Chapter 18
Role of Neutral Sphingomyelinases in Aging and Inflammation

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Abstract Aging is characterized by changes in the organism's immune functions and stress response, which in the elderly leads to increased incidence of complications and mortality following inflammatory stress. Alterations in the neuro-endocrine axes and overall decline in the immune system play an essential role in this process. Overwhelming evidence however suggests that many cellular cytokine signaling pathways are also affected, thus underscoring the idea that both, "cellular" and "systemic" changes contribute to aging. IL-1β for example, induces more potent cellular responses in hepatocytes isolated from aged animals then in hepatocytes from young rats. This phenomenon is referred to as IL-1β hyperresponsiveness and is linked to abnormal regulation of various acute phase proteins during aging.

Evidence has consistently indicated that activation of neutral sphingomyelinase and the resulting accumulation of ceramide mediate cellular responses to LPS, IL-1β, and TNFα in young animals. More recent studies identified the cytokine-inducible neutral sphingomyelinase with nSMase2 (smpd3) that is localized in the plasma membrane and mediates cellular responses to IL-1β and TNFα. Intriguingly, constitutive up-regulation of nSMase2 occurs in aging and it underlies the hepatic IL-1β hyperresponsiveness. The increased activity of nSMases2 in aging is caused by a substantial decline in hepatic GSH content linking thereby oxidative stress to the onset of pro-inflammatory state in liver. nSMase2 apparently follows a pattern of regulation consisting with "developmental-aging" continuum, since in animal models of delayed aging, like calorie-restricted animals, the aging-associated changes in NSMase activity and function are reversed.

Keywords IL-1β · oxidative stress · GSH · ceramide · nSMase2

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18.1 The "Inflamm-Aging" Theory of Aging

The contemporary understanding of aging is based on a wealth of theories involving evolutionary, genetic, metabolic, and environmental principles. The "inflamm-aging" hypothesis (Chung et al., 2001), in particular, arises from some phenomenological clinical observations that identify inflammation not only as an underlying companion of aging-related diseases, but also as a factor that in itself often defines the systemic aging process (Han et al., 1995; Sly et al., 2001; Spaulding et al., 1997; Tang et al., 2000; Wu et al., 2003; Yamamoto et al., 2002). In youth, inflammation is a complex host defense mechanism against environmental stresses, like chemicals, drugs, oxidants, and microbial organisms. During aging however, a sustained elevation in a variety of inflammatory markers occurs even in the absence of clinically relevant stimulants of host defense, adversely affecting basal homeostasis of different organs and the ability to cope with environmental challenges. Such changes are well documented for (among others) the cardiovascular system (Yamamoto et al., 2002), macrophage functions (Claycombe et al., 2002; Tang et al., 2000; Wu et al., 2003), liver (Hsieh et al., 1998, 2003; Rabek et al., 1998; Suh, 2001) and brain (Kalehua et al., 2000; Sly et al., 2001; Suh, 2001).

The mechanisms underlying the onset of pro-inflammatory state in aged organisms are complex. Changes in the neuro-endocrine axis, overall decline in the adaptive component of the immune system, and upregulation of the innate immune response are all well established phenomena associated with the aging process that certainly are involved in onset of inflammatory state. The widely accepted "free radical theory of aging" postulates that aging-associated decay in the mitochondrial functions of various cells gradually leads to increased production of reactive oxygen species and to the activation of redox-sensitive pro-inflammatory molecules, mainly, Nuclear Factor κB (NFκB) and c-Jun N-Terminal Kinase (JNK) (Chung et al., 2001; Franceschi et al., 2000; Franceschi et al., 2007). The constitutive activation of these molecules causes sustained increase in the basal levels of pro-inflammatory cytokines, such as TNFα, IL-1β and IL-6, which consequently stimulate inflammatory reactions in various organs. However, for many organisms, including humans, aging-associated increases in cytokines never reach the levels necessary to evoke cellular responses in young organisms.

Recent studies have given indications to the fact that in many, but not all cell types, various signaling pathways are also affected by aging (Hsieh et al., 1998; Hsieh et al., 2003; Rutkute et al., 2007b; Tang et al., 2000; Wu et al., 2003). For example, when treated with bacterial endotoxin, lipopolysaccharide (LPS), tumor necrosis factor α (TNFα) or pharmacological inducers of oxidative stress, peritoneal macrophages (Tang et al., 2000; Wu et al., 2003), hepatocytes (Hsieh et al., 1998), and glial cells isolated from aged animals exhibit more severe and prolonged responses as compared to cells isolated from young animals. This is evidenced by substantial differences in the magnitude and

temporal pattern of activation of cyclooxygenase-2 (Cox-2) (Wu et al., 2003), JNK (Hsieh et al., 2003; Rutkute et al., 2007b), NF-κB (Wu et al., 2003), CCAAT enhancer-binding protein (C/EBP) (Hsieh et al., 1998). Therefore, onset of the pro-inflammatory state in aging is apparently not only due to systemic factors, but also to changes in cellular responsiveness caused by, among others, a decreased capacity of anti-oxidant defense, up- or down-regulation of proteins functioning as rate-limiting factors in signaling cascades, and disruption of the balance between kinase and phosphatase activities.

18.2 Hepatic IL-1 β Signaling Pathway During Inflammation and Aging

IL-1β is a prototypic inflammatory cytokine that mediates the host response to infection, and its basal systemic levels are relatively constant with age (Di Iorio et al., 2003). Various IL-1β-related functions however, like the regulation of acute phase protein (APP) expression in liver appear to be age-dependent. The levels of acute phase reactants increase with age in a variety of species, from *D. melanogaster* (Zerofsky et al., 2005) to mammals (Berk et al., 1990; Rosenthal et al., 1975). Most importantly, these increases have been linked to aging-related disorders, like Alzheimer's disease (Abraham et al., 1988), rheumatoid arthritis (Rosenthal et al., 1975), cardiovascular diseases (Berk et al., 1990), impaired tissue renewal, frailty and reduced glucose tolerance (Ceda et al., 2005; Walston and Fried, 1999).

Cellular responses to IL-1β are mediated through the interleukin-1 receptor type I (IL-1RI). Together with TLR-4, the LPS receptor, IL-1RI is a prototypic member of the Toll-Like Receptor (TLR) family that shares a conserved signaling pathway (Fig. 18.1). Ligand binding to TLR results in the recruitment of several adaptor proteins including MyD88 (Wesche et al., 1997), followed by IL-1R-associated kinase-1 (IRAK-1) and IRAK-4 binding to the receptor complex. The subsequent IRAK-1 phosphorylation facilitates binding of tumor necrosis factor-associated factor-6 (TRAF-6) and the IRAK-1/IRAK-4/TRAF-6 complex then separates from the receptor and interacts with the transforming growth factor-β-activated kinase-1 (TAK-1) (Cao et al., 1996). Activation of TAK-1 apparently initiates the mitogen activated protein kinase kinase cascade, leading to the activation of transcription factors like activator protein-1 (AP-1) and NF-κB through JNK and inhibitory κB kinases respectively, resulting in the induction of APP mRNA transcription (Ninomiya-Tsuji et al., 1999).

IRAK-1 plays a central role in the TLR signaling cascade and it has recently been suggested that the rate of IRAK-1 degradation determines the magnitude of the response. Initially, phosphorylation of IRAK-1 is essential for its release from the receptor and the activation of downstream signaling molecules, but IRAK-1 phosphorylation also leads to its ubiquitination and proteasome-mediated

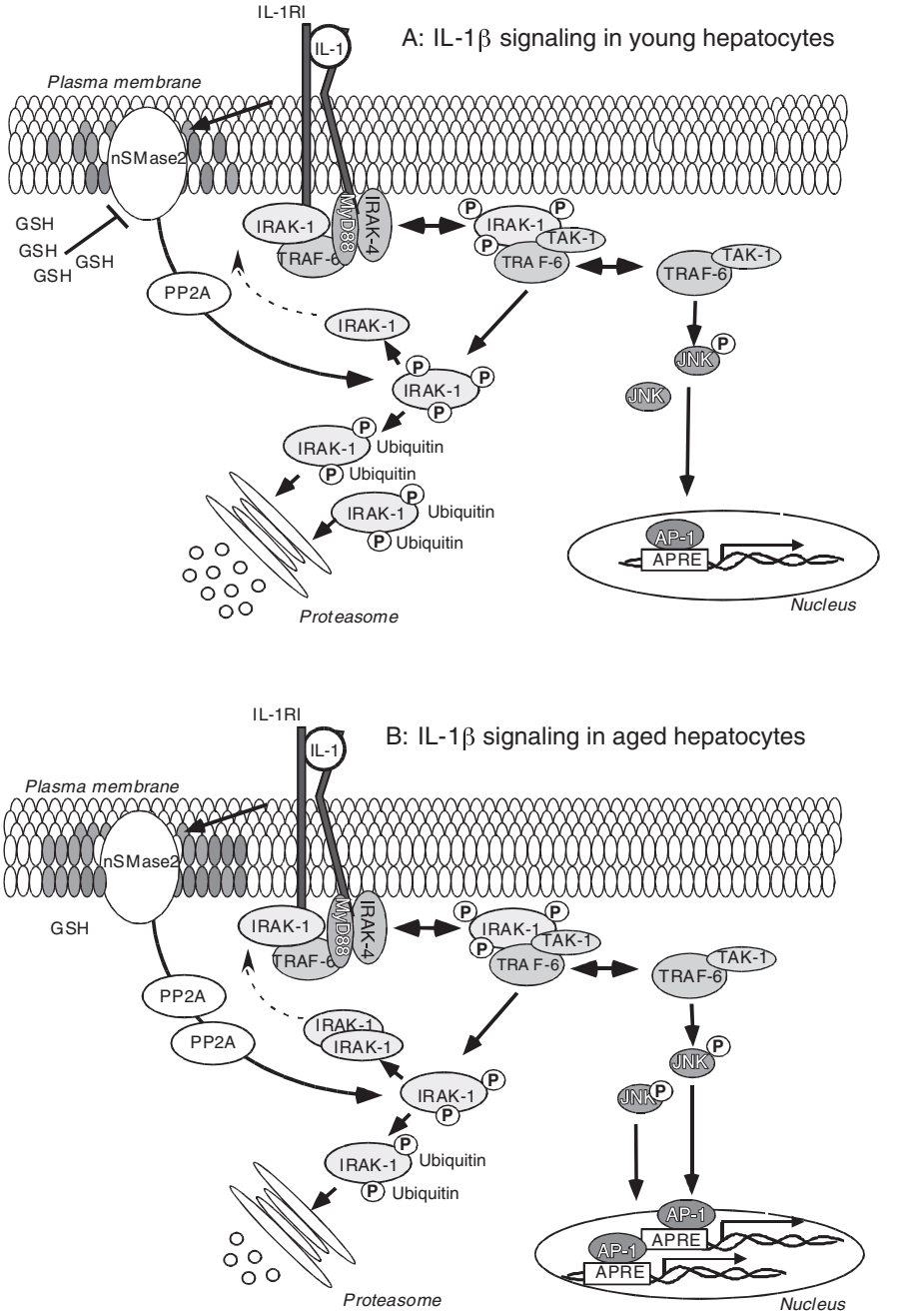

Fig. 18.1 Mechanism for the age-related hyperresponsiveness to IL-1β. Binding of IL-1β to the IL-1RI induces the formation of a signaling complex containing IRAK-1, IRAK-4, MyD88 and other adapter molecules. IRAK-4 and other, unidentified kinases phosphorylate

degradation, resulting in the termination of the signaling cascade (Yamin and Miller, 1997). Suppression of IRAK-1 degradation in macrophages through the use of ubiquitin ligase inhibitors has been shown to potentiate the inflammatory response, while decreased stability of IRAK-1 is the hallmark of endotoxin tolerance exemplified by the muted response to secondary LPS administration (Cuschieri et al., 2004; Li et al., 2000). Interferon γ and granulocyte-macrophage colony-stimulating factor can prevent this LPS tolerance by inhibiting IRAK-1 degradation (Adib-Conquy and Cavaillon, 2002).

Numerous studies have now confirmed that the IL-1β signaling cascade in liver is strongly affected by aging (Rutkute et al., 2007a,b; Rutkute and Nikolova-Karakashian, 2007c). The basal expression levels of IL1RI, IRAK-1, TAK-1 and JNK remain unchanged; yet stimulation with IL-1β evokes a more potent JNK phoshorylation in primary hepatocytes isolated from aged rats than in hepatocytes isolated from young ones. Importantly, concentrations of IL-1β that are too low to appreciably affect JNK activation in young animals are capable of inducing significant JNK phosphorylation in aged ones (Rutkute et al., 2007b). The increased phosphorylation of JNK leads to increased phosphorylation of c-jun, the major JNK substrate, and to more potent stimulation of expression of Insulin-Like Growth Factor Binding Protein 1 (IGFBP1) (Rutkute et al., 2007c). The latter is a hepatic acute phase protein and its production is potently upregulated in response to sepsis (Lang et al., 1996), endotoxin injection (Fan et al., 1994; Lang et al., 1997), and other inflammatory conditions. In young organisms increased circulating levels of IGFBP-1 during inflammation maintain the inflammatory catabolic state by diminishing the levels of bioavailable Insulin-Like Growth Factor-I (IGF-I) and thereby counteracting its anabolic effects (Lee et al., 1993). Notably, IGFBP1 serum concentrations are significantly elevated in aged organisms and are linked to declining levels of bioactive IGF-I (Yang et al., 2005), which in turn is

Fig. 18.1 (continued) IRAK-1 at multiple residues, thus activating its own kinase activity leading to further autophosphorylation. The hyper-phosphorylation of IRAK-1 leads to disassociation of the complex from the IL-1β receptor and facilitates its interaction with TAK-1, which is upstream of JNK. Phosphorylation of JNK ultimately leads to activation of AP-1 transcription factor that regulates APP transcription through the acute phase response element (APRE) in the promoter(s). Phosphorylation of IRAK-1 also serves as a signal for its ubiquitination and rapid degradation in proteasomes, which effectively terminates the signaling cascade. IL-1β binding to its receptor however, also transiently activates the plasma membrane-localized nSMase2, which regulates the rate of IRAK degradation in a PP2A-dependent manner. Most likely, PP2A de-phosphorylates IRAK-1, thus preventing its ubiquitination and degradation and allowing further receptor binding of non-phosphorylated IRAK-1. In young hepatocytes, the high concentrations of GSH, which is a reversible inhibitor of nSMase2, limit the activity of nSMase2. During aging, depletion of cellular GSH content leads to constitutive activation of nSMase2, increased PP2A activity, and higher abundance of IRAK-1 available for receptor activation. These changes lead to higher abundance of phosphorylated JNK molecules and respectively more pronounced stimulation of gene expression

associated with slow cell growth, impaired tissue renewal, frailty and reduced glucose tolerance (Ceda et al., 2005; Walston and Fried et al., 1999). IL-1β-induced hyper-production of IGFBP1can be substantially diminished *in vitro* by inhibiting JNK activity (Rutkute et al., 2007c). This indicates that differences in the magnitude of JNK phosphorylation in aged organisms could have important consequences for the hepatic physiological responses.

Recent studies into the mechanisms underlying aging-associated hyperresponsiveness to IL-1β in liver have revealed that the lipid-metabolizing enzyme, neutral sphingomyelinase (NSMase), and its product, ceramide, are implicated (Fig. 18.1). In young animals, NSMase is known to mediate the cellular response to IL-1β and other inflammatory cytokines; in aging, however, its activity is constitutively elevated in a redox-sensitive manner. The higher basal NSMase activity (and higher ceramide content at the plasma membrane) in hepatocytes from aged animals is responsible for slower IRAK-1 degradation, which keeps IRAK-1 available for recruitment to various signaling complexes, in turn causing more abundant activation of downstream signaling molecules, such as JNK. The experimental evidence for the role of NSMase, and more specifically the role of nSMase2 in the onset of hepatic IL-1β hyperresponsiveness during aging and the regulation of nSMase2 by oxidative stress and GSH are discussed below.

18.3 Functions of Neutral Sphingomyelinase-2 (NSMASE2) as Mediator of Inflammatory Responses in Young Organisms

The sphingomyelinase (nSMase2) family is a group of biochemically and genetically different enzymes all of which hydrolize Sphingomyelin (SM) to ceramide. SMase activities with neutral and acidic pH optima are found in most mammalian cells, and an enzyme active in alkaline pH is localized in the intestinal wall. Overwhelming evidence shows that pro-inflammatory cytokines like IL-1β and TNFα, as well as other inducers of host immune response, like LPS, activate either the neutral or acidic SMase activity causing transient elevation in the concentration of cellular ceramide. Ceramide has been shown to play an important role as a mediator of cellular responses to stress and its downstream targets are known to include kinases, phosphatases and transcription factors. Taken together, these observations have resulted in postulation of the "SM signaling pathway", which is essential part of the mechanism by which cells of young organisms cope with stress (Hannun and Obeid, 2002).

The cytokine-induced neutral SMase (NSMase) activity is associated with the plasma membrane and is $Mg2+$-dependent. However, a more detailed understanding of its role in signaling at the molecular level was hampered until recently, due to the lack of knowledge about the encoding gene(s). Studies from various groups have now identified the smpd3 gene as the one responsible for this NSMase activity. It encodes a 71 kDa protein termed nSMase2 with two

putative transmembrane domains in the N-terminus and a catalytic domain within the C-terminal region (Hofmann et al., 2000). Extensive biochemical studies (reviewed recently by Clarke et al (Clarke et al., 2006)) have shown that nSMase2 is a *bona fide* NSMase that is Mg dependent and exclusively utilizes SM as a substrate (Luberto et al., 2002; Sawai et al., 1999). Moreover, it shows substrate specificity for very long chain SM, indicating that only a certain subset of cellular SM may be accessible as a substrate (Marchesini et al., 2004). Of particular interest is the observation that nSMase2 is activated by phosphatidylserine and other anionic phospholipids, a property that could be of importance in the regulation of NSMase activity taking into account the intrinsic asymmetry of the plasma membrane where phosphatidylserine is enriched in the inner leaflet. It is noteworthy that the plasma membrane asymmetry is disrupted during apoptosis and other pathophysiological conditions, as well as during aging.

Stoffel and co-workers reported that endogenous and overexpressed nSMase2 localized at the Golgi apparatus in several cell lines (Stoffel et al., 2005). However, studies with highly differentiated primary hepatocytes cultured in three-dimentional matrix of extracellular proteins, Matrigel™, in the absence of growth factors except insulin, have shown that the overexpressed nSMase2 is localized primary at the plasma membrane (Karakashian et al., 2004). Other studies have shown that nSMase2 probably translocates to the plasma membrane when cell confluence is reached (Marchesini et al., 2004) and that nSMase2 is required for the confluence-induced cell cycle arrest to ensue (Hayashi et al., 1997). Lending further support as to the role of nSMase2 in signaling has been the observation made in oligodendroma-derived cells of regulated translocation of nSMase2 to the caveolae, which are the signaling domains of the plasma membrane (Goswami et al., 2005).

Published data from different labs have unequivocally shown that nSMase2 is regulated by cytokines like IL-1β and TNFα, but also that it mediates some of the cytokine effects in young organisms (De Palma et al., 2006; Karakashian et al., 2004; Marchesini et al., 2004). Treatment of hepatocytes with IL-1β leads to increased cellular NSMase activity and accumulation of ceramide (Chen et al., 1995). Increases in the ceramide level on the other hand have been shown to stimulate expression of the hepatic APP, like α-1-acid glycoprotein and C-reactive protein (Chen et al., 1995; Lozanski et al., 1997). Ceramide accumulation is also linked to the activation of TAK-1, JNK and NF-κB, all of which have important roles in the IL-1β cascade (Shirakabe et al., 1997; Verheij et al., 1996; Westwick et al., 1995).

In hepatocytes, nSMase2 is one of the two so far described neutral, Mg^{2+}-dependent sphingomyelinases, the other being nSMase1. Specific silencing of nSMase2 with siRNA has only a minimal effect on the basal cellular NSMase activity, however it results in a complete inhibition of the cytokine-stimulated NSMase activity (Rutkute et al., 2007b). Therefore, nSMase2 is probably an inducible enzyme that contributes little to the basal turnover of SM, but at the same time it is also the only neutral SMase activated by IL-1β. The IL-1β-induced

activation of NSMase2 is required for stimulation of JNK phosphorylation (Rutkute et al., 2007b); however, overexpression of NSMase2 is not sufficient to mimic IL-1β-induced JNK phosphorylation (Karakashian et al., 2004). This indicates that the role of nSMase2 in the IL-1β signaling cascade is rather complex. Indeed, it has been show that activation of nSMase2 regulates the extent of IRAK-1 phosphorylation and degradation following receptor activation. Increased NSMase-2 activity attenuates IL-1β-induced IRAK-1 phosphorylation, and its subsequent ubiquitination and degradation (Karakashian et al., 2004). In contrast, silencing of nSMase2 using siRNA has the opposite consequences and leads to more rapid degradation of IRAK-1. Also, these effects strictly correlate with higher, or respectively, lower JNK phosphorylation (Rutkute et al., 2007b). Apparently, the IL-β-induced stimulation of NSMase, which peculiarly happens after the initial burst in MAP kinase activation, serves to slow down IRAK-1 degradation, keeping IRAK-1 available for recruitment to various signaling complexes, leading to potentiation of JNK phosphorylation. Such a scenario is further supported by reports of increased JNK activation in the presence of inhibitors of proteasome functions which serves to slow down IRAK degradation (Cuschieri et al., 2004). nSMase2 induced stabilization of IRAK-1 most likely involves a phosphatase, like the ceramide-activated protein phosphatase 2A (Chalfant et al., 1999). Indications to that effect come from earlier studies where increased IRAK-1 stability in nSMase2 overexpressing cells was protein phosphatase 2A-dependent (Karakashian et al., 2004).

TNFα has also been shown to activate nSMase2 (Clarke et al., 2007; Marchesini et al., 2004; Tellier et al., 2007). TNFα-induced nSMase2 activation is a prerequisite for endothelial nitric oxide synthase activation in HUVEC cells (De Palma et al., 2006), as well as for the up-regulation in A549 lung epithelial cells of vascular cell adhesion molecule (VCAM) and intracellular adhesion molecule 1 (ICAM) (Clarke et al., 2007) all of which have prominent roles in vascular inflammatory responses. Taken together, the studies described above firmly establish nSMase2 and its product, ceramide, as important factors in the cytokine signaling networks of young organisms.

18.4 Hepatic Neutral Sphingomyelinase: The Link Between Oxidative Stress and IL-1β Hyperresponsiveness in Aging

The functions of ceramide as mediator of host response to infection and stress are well preserved among species, from drosophila and yeast to mice, and are important for organism survival; however, mounting evidence suggests that in the course of aging, these functions change. Studies in the late 80s and early 90s found increased basal levels of ceramide during aging in a variety of cells, including hepatocytes (Cutler and Mattson, 2001; Lightle et al., 2000; Petkova et al., 1988). Studies in cellular models of senescence using human diploid fibroblast related these increases to retardation in the rate of cell growth via

inhibition of rB hyperphosphorylation and the appearance of markers of senescence, such as β-galactosidase (Mouton and Venable, 2000; Venable et al., 1995), suggesting a fundamental role for ceramide in the mechanisms leading to cellular aging. In rodents, ceramide concentrations also change following a "development-aging" continuum: normal aging is associated with accumulation of ceramide, while caloric restriction (CR) that extends the rat life span has been found to decrease the levels of ceramide (Algeri et al., 1991).

Mechanistic understanding of the role of ceramide in aging comes from studies in cultured hepatocytes isolated from young and old rats. In rodents, age-associated changes in ceramide are paralleled by a gradual increase in hepatic NSMase activity (Lightle et al., 2000; Petkova et al., 1988). These changes seem to be limited to the plasma membrane, implying that nSMase2 activity might be involved (Lightle et al., 2000). Indeed, suppression of nSMase2 in hepatocytes from aged animals, either by siRNA silencing or by pharmacological inhibitors, reduces NSMase activity to levels similar to those found in young animals (Rutkute et al., 2007b). Recent evidence has emerged suggesting that these age-associated changes in NSMase-2 activity are due to a decline in hepatic GSH content, providing a novel link between oxidative stress and inflammation during aging.

Oxidative stress is known to be a major factor leading to inflammation during aging. The onset of oxidative stress in aging animals is accompanied by a net decrease in the concentrations of GSH, the major scavenger of free radicals, in different organs, but most notably, in liver. In isolated primary hepatocytes, a dramatic age-dependent decline (40 to 70%) in GSH levels has been reported (Hagen et al., 2000; Vericel et al., 1994). This is noteworthy, because GSH has been found to be a reversible inhibitor of cellular NSMase activity (Liu and Hannun, 1997, 1998). The modulation of NSMase activity by GSH was first established in the context of regulation of TNFα signaling and apoptosis (Liu and Hannun, 1997, 1998). Later, the ability of GSH to affect the sensitivity of T47D/H3 breast cancer cells to doxorubicin was attributed to the inhibitory effect GSH has on NSMase activity (Gouaze et al., 2001). A correlation between oxidative stress and NSMase activity was also found in long-lived rats on vitamin Q10 enriched diet (Bello et al., 2005), and in astrocytes treated with vitamin E (Ayasolla et al., 2004). Finally, recent research has shown that specific downregulation of nSMase2 with siRNA blocks H_2O_2-induced apoptosis of human aortic endothelial cells, identifying nSMase2 as a redox-sensitive protein (Castillo et al., 2007).

Apparently, in the liver the activation of nSMase2 in the process of aging is linked to the substantial age-related decline in the hepatic GSH concentrations. Indeed, supplementation of aged hepatocytes with N-Acetyl cysteine (NAC), a precursor of GSH synthesis, significantly decreases the endogenous NSMase activity to levels typically found in young hepatocytes, while the addition of L-buthionine-S,R-sulfoximine (BSO), an inhibitor of GSH synthesis, to young hepatocytes lowers GSH levels causing sharp increases in NSMase activity (Rutkute et al., 2007a). Notably, GSH depletion exerts its effect on NSMase

activity in a biphasic manner: experiments in hepatocytes from young animals, in which gradual depletion of GSH was achieved by varying the time and dose of BSO treatment, reveal that only when hepatic GSH concentration drops below 30% of its basal level (which coincides remarkably well with the decline typically found in aged animals), is a sharp, dose-dependent activation of NSMase observed. This is consistent with the existence of either a threshold for NSMase activation or of a specific GSH pool, possibly within the immediate surroundings of the enzyme, that influences NSMase activity. Since the direct, *in vitro,* effect of GSH on the overexpressed nSMase2 is also biphasic, the former possibility appears more likely. Finally, evidence supporting the role of GSH depletion in NSMase activation during aging comes also from experiments where the sensitivity of NSMase to inhibition by exogenously added GSH was tested *in vitro*. These studies found that NSMase activity in aged hepatocytes, as compared to young ones, is much more sensitive to direct inhibition by GSH, indicating that in hepatocytes from young rats, the activity is already inhibited by the endogenous GSH (Rutkute et al., 2007a). Interestingly, nSMase1, a NSMase that is genetically distinct from nSMase2, is also sensitive to changes in the GSH/GSSG ratio, but it is not affected by direct *in vitro* treatment with GSH (Martin et al., 2007). This suggests that redox sensitivity might be a common property of neutral sphingomyelinases. It should be noted that, while the increase in NSMase activity during aging is apparently caused by GSH depletion, the IL-1β-induced NSMase activation is GSH-independent (Rutkute et al., 2007a). This is consistent with the observations that IL-1β (unlike TNF-α) induces only modestly ROS production in the liver (Lang et al., 1999), without substantially altering the liver GSH content. Based on all these findings it could be hypothesized that in the process of aging, nSMase2 activity plays the role of a converging point that integrates the effects of oxidative stress into the IL-1β signaling cascade, and by doing so essentially modulates cellular responsiveness to IL-1β.

It has been reported that basal JNK phosphorylation in whole livers increases with aging due to increased oxidative stress (Bose et al., 2005). However, this observation does not hold true in primary hepatocyte cultures, where basal JNK phosphorylation is undetectable in cells from both young and aged animals, despite a significant difference in their GSH content. These observations suggest that at least in hepatocytes, oxidative stress alone is not sufficient to induce JNK activation. However, like the increase in nSMase2 activity, the drop in GSH content in aging is sufficient to stabilize IRAK-1 and potentiate JNK phosphorylation in the presence of external stimulus such as IL-1β, leading to IL-1β hyperresponsiveness (Rutkute et al., 2007a). The addition of BSO to young hepatocytes lowers their GSH levels causing sharp increase in NSMase activity and, notably, IL-1b hyperresponsiveness. Vice versa, restoration of normal GSH concentration and NSMase activity in aged hepatocytes by NAC supplementation also restores normal levels of IRAK-1 degradation and JNK phosphorylation. These results suggest that GSH depletion in aged hepatocytes might induce IL-1β hyperresponsiveness by activating nSMase2.

Accordingly, overexpression of nSMase2 in hepatocytes from young rats induces hyperresponsiveness to IL-1β stimulation; but more importantly, inhibition of nSMase2 activity in hepatocytes from aged animals, either by siRNA silencing or by pharmacological inhibitors, is sufficient to restore normal IL-1β response (Rutkute et al., 2007b). Finally, in NAC-treated aged hepatocytes that show "youthful" GSH levels, NSMase activity, and IL-1β responsiveness, the overexpression of NSMase-2 rescues the aging phenotype (Rutkute et al., 2007a). Thus, in the pathway responsible for hepatic IL-1β hyperresponsiveness during aging, nSMase2 is probably the only factor acting downstream of GSH depletion.

Important data supporting the hypothesis that NSMase is the link between oxidative stress/GSH depletion and IL-1β hyperresponsiveness in the liver comes from studies using calorie restricted aged rats (Rutkute et al., 2007a). Calorie restriction extends the lifespan of many species, from yeast to mammals. Animals, subjected to calorie-restricted diet at early maturity, exhibit delayed aging in terms of their physiological functions, and a marked decrease in the incidence of aging-related diseases. Notably, hepatocytes from aged, calorie restricted rats as compared to those from age-matched *ad libitum* fed rats, have attenuated IL-1β responsiveness resulting from higher GSH content and lower NSMase activity. These observations indicate that the IL-1β response and NSMase activity are modulated in age-specific manner in correlation with the lifespan of the organism.

18.5 Importance of Other Ceramide-Metabolizing Enzymes for Aging

18.5.1 Acid Sphingomyelinase and Brain Aging

Acid sphingomyelinase (ASMase) is a lysosomal enzyme with some functional resemblance to nSMase2 in regards to its ability to generate ceramide and be activated during various stress conditions. Importantly, ASMase is one out of 15 genes that are up-regulated during senescence as determined by subtraction library studies of cultured young and senescent fibroblasts and of fibroblasts from normal and Werner syndrome subjects (Lecka-Czernik et al., 1996). The functional significance of these changes in the ASMase activity of brain is underscored by recent studies describing ASMase knockout mice as being protected against hypoxia-induced neuronal damage (Yu et al., 2000). This could indicate that ASMase is important in the onset of neuronal loss. Indeed, elevation in cellular ceramide has been implicated in the onset of pathophysiological amyloidosis associated with Alzheimer Disease (Puglielli et al., 2003) . Furthermore, increases in ASMase activity were found in the brain of a senescence–accelerated mouse, the enzyme activity correlating with the onset of premature aging (Kim et al., 1997).

18.5.2 De Novo Synthesis of Ceramide and Yeast Longevity

The most direct evidence substantiating the importance of ceramide in the process of aging comes from studies identifying the yeast Longevity Assurance Gene-1 (LAG-1) and its homologue, LAC-1 as regulators of ceramide biosynthesis (Riebeling et al., 2003; Venkataraman and Futerman, 2002a). De novo synthesis is the major pathway for ceramide generation in yeast. The LAG-1 and LAC-1 proteins together with a third protein, Lip1, form a complex that acylates sphinganine (dihydrosphingosine) to form dihydroceramide and also acylates sphingosine to form ceramide (Pewzner-Jung et al., 2006). LAG-1 has previously been identified as a regulator of yeast life span and its deletion has been shown to prolong the life of S. Cerevisiae by 50%. The role of ceramide synthesis in aging is further underscored by the observation that the mammalian homology of LAG-1, UOG 1 (currently re-named to LASS1) which is known to regulate ceramide synthesis in mammalian cells can normalize the life span of Lag-1ΔLac-1Δ strain 85% as effective as LAG-1 itself (Venkataraman et al., 2002b). The mechanism by which LAG-1 affects longevity is unclear. However, *de novo* ceramide synthesis in yeast is a major signaling mechanism involved in regulating the yeast response to heat or osmotic chock (Jenkins and Hannun, 2001).

18.5.3 Ceramidase and Drosophila Retinal Degeneration

A recent study in drosophila showed that modulation of cellular ceramide content might play an important role in the onset of aging associated retinal degeneration (Acharya et al., 2003). Reduction in the levels of ceramide through the targeted expression of the Drosophila Neutral Ceramidase (an enzyme that catalyzes the degradation of ceramide to sphingosine and fatty acid) led to the rescue of retinal degeneration, underlying a possible functional significance for the aging-associated accumulation of ceramide.

18.6 Concluding Remarks

NSMase2 has now been identified as the cytokine-regulated SMase that is required for hepatic acute phase response during infection. NSMase2 is not only a *bona fide* neutral SMase, but in the liver, it exhibits characteristics typical of a signaling enzyme: its very low basal activity is substantially increased by cytokine treatment. The role that nSMase2 activation plays in the IL-1β signaling cascades is rather complex: nSMase2 activity is required, but not sufficient for IL-1β induced activation of JNK. This indicates that nSMase2 activity and its product, ceramide, mediate a regulatory step in the IL-1β signaling pathway. Thus, it is plausible that nSMase2 is a converging point in the cell signaling

cascades that integrates the effects of various stress stimuli, thereby modifying the magnitude of the response. This hypothesis is further supported by the reported role of nSMase2 during aging in the onset of the hepatic hyperresponsiveness to IL-1β, where the sensitivity of nSMase2 towards GSH provides a novel mechanism by which oxidative stress modifies the normal hepatic response to IL-1β.

Apparently, the age-related increases in NSMase activity observed in liver play a crucial role in the onset of the pro-inflammatory state that epitomizes the process of aging. The dual role of nSMase2: as an essential component of the host defense against infection in young organisms; and at the same time as facilitator of the onset of pro-inflammatory state in the elderly, fits with the "antagonistic pleiothropy" view of the aging process, according to which evolution only favors traits that are beneficial during the reproductive age, even though the same traits may be deleterious later in life (Bonsall, 2006; Kirkwood and Austad, 2000).

It is also quite possible that nSMase2 has much broader function in the process of aging. New evidence to that effect comes in the face of nSMase2 knockout mice (Stoffel et al., 2005) that develop a novel form of dwarfism, exhibit delayed puberty, and have reduced levels of IGF1.

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