerged recently from studies aimed at understanding how this molecule is secreted from the cells. Since sphingosine-1-phosphate has a negatively charged head group, it cannot pass the hydrophobic plasma membrane. In mast cells, ABCC1 has been shown to facilitate the secretion of endogenously produced sphingosine-1-phosphate through the plasma membrane (91). A novel multipass transmembrane protein, spinster homolog 2 (Spns2), has been identified recently as a sphingosine-1-phosphate transporter in Zebra fish. Notably, mutation in Spns2 causes migrational defects in myocardial precursor cells and the development of cardia bifida (two hearts) (65). A similar phenotype was also reported for *S1P receptor-2* mutant (69).

Sphingosine-1-phosphate signaling *via* the $S1P_2$ receptor stimulates a transient burst of ROS production by skeletal muscle cells. This response is linked to ligand-independent trans-phosphorylation of the insulin receptor and a substantial increase in glucose uptake (103). These data support sphingosine-1-phosphate as a regulator of insulin responsiveness in muscle. This model is consistent with the action of palmitic acid, a dietary saturated fat linked to the development of diabetes that increases SK1 mRNA in C2C12 myotubes by approximately four-fold (58).

Sphingolipids and Oxidative Stress

Products of sphingolipid metabolism are associated with oxidative stress. Also, stimuli of ceramide production, for example, TNF, chemotherapeutic agents, and radiation, increase oxidant production. These associations suggest that sphingolipids may act as second messengers to increase oxidant production. A large number of studies have been conducted over the past decade to test this thesis. However, the cellular mechanism remains elusive. There appears to be a complex, two-way interaction between sphingolipids and oxidant production. Increased ROS activity, decrements in antioxidant defenses, and activation of NO synthase or NADPH oxidase can stimulate turnover of complex sphingolipids and generation of bioactive sphingolipid metabolites, for example, ceramide, sphingosine, and sphingosine-1-phosphate. Conversely, ceramide analogs act directly on isolated mitochondria to inhibit mitochondrial electron transport at complex III, increasing ROS production, and agents that inhibit complex III (*e.g.*, TNF, adriamycin, tamoxifen) may do

so by increasing mitochondrial ceramide levels (117). The interplay between sphingolipid signaling and oxidant activity likely contributes to the onset and propagation of oxidative stress in inflammatory disease.

Regulation of ceramide metabolism by oxidative stress

Initial indications that oxidative stress and ceramide accumulation are linked came from studies in the early 1980s when reduced glutathione (GSH) was found to be a reversible inhibitor of cellular nSMase activity (77, 78). The modulation of nSMase activity by GSH was first established in the context of TNF signaling and apoptosis (77, 78). Later, the ability of GSH to affect the sensitivity of T47D/H3 breast cancer cells to doxorubicin was attributed to GSH inhibition of nSMase activity (49). A correlation between oxidative stress and nSMase activity was also found in long-lived rats on vitamin Q10 enriched diet (9) and in astrocytes treated with vitamin E (8).

Most results suggest that ROS sensitivity is a common feature of all SMases. In parallel, SMase isoforms are activated by different stressors through different mechanisms. Research on this topic has focused on lung biology (17), because airway epithelial cells are constantly exposed to environmental insults such as air pollution or tobacco smoke that may contain high levels of reactive nitrogen species and ROS. For example, H_2O_2 exposure activates nSMase2 in the lung, generating ceramide and inducing apoptosis. Peroxynitrite exposure also induces apoptosis, increasing ceramide levels by activating acid SMase (17). Animal models of pulmonary emphysema reinforce the links between oxidative stress, ceramide accumulation, and lung injury (46).

In the liver, activation of nSMase2 during oxidative stress seems to be caused by GSH depletion. This is evident in the aging-related increase of nSMase2 activity that occurs in hepatocytes. Inhibition of GSH synthesis in young hepatocytes activates nSMase, whereas restoration of GSH content in hepatocytes from aged animals by treatment with *N*-acetylcysteine (NAC) inhibits nSMase activity. Importantly, the GSH decline and nSMase activation are not observed in aged, calorie-restricted animals that have low levels of oxidant activity. Dietary depletion of hepatic GSH content in mice fed a methionine-deficient diet also results in ceramide accumulation (15); in this case, acid SMase was activated and not nSMase. Interestingly, the methionine-deficient diet selectively depressed mitochondrial GSH with no effect on cytosolic GSH levels. These changes were associated with hepatocellular injury, oxidative stress, inflammation, and fibrosis. When GSH content was depleted *via* oral CCl_4 administration, total ceramide in the liver increased in parallel with nSMase activity (61). Similar changes occurred in plasma, kidney, and the brain, suggesting that CCl_4 intoxication causes oxidative stress and ceramide accumulation in tissues beyond the liver. Together, these observations support the hypothesis that all SMase isoforms are sensitive to oxidative stress and that the stressor determines which isoform is affected.

Oxidants also stimulate ceramide accumulation in cardiomyocytes. Hernandez *et al.* (55) identified nSMase activation as one of the earliest responses of cardiac myocytes to redox stress imposed by hypoxia-reoxygenation. These changes were linked to JNK activation and led to apoptosis. nSMase activation is associated with the progression of chronic heart failure after myocardial infarction (2). GSH deficiency is evident in the myocardium of the failing human heart or the heart of rats 2 months after myocardial infarction. In the latter case, GSH levels can be restored by oral treatment with the glutathione precursor NAC. This inhibits nSMase activation, Bcl-2 depletion, and caspase-3 activation, thereby improving cardiac function. Pchejetski at al. (99) documented an alternative cellular mechanism In H9c2 cardiomyoblasts. They found that oxidative stress inhibits sphingosine-1-kinase, decreasing cellular levels of the pro-survival sphingosine 1-phosphate rather than activating nSMase. This is the first evidence that ceramide clearance is also sensitive to oxidants.

Finally, several studies indicate that oxidative stress causes ceramide accumulation in neuronal tissues. The nSMase-ceramide pathway mediates oxidative stress-induced apoptosis in human primary oligodendrocytes (62). Cells were challenged with a superoxide-generating system (hypoxanthine and xanthine oxidase), hydrogen peroxide, a catalase inhibitor (aminotriazole), and diamide (depletes GSH), which activated nSMase and stimulated ceramide production. The pathogenesis of Alzheimer's disease is tightly linked to oxidative stress-induced accumulation of ceramides and cholesterol, which, in turn, can trigger a neurodegenerative cascade that leads to clinical disease (23).

Several mechanisms may contribute to oxidant-stimulated ceramide production. Coenzyme Q is a plasma membrane-localized antioxidant that regenerates antioxidants such as ascorbate and α-tocopherol and is a noncompetitive inhibitor of the Mg^{2+}-dependent nSMase (95). *Ex vivo* studies have explored mechanisms by which GSH might directly interact with nSMase1 and nSMase2 to inhibit enzyme activity. Katan and colleagues (36) have proposed that reversible inactivation of nSMase1 by H_2O_2 and oxidized glutathione reflects the formation and breakage of disulfide bonds among eight cysteine residues that are located outside the enzyme's catalytic site. In contrast, peroxynitrite causes irreversible inactivation of nSMase1 that likely reflects covalent modification of thiol residues.

Alternative mechanisms have also been proposed. Filosto *et al.* (39) found that nSMase2 interacts directly with calcineurin, a phosphatase that functions as a redox switch for nSMase2. Specifically, oxidative stress causes inhibition or degradation of calcineurin, allowing phosphorylative activation of nSMase2. Dumitru *et al.* (33) have proposed that ROS activate acid SMase to promote the formation of large, ceramide-rich membrane platforms. These platforms are thought to stabilize associations among cytokine receptors, ROS-generating enzyme complexes, and acid SMase. The physical association facilitates ROS/sphingolipid signaling in a feed-forward mechanism.

Ceramide accumulation, ROS generation, and oxidative stress

The ability of ceramide to induce ROS production has been extensively studied in the context of apoptosis [for a detailed review please see Andrieu-Abadie *et al.* (7)]. Ceramide analogs, C2, C6, and C8, were found to stimulate H_2O_2 generation by intact hepatocytes and isolated mitochondria when applied at concentrations less than 1 m*M*, mimicking the effects of TNF (42). No such responses were evoked by

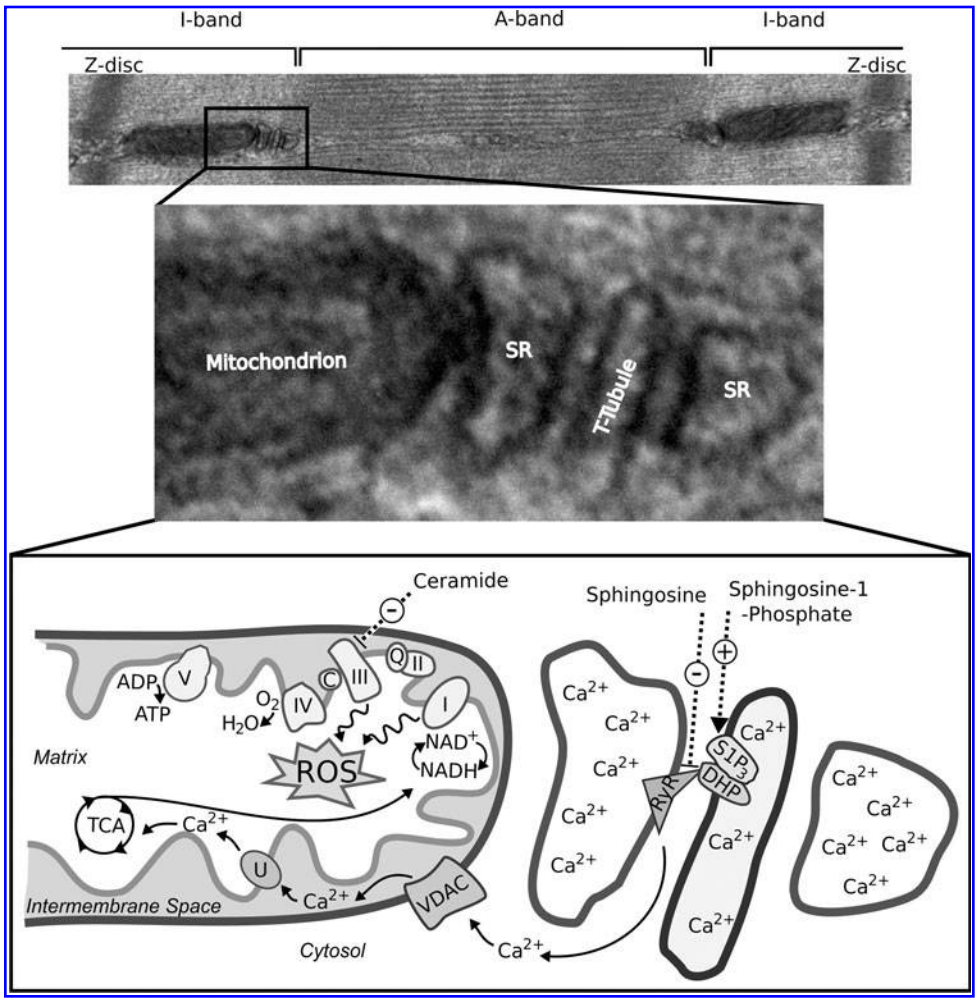

FIG. 4. Actions of sphingolipids on mitochondrial reactive oxygen species (ROS) production. *Upper panel*: electron micrograph of sarcomere from murine soleus muscle. *Middle*. Expanded image of intermyofibrillar mitochondrion apposed to a sarcoplasmic reticulum (SR) terminal cisternae/t-tubule junction. *Lower panel*: Diagram depicts mechanisms by which sphingolipids are proposed to act on the electron transport system (ceramide) and on calcium regulation (sphingosine, sphingosine-1-phosphate) to alter mitochondrial ROS production; see text for explanation.

dihydroceramide or sphingosine at these concentrations, arguing for ceramide specificity. It should be noted, however, that delivery efficiency of the sphingolipids was not monitored. Therefore, the question of whether ceramide or its metabolic product was responsible is not completely answered. The mechanism of ceramide action seems to involve inhibition of respiratory complex III rather than a transition in mitochondrial permeability (42, 102). Notably, mitochondria from TNF-treated cells contain two- to threefold more ceramide than mitochondria from untreated cells. This finding suggests that mitochondria are a target of TNF/ceramide signaling and that ceramide acts on the electron transport chain to stimulate H_2O_2 production. This may account for the increase in ROS generation during TNF cytotoxicity (50).

The mechanism of ceramide action is not fully resolved. However, ceramide has been shown to interact with the oxidized form of cytochrome c leading to its displacement from the mitochondria membrane, a fall in oxygen consumption, and diminished mitochondrial transmembrane potential ($\Delta\Psi$). It has been suggested that ceramide-induced displacement of cytochrome c produces a gap between mitochondrial respiratory complexes III and IV, creating a site for electron leak (7, 43, 107).

Altogether, these studies suggest that the respiratory electron chain of mitochondria is a ceramide target (Fig. 4). The source of ceramide in the mitochondria remains elusive. Recent studies have begun to identify enzymes in mitochondria that may enable ceramide generation, including ceramide synthase and SMase. Intriguingly, knockout of the ceramide transfer protein CerT1 causes ceramide to accumulate in mitochondria (122), suggesting that CerT1 might regulate mitochondrial ceramide content.

Ceramide has been implicated in the induction of oxidative stress in other model systems. Both TNF- and IL-1β-induced generation of ceramide parallels the ROS production that potentiates central nervous system injuries and certain neurological disorders (3). In the lung, oxidative stress increases ceramide levels, and sphingolipids trigger proapoptotic signaling that promote alveolar space enlargement. These observations made in an experimental model of emphysema led to the hypothesis that oxidative stress generated by ceramide is required for its pathogenic effect (100). Transgenic mice that overexpress human Cu/Zn SOD are protected from ceramide-induced superoxide production, apoptosis, and air space enlargement. This implicates the excessive lung ceramides as amplifiers of lung injury through redox-dependent mechanisms.

Ceramide has also been shown to activate ROS, mitochondrial oxidative damage, and apoptosis in vascular cells (82). An intriguing bi-modal effect was observed in these experiments. Ceramide stimulated NO production at low

concentrations. At high concentrations, ceramide stimulated H_2O_2 production and apoptosis.

Several articles provide evidence that ceramide accumulation in striated muscle is sufficient to induce oxidative stress. Suematsu *et al.* (116) found that TNF increases ROS production in cultured neonatal rat ventricular myocytes and that damage of mitochondrial DNA can be prevented using an aSMase inhibitor, thus implicating ceramide as mediator. Exogenous ceramide reproduced the effects of TNF, suggesting that ceramide-induced oxidative stress plays a role in heart failure.

Ferreira *et al*. (38) used a live-cell fluorescence assay to test ceramide effects on cytosolic oxidant activity in skeletal muscle preparations. In mature C2C12 myotubes, direct exposure to either C_2-ceramide or C_6-ceramide increased oxidant activity. A similar increase was seen in murine diaphragm fiber bundles. In companion experiments, biological ceramide was generated by exposing muscle preparations to recombinant bacterial SMase. This also increased cytosolic oxidant activity in myotubes and muscle fibers. A separate assay was used to selectively evaluate cytosolic NO activity. Basal NO activity was not altered by SMase exposure, suggesting that the rise in overall oxidant activity is NO independent. These observations show that ceramide acts on skeletal muscle to rapidly increase cellular production of oxidants, probably ROS.

Sphingolipids and Contractile Function

A growing body of evidence suggests that sphingolipid signaling may have important effects on skeletal muscle contraction. In unfatigued muscle, sphingolipids are suggested to alter force *via* oxidant-dependent and oxidant-independent mechanisms. Strenuous exercise alters sphingolipid metabolism and the composition of sphingolipid bases in muscle. Fatigue of exercising muscle is sensitive to selected sphingolipids, notably sphingosine-1-phosphate and ceramide, which have complex effects on muscle performance. Finally, over the long term, muscle strength and fatigue characteristics may be influenced by sphingolipid effects on muscle mass and muscle metabolism. The following sections outline our knowledge in this emerging field.

Force depression via *ceramide-derived oxidants*

As detailed in previous sections, sphingolipids can stimulate cellular production of oxidants, both ROS and NO derivatives. Cytosolic oxidant activity has complex effects on contractile function of skeletal muscle. Under basal conditions, muscle-derived NO depresses force (67), whereas ROS effects are biphasic, increasing or decreasing force in a concentration- and time-dependent manner (105). During strenuous exercise, ROS accumulate in the working muscle and contribute to fatigue-related decrements in force (104). By stimulating oxidant production, sphingolipids are predicted to influence contractile function indirectly, altering force *via* redox mechanisms.

This prediction is supported by recent data from Ferreira *et al.* (38). Direct exposure to recombinant SMase depressed isometric force of intact-fiber bundles from murine diaphragm. This effect was dose and time dependent and could be detected across the full range of physiologic stimulus frequencies. C6 ceramide had a similar effect, progressively depressing maximal tetanic force over 30 min. The depression of force by SMase exposure was blunted by pretreating fiber bundles with NAC, a thiol donor and nonspecific antioxidant; thus, muscle-derived oxidants played a causal role in the force loss caused by SMase. These oxidants did not appear to be NO derivatives, as pretreatment with an NO synthase inhibitor (L-NAME) did not preserve force.

Sphingolipids and excitation-contraction coupling

Skeletal muscle contraction is controlled by motor input from the central nervous system. Neural input stimulates sarcolemmal action potentials. These propogate into the cell *via* T-tubules, activating voltage sensors that cause calcium release from ryanodine-sensitive channels in the SR. Calcium efflux from the SR activates myofilament proteins, resulting in muscle contraction. A number of reports show that this process is sensitive to sphingolipids, notably sphingosine and sphingosine-1-phosphate.

In 1992, Sabbadini *et al*. (108) showed that sphingosine acts directly on the SR calcium release channel to inhibit calcium release. Sphingosine inhibited caffeine-induced calcium release from chemically permeabilized single fibers and terminal cisternae vesicles from rabbit psoas muscle. This response was dose dependent and near complete at 3 μM, whereas SM and sphingosylphosphorylcholine (SPC) were inactive at this concentration, demonstrating specificity. In studies of SR vesicles, sphingosine increased the equilibrium dissociation constant (K_d) of [^{3}H]ryanodine binding to calcium release channels and depressed the amount of [^{3}H]ryanodine required to saturate channel binding (B_{max}), demonstrating that sphingosine acts directly on channels. In contrast, sphingosine did not alter the pCa-force relationship in permeabilized fibers, eliminating myofilament proteins as the site of sphingosine action. The authors concluded that locally generated sphingosine might be a negative modulator of SR calcium release under physiological conditions.

Needleman *et al*. (96) used rabbit muscle SR membranes, purified SR calcium release channels, and calcium release channels in planar lipid bilayers to assess the molecular mechanism of sphingosine action in greater detail. They confirmed that sphingosine interacts directly with a site on the calcium channel located between Arg^{4475} and the carboxyl terminus. Sphingosine interaction greatly enhances the rate of [^{3}H]ryanodine release from the high-affinity binding site, reflecting noncompetitive inhibition of [^{3}H]ryanodine binding. A follow-up study (110) tested the properties of other sphingolipid bases on channel function. *N,N*-dimethylsphingosine, dihydrosphingosine, and phytosphingosine inhibited [^{3}H]ryanodine binding, albeit less effectively than sphingosine, and stabilized channels in the closed state. In contrast, sphingosine-1-phosphate and C6-to-C18 ceramides had no effect on [^{3}H]ryanodine binding, suggesting that these sphingolipids do not influence calcium channel function.

Sphingosine-1-phosphate has the opposite effect on calcium regulation, acting on distinct target proteins to promote calcium mobilization. In undifferentiated C2C12 myoblasts, exogenous sphingosine-1-phosphate acts *via* G(i)-coupled, agonist-specific surface receptors, $S1P_3$ (Edg3) and $S1P_2$ (Edg5), to increase cytosolic calcium levels (84). Sphingosine-1-phosphate binding to Edg receptors activates voltage-independent L-type

calcium channels plus the inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]-mediated calcium pathway, resulting in calcium mobilization from both extracellular and intracellular pools (40). Muscle differentiation alters the expression of S1P receptors and calcium channels. This led Bencini *et al.* (10) to test sphingosine-1-phosphate effects on voltage-dependent calcium release in segments of skeletal muscle fibers from adult mice. Sphingosine-1-phosphate (100 n*M*) altered intramembrane charge movements across the sarcolemma and increased electrically evoked L-type calcium channel current (I_{Ca}) by shifting the I_{Ca} activation curve. These responses were largely attributed to the $S1P_3$ (Edg3) receptor isoform.

Interestingly, high (10–25 μM) concentrations of exogenous sphingosine-1-phosphate trigger intracellular calcium transients (10). This resembles an earlier observation that high concentrations of sphingosine (30–50 μM) or SPC (10–20 μM) can paradoxically activate SR calcium release channels (108). Such findings emphasize the complex dose dependence of sphingolipid effects on calcium handling and illustrate the potential for nonphysiological responses at high concentrations.

Data from cultured myoblasts and reduced muscle preparations suggest that sphingosine and sphingosine-1-phosphate have the capacity to modulate muscle force. By inhibiting SR calcium release, sphingosine is predicted to depress excitation-contraction coupling and diminish force. Sphingosine-1-phosphate is predicted to have opposite effects. These predictions have been tested in only one study. Danieli-Betto *et al.* (26) evaluated exogenous sphingosine and sphingosine-1-phosphate for effects on unfatigued muscle. Murine EDL was electrically stimulated under isometric conditions; twitch properties and maximal tetanic (150 Hz) force were measured. Contractile function was unaffected by sphingosine (1, 10, 100 μM) or sphingosine-1-phosphate (1, 10, 20 μM) at any concentration tested. The inconsistency between this finding and previous results in reduced preparations is unresolved. Additional studies are clearly needed to define sphingolipid effects on excitation-contraction coupling in intact muscle fibers.

Sphingolipids and fatigue

Sphingolipids alter cellular processes that contribute to muscle fatigue. This includes oxidant production by muscle fibers, which is elevated during fatiguing exercise (104). Antioxidant pretreatment can blunt muscle-derived oxidants during exercise, slowing the development of fatigue (88, 106, 111). Conversely, exogenous oxidants and proinflammatory stimuli augment oxidants in exercising muscle, increasing total oxidant activity, and accelerating fatigue (45, 70, 73). In combination, these observations identify muscle-derived oxidants, particularly ROS, as physiologic mediators of fatigue and suggest that exogenous stimuli which increase ROS production will promote fatigue. Sphingolipids are clearly in this category.

Failure of calcium release contributes importantly to fatigue. Calcium release is compromised in fatiguing muscle due to conductance failure of sarcolemmal and T-tubule action potentials, voltage sensor inactivation, and inhibition of SR calcium release channels (4). Exogenous sphingolipids stimulate similar changes in reduced muscle preparations, suggesting a potential role in fatigue.

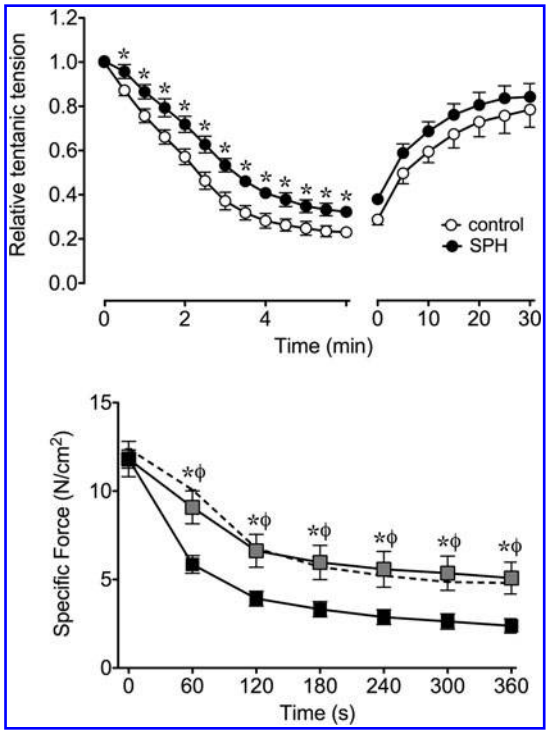

FIG. 5. Sphingolipid effects on muscle fatigue. *Upper panel*: relative forces (% initial force at time 0) developed by murine extensor digitorum longus muscles in oxygenated buffer containing 5 μM bovine serum albumin (control; open symbols) or 10 μM sphingosine (SPH, closed symbols); muscles were studied at 30°C using repetitive, maximal tetanic stimuli (150 Hz, 200 ms trains, 0.2 trains/s); sphingosine increased relative force during fatigue and recovery; $n = 4$/group; $^*p < 0.05$ *versus* time-matched control; reproduced with permission from (26). *Lower panel*: specific force (N/cm^2) of fiber bundles from murine diaphragm in oxygenated buffer (dashed line), buffer containing recombinant bacterial SMase 0.5 U/ml (*closed squares*), or buffer containing SMase plus *N*-acetylcysteine 10 m*M* (NAC, *shaded squares*) at 37°C during submaximal tetanic stimulation using a matched-force protocol (40–71 Hz, 500 ms trains, 0. 5 trains/s); SMase promoted fatigue, an effect abolished by NAC; $n = 5$–7/group; $^*p < 0.05$ control *versus* SMase; $^{\varphi}p < 0.05$ SMase *versus* SMase + NAC; reproduced with permission from Ferreira *et al.* (38).

Several studies have tested this postulate (Fig. 5). Danieli-Betto *et al.* (27) demonstrated the capacity of sphingosine-1-phosphate to oppose fatigue. *In vitro* exposure of murine EDL to sphingosine-1-phosphate slowed the decline in force during fatiguing contractions; final force was 40% greater than control. This response was attributed, in part, to preservation of action potential amplitude during fatigue. Sphingosine also slowed force loss during fatigue. However, this effect was abolished by sphingosine kinase inhibition, leading investigators to conclude that sphingosine-1-phosphate synthesis was essential and that sphingosine did not directly influence fatigue.

In contrast, Ferreira *et al.* (38) showed that SMase/ceramide signaling promotes fatigue. Bundles of intact fibers from murine diaphragm were pretreated with recombinant SMase and subjected to standardized fatigue protocols. SMase-treated

muscles developed less force throughout the fatigue protocols and final force was one-half of control values. Recognizing that SMase stimulates oxidant activity, the investigators tested effects of the antioxidant NAC on muscle fatigue. Antioxidant pretreatment preserved the function of SMase-treated muscle; force remained at control levels throughout the fatigue protocol. The investigators concluded that SMase/ceramide signaling promotes fatigue by stimulating oxidant production.

A series of reports by Górski and associates demonstrate the effects of exercise and exercise training on sphingolipid metabolism in skeletal muscle. Major sphingolipid mediators (sphinganine, SM, ceramide, sphingosine, and sphingosine-1-phosphate) and key regulatory enzymes (serine palomitoyltransferase, nSMase, aSMase, neutral, and ACER) are detectable in limb muscles of rats (11, 29, 30) and humans (54). Under resting conditions, sphingolipid markers differ modestly between aerobically adapted muscles (soleus, red gastrocnemius) and anerobic muscle (white gastrocnemius) of rats. Aerobic muscle tends to have higher levels of sphingolipid mediators, more SMase activity, and less ceramidase activity (11, 29, 30). In contrast, serine palomitoyltransferase activity is similar between muscle types (11).

Exercise has complex effects on sphingolipid metabolism that vary with exercise duration and muscle type. Serine palomitoyltransferase activity and sphinganine levels increase progressively during exercise in both muscle types (11, 30), evidence of greater *de novo* biosynthesis. Sphingosine responds similarly (11, 30). Changes in SM, ceramide, and sphingosine-1-phosphate are less consistent among intermediate exercise levels (11) but are generally elevated after exhaustive exercise (11, 29, 30, 54). Accumulation of these metabolites is favored by higher rates of *de novo* biosynthesis (above) combined with decrements in the activities of key pathway enzymes. Exhaustive exercise depresses the activities of both SMase isoforms (11, 29, 54) and both ceramidase isoforms (11) across muscle types.

The contrast between aerobic and anerobic muscles suggests that endurance training may alter sphingolipid metabolism. To address this issue, Helge *et al.* (54) tested the effect of prolonged exercise on vastus lateralis muscles of trained and untrained humans. Training caused subtle differences in total SM fatty acid regulation. Total ceramide fatty acids and nSMase activity were unaffected.

Muscle adaptation and metabolism

Sphingolipids are likely to influence force production and fatigue indirectly *via* effects on other cellular processes. Sphingolipids can alter muscle development, adaptation, and repair after injury. These processes determine muscle fiber type and muscle mass, factors that strongly influence contractile function. Sphingolipids also exert complex effects on uptake and utilization of metabolic substrates. Substrate availability can limit muscle energetics, growth, and fatigue characteristics. As outlined next, we have a growing understanding of the mechanisms by which sphingolipids regulate these processes and an appreciation for their potential importance in pathologic settings.

Sphingolipids modulate proliferation, differentiation, growth, and apoptosis of skeletal muscle cells. This is best understood in the context of sphingosine-1-phosphate/$S1P_2$ signaling in the skeletal muscle-derived C2C12 cell line. Exogenous sphingosine-1-phosphate inhibits proliferation of C2C12 myoblasts and stimulates differentiation (32), responses mediated by the $S1P_2$ receptor isoform (85, 115). In part, sphingosine-1-phosphate promotes differentiation by inducing expression of connexin-43, a gap junction protein, and stimulating cytoskeletal remodeling (41, 115). Meacci *et al.* (85) used a series of genetic interventions to identify SK1 as the isoform that regulates this response. Studies by Nagata *et al.* (94) suggest that sphingosine-1-phosphate signaling is also essential for satellite cell activation. Exogenous sphingosine-1-phosphate stimulates entry into the cell cycle and satellite cell proliferation, whereas inhibition of sphingosine-1-phosphate synthesis impairs muscle regeneration after cardiotoxin injury. The available data suggest that ceramide signaling has opposing effects. Exposure to exogenous ceramide slows myoblast differentiation, whereas inhibition of ceramide synthesis *via* the *de novo* pathway promotes differentiation (86, 87). Ceramide is also a proapoptotic stimulus for differentiated myotubes (120), which mimics ceramide effects in nonmuscle cell types (53).

Sphingolipid effects on muscle adaptation may contribute to weakness and fatigue in chronic inflammatory diseases. Perhaps the best example is heart failure, in which TNF serum levels are persistently elevated (97). Serum TNF is thought to exert endocrine effects on skeletal muscle, acting *via* sarcolemmal TNF subtype 1 receptors to stimulate neutral SMase activity and increase ceramide synthesis (1). Circulating SMase is also elevated in patients with heart failure (31), providing a more direct endocrine stimulus for ceramide synthesis. Serum SMase could by-pass receptor regulation, acting directly on the outer leaflet of the sarcolemma to generate ceramide at the cell surface. By either stimulus, the rise in ceramide signaling is suggested to promote pathologic changes in skeletal muscle that occur in heart failure, that is, muscle apoptosis, fiber atrophy, and contractile dysfunction (24, 25, 121).

Sphinolipids regulate glucose and amino acid uptake by skeletal muscle. Most notably, ceramide decreases insulin-stimulated glucose uptake (48). The cellular response involves failure of glucose transporter type 4 (GLUT4) translocation (63), impaired protein kinase B recruitment (51), loss of Rac activation (63), and actin remodeling (63). This mechanism contributes to insulin resistance caused by palmitate supplementation (124) and diets rich in saturated fatty acids (12), experimental interventions that stimulate ceramide synthesis. Conversely, sphingosine-1-phosphate facilitates insulin-stimulated glucose uptake *via* a separate, oxidant-mediated mechanism (103). Sphingosine-1-phosphate acts *via* $S1P_2$ to transiently stimulate ROS production, inhibiting the activity of protein tyrosine phosphatase-1B. This enables ligand-independent trans-phosphorylation of the insulin receptor, increasing glucose uptake. In addition, amino acid metabolism is sensitive to ceramide signaling. Ceramide exposure downregulates System A amino acid transporters in L6 myotubes, decreasing amino acid uptake and protein synthesis (60).

The metabolic impact of sphingolipid signaling has clinical implications for muscle function as recently reviewed by Holland and Summers (57). In obesity and type II diabetes, changes in the metabolic profile and elevation of serum

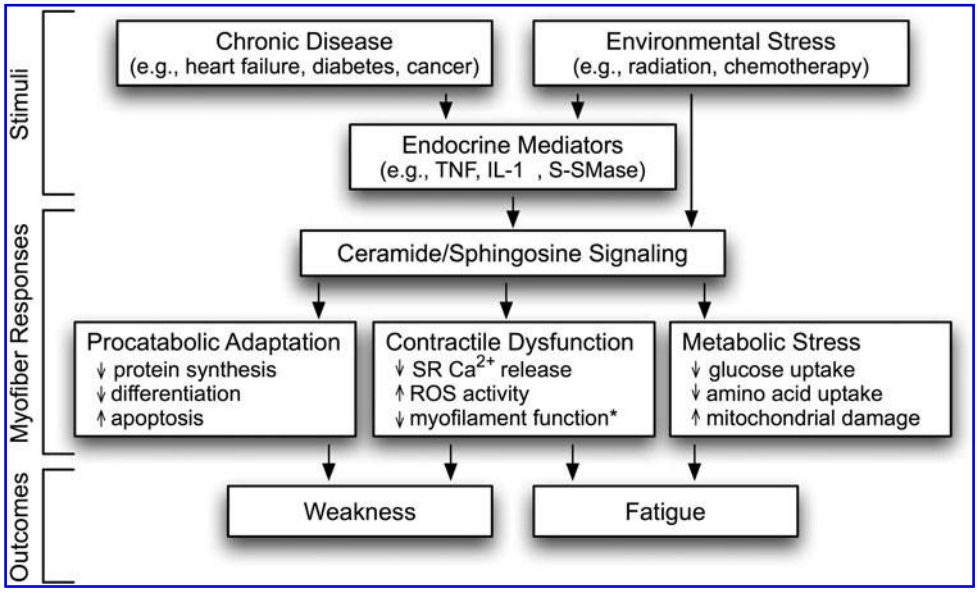

FIG. 6. Sphingolipids as mediators of muscle dysfunction. Global model depicts events by which sphingolipid metabolites may promote weakness and fatigue caused by chronic inflammatory disease or environmental stress; model integrates data from published reports as detailed in text.

cytokines promote sphingolipid synthesis in peripheral tissues, including skeletal muscle. Greater ceramide production favors insulin resistance, which limits glucose uptake and exacerbates the disease. Elevated ceramide activity also opposes amino acid uptake and protein synthesis by skeletal muscle. This promotes loss of lean body mass and exercise intolerance, common clinical complications of diabetes.

Conclusion

The available data paint an intriguing picture of sphingolipid biology in skeletal muscle. Sphingolipid metabolism can vary importantly from nonmuscle tissues, both in the expression of regulatory proteins and in the tissue levels of individual metabolites. Within muscle cells, these components differ among intracellular compartments, suggesting localized regulation of organelle function or signaling events. Sphingolipids have complex effects on myocyte function, acting to increase cellular oxidant activity, inhibit proliferation of precursor cells, stimulate apoptosis, depress insulin receptor signaling and glucose uptake, oppose amino acid uptake, and depress protein synthesis.

Effects on contractile function are less well defined. Biochemical and subcellular studies show that sphingosine and spingosine-1-phosphate have the potential to influence Ca^{2+} regulation *via* several mechanisms. However, these mediators have not been shown to alter force of unfatigued muscle, raising questions about physiological relevance. In contrast, SMase/ceramide signaling clearly depresses force. This response involves muscle-derived oxidants, probably ROS, as downstream effectors. During repetitive muscle contractions, sphingosine-1-phosphate and ceramide have opposing effects on fatigue. Sphingosine-1-phosphate slows fatigue, preserving force over time, whereas ceramide promotes fatigue. The response to ceramide is abolished by antioxidant pretreatment, reinforcing the role of cellular oxidants as essential mediators of ceramide action.

Integrating these findings, we speculate that sphingolipid signaling may promote the muscle wasting and contractile dysfunction that occurs in chronic disease (Fig. 6). Inflammatory mediators stimulate sphingolipid metabolism, elevate sphingolipid tissue levels, and alter the relative abundance of ceramide, sphingosine, and sphingosine-1-phosphate. In skeletal muscle, sphingolipid mediators stimulate cellular responses (above) that predispose skeletal muscle to weakness, premature fatigue, and cachexia. These symptoms plague individuals with heart failure, cancer, diabetes, and other chronic inflammatory diseases, amplifying illness and contributing to death. Research is needed to define sphingolipid effects in these conditions. Greater understanding of sphingolipid pathobiology may identify novel therapeutic strategies to preserve muscle mass and contractile function, enhancing the quality and duration of life for affected individuals.

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Abbreviations Used

ABCC1 = ATP-binding cassette, sub-family C (CFTR/MRP), member 1
ACER = alkaline ceramidases
ASAH1 = acid ceramidase
ASAH2 = neutral ceramidase
CERK = ceramide kinase
CerS = dihydroceramide synthase
CerT1 = ceramide transfer protein
CPP = ceramide-1-phosphate phosphatase
ER = endoplasmic reticulum
GLUT4 = glucose transporter type 4
GSH = reduced glutathione
IL-1β = interleukin-1β
JNK = c-Jun *N*-terminal kinase
NAC = *N*-acetylcysteine
NF-κB = nuclear factor-kappa B
NO = nitric oxide
PKCζ = protein kinase C zeta
PP2A = protein phosphatase 2
Rac = Rho family GTPase
ROS = reactive oxygen species
RyR = ryanodine receptor
$S1P_{1-5}$ = sphingosine-1-phosphate receptors 1–5
SK1 and SK2 = sphingosine kinase 1 and 2
SM = sphingomyelin
SMase = sphingomyelinase
SMS1 SMS2 = SM synthase 1 and 2
SPC = sphingosylphosphorylcholine
SPL = sphingosine phosphate lyase
Sply = sphingosine-1-phosphate lyase gene
Spns2 = spinster homolog 2
SPT = serine palmitoyltransferase
SR = sarcoplasmic reticulum
TGFβ1 = transforming growth factor β1
TNF = tumor necrosis factor-α

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1. Chiara Donati, Francesca Cencetti, Paola Bruni. 2012. New insights into the role of sphingosine 1-phosphate and lysophosphatidic acid in the regulation of skeletal muscle cell biology. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* . [CrossRef]
2. Marta Ugarte, Marie Brown, Katherine A Hollywood, Garth J Cooper, Paul N Bishop, Warwick B Dunn. 2012. Metabolomic analysis of rat serum in streptozotocin-induced diabetes and after treatment with oral triethylenetetramine (TETA). *Genome Medicine* **4**:4, 35. [CrossRef]