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Review

The emerging role for sphingolipids in the eukaryotic heat shock response

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Abstract. Eukaryotic cells have a highly conserved response to an increase in temperature, termed the heat shock response. Recent research has revealed multiple roles for various sphingolipids in the heat shock responses of both yeast and mammalian cells. Heat stressed or shocked yeast and mammalian cells have an acute activation of serine palmitoyltransferase, resulting in the de novo biosynthesis of sphingolipids. Also, both mammalian and yeast cells were shown to increase ceramide levels upon heat stress or shock. In yeast cells, several functions have emerged for the de novo produced sphingoid bases in terms of the heat stress response. These functions include a role in accumulation of trehalose, a role in the heat-induced transient G0/G1 cell cycle arrest and phytosphingosine activation of a ubiquitin protein degradation pathway. However, in mammalian systems, ceramides have been demonstrated as bioactive lipids. Ceramides produced in response to heat shock were demonstrated to induce the production of *c-jun*, leading to apoptosis, and to be upstream of dephosphorylation of serine-rich proteins. Increasingly, sphingolipids are emerging as bioactive signaling molecules involved in numerous aspects of the eukaryotic heat shock response.

Key words. Heat shock; sphingolipids; ceramide; yeast; mammalian; eukaryote; and sphingoid bases.

Introduction

The emergence of lipids as signaling molecules has led to an increasing interest in their roles and functions in eukaryotic responses to a wide range of stimuli. One aspect of these investigations has focused on the signaling role of sphingolipids, whose complex forms are major components in membranes of both mammalian and yeast cells. Sphingolipids are characterized by a sphingoid base, such as sphingosine (inset, fig. 1 A), which can subsequently have a fatty acid linked to its free amine, thus forming the ceramides. Ceramides can be further modified with the addition of a head group to form the complex sphingolipids, such as sphingomyelin in mammalian cells (fig. 1 A) and the inositol phosphoceramides in yeast (fig. 1 B). Conversely, the complex sphingolipids can be broken down to ceramide by removal of their head group. Ceramide can be further broken down by ceramidases, which remove the amide-linked fatty acid. Sphingoid bases may be degraded after phosphorylation into hexadecanal or hexadecenal and ethanolamine phosphate (figs 1 A, B), depending on the precursor involved. In mammalian cells, sphingolipids have been shown to have roles in differentiation [1], apoptosis [2] and proliferation [3]. More specifically, ceramide has been proposed as a stress-responsive lipid [4]. Ceramides mediate numerous cellular responses to a wide range of stimuli and can be produced by either de novo biosynthesis or the breakdown of complex sphingolipids (figs 1 A, B). However, research has defined signaling roles for the sphingoid bases, and sphingoid base phosphates among others in response to various cellular stresses, including heat shock. The eukaryotic heat shock response is characterized by the production of heat shock proteins [5] that function as

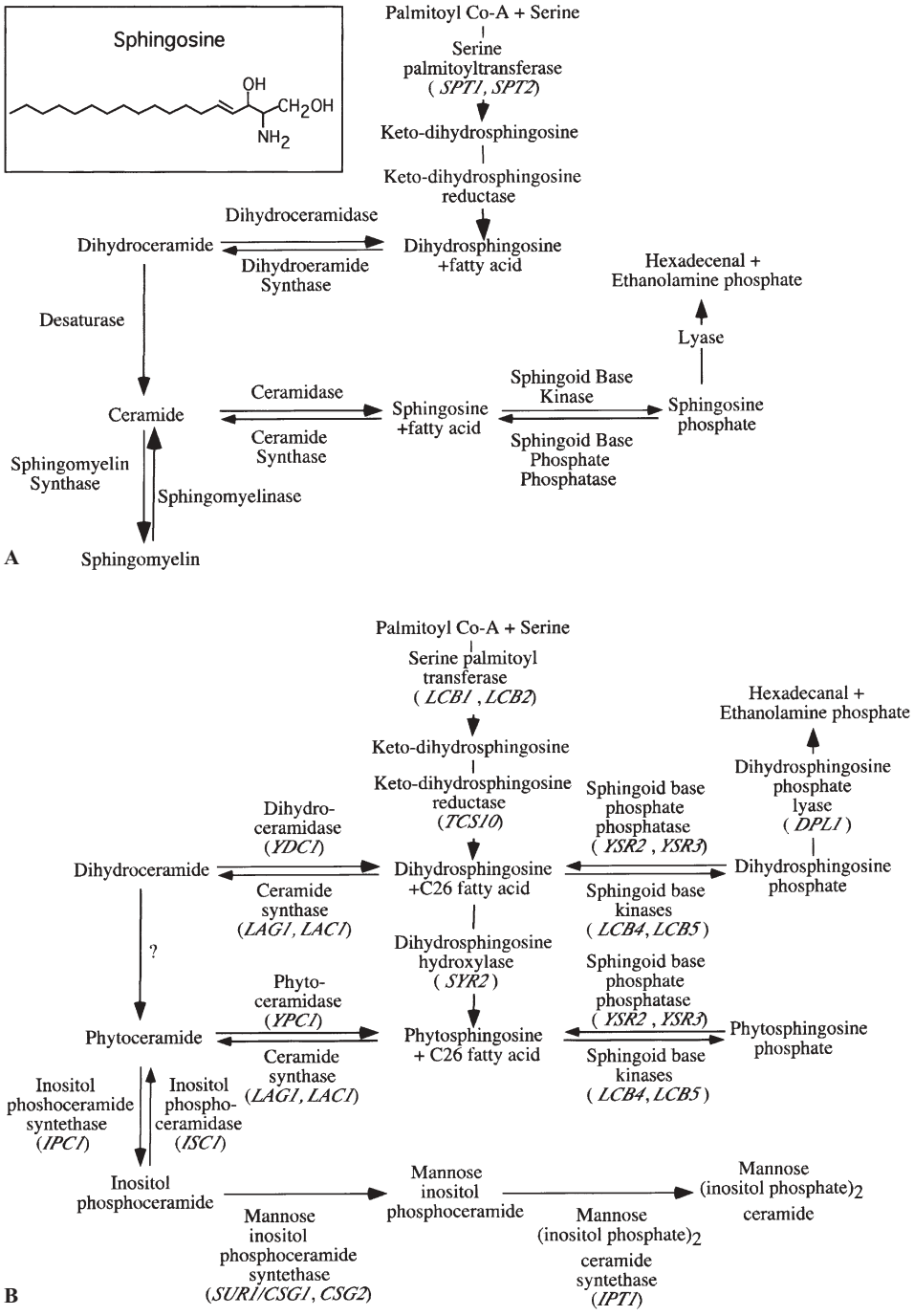

Figure 1. (*A*) Mammalian sphingolipid biosynthetic and breakdown pathways. Scheme of the major enzymes and sphingolipid species produced in mammalian cells. Enzyme and encoding gene are given if known. Inset is the structure of sphingosine. (*B*) Yeast sphingolipid biosynthetic and breakdown pathways. Scheme of the major enzymes and sphingolipid species produced in yeast cells. Enzyme and the encoding gene are given.

chaperonins, and in the processes of protein degradation. Yeast and mammalian cells produce the majority of the heat shock proteins through transcriptional upregulation caused by activation of heat shock factors. Mammalian cells have two heat shock factors, while yeast have only one [6]. Conversely, a subset of heat shock proteins has been determined to be produced by other signaling pathways, such as the RAS/cAMP signaling cascade in yeast. The exposure of eukaryotic cells to an increased ambient temperature also induces a transient cell cycle arrest in the G0/G1 phase of the cell cycle [7]. In yeast, the heat-induced arrest is thought to be mediated by a decrease in the levels of G1 cyclins, which are regulators of entry into the S phase at the point known as START [7]. Also, heat-

stressed yeast cells accumulate large quantities of trehalose, a disaccharide, that has been determined to be a thermoprotectant [8]. Trehalose levels are controlled in part by regulation of its biosynthetic and breakdown enzymes through the RAS/cAMP signaling cascade [9]. Thus, the signaling pathways involved in the generation of thermotolerance in eukaryotic cells are quite complex. This review will focus on yeast and mammalian cells, as these are the two systems in which heat stress and heat shock studies have been published.

Yeast and mammalian cells vary in the manner in which they react to challenges of increased ambient temperature. Yeast are able to react to an increase in ambient growing temperature from 25 to 37 or 39 °C, heat stress, by gaining thermotolerance through the accumulation of trehalose, a transient G0/G1 arrest and the production of heat shock proteins. Once thermotolerance is achieved, yeast degrade the accumulated trehalose in an HSP-70-dependent process [10], and resume a normal cell cycle. Finally, yeast will begin to grow at the elevated temperature. However, larger increases in ambient temperature to 50–55 °C result in heat shock of the yeast, and they try to survive until the temperature returns to 30 °C. This situation is more akin to mammalian cells that have a heat shock response to an increase of temperature from 37 to 42.5 °C, heat shock. Mammalian cells cannot grow at 42.5 °C and must try to survive until the temperature returns to 37 °C. The length of exposure of mammalian cells to heat shock is critical in determining whether cells can regrow or are committed to apoptosis or undergoing necrosis. Interestingly, yeast cells are able to withstand much larger changes in ambient temperature than are mammalian cells. Recently, research has revealed roles for diverse sphingolipids in both the mammalian and yeast heat stress and shock responses. These roles have been investigated in several laboratories, and the results of the recent findings in yeast and in mammalian cells are summarized below.

Sphingolipids in the yeast heat stress response

Isolation of yeast mutants that lacked sphingolipids altogether (1Δ4, Δ*lcb1*), and subsequent isolation of their suppressor strains 4R3 and 7R4 [11], provided the initial indication for the significance of sphingolipids in the yeast heat stress response. The suppressor strains 4R3 and 7R4 have a point mutation in a yeast homolog of the 1-acyl-*sn*-glycerol-3-phosphate acyltransferase of bacteria that allows them to produce novel inositol glycerolipids containing the C26 fatty acid found exclusively in the yeast sphingolipids. These novel inositol glycerolipids apparently fulfill the physiostructural membrane functions of the yeast inositol phosphoceramides [12] and allow for slow growth of these strains at 30 °C. However, these suppressor strains were found to be sensitive to heat stress (37–39 °C) [11], indicating a possible role for the sphingolipids in yeast growth at the elevated temperature. In addition, supplementation of the strains 4R3 and 7R4 with 1, 5 μM phytosphingosine or 1 μM dihydrosphingosine allowed for some growth of these strains in liquid cultures at the elevated 39 °C [13]. Combined, these data point to a necessity for sphingolipids in the yeast heat stress response.

The need for sphingolipids in the yeast heat stress response was shown genetically with studies utilizing strain 7R4-LCB1 (which contains *LCB1* and the suppressor mutation *SLC2-1*). Strain 7R4-LCB1 was able to grow as well as the wild-type strain SJ21R at the elevated temperature of 39 °C, demonstrating that the heat-sensitive phenotype of the suppressor strains was due to the lack of sphingolipids and not an effect of the suppressor mutation. Furthermore, the pharmacologic data in which only the endogenous sphingoid bases, phytosphingosine and dihydrosphingosine and not other sphingolipids, allowed for growth of the suppressor strains at the elevated temperature, support the idea of a key role for endogenous yeast sphingolipids in their heat stress response [13]. Finally, the serine palmitoyltransferase temperature sensitive mutant strain, lcb1-100, was incapable of growth at 37–39 °C, meaning de novo biosynthesis of sphingolipids specifically was needed for yeast to gain thermotolerance and grow at the new elevated temperature [14, 15].

Findings of the necessity of sphingolipids in the yeast heat stress response led to an investigation of possible roles for sphingolipids not only in this response, but also in the heat shock response. Yeast strains with knockouts of the many sphingolipid biosynthetic and breakdown enzymes were studied for their sensitivity or resistance to heat stress or heat shock. Interestingly, no other sphingolipid enzyme knockout strains were found to be sensitive or resistant to heat stress (37–39 °C); however, several sphingolipid enzyme knockout strains were determined to be resistant to heat shock. The strain containing a double knockout of the sphingoid base phosphate phosphatases (*YSR2, YSR3*) was shown to have better growth after exposure to 50 °C for 40 min [16, 17] as compared with its isogenic wild-type strain. Research also demonstrated that a strain containing the double knockout of a sphingoid base phosphate phosphatase and the dihydrosphingosine phosphate lyase was able to survive a short exposure to 44 °C tenfold better than its wild-type strain [18]. The aforementioned knockout strains were shown to accumulate large amounts of sphingoid base phosphates, leading to the proposal of these sphingolipids as possible mediators of thermotolerance in the yeast heat shock response [18]. However, a yeast strain lacking the dihydroceramidase grew better than the isogenic wild type after a 40-min exposure to 50 °C [19]. Overall, the hyperaccumulation of sphingoid base phosphates and

perhaps sphingoid bases may confer some resistance to the exposure of yeast cells to heat shock.

In light of the need for sphingolipids in the yeast heat stress response, the levels of various sphingolipid species and sphingolipid enzyme activities were analyzed throughout the yeast heat stress response. Heat stress was shown to induce a transient increase in the yeast sphingoid bases of phytosphingosine and dihydrosphingosine. Yeast produces both 18-carbon (C18)-long and 20-carbon (C20)-long sphingoid bases. Heat stress resulted in a moderate increase (1.4- to 3-fold) of the C18 dihydrosphingosine and phytosphingosine, but a large increase in the C20 dihydrosphingosine and phytosphingosine. The reported data vary on the fold change of the C20 sphingoids, with one group reporting 6-fold and 10-fold increases [13], while the other reported over 100-fold increases [20]. The discrepancy in these increases may arise from the different methods used to analyze the sphingoid bases. Regardless, these data indicate a change in either the fatty acid preference of the serine palmitoyltransferase enzyme or in the available pools of fatty acids for condensation to 3-ketodihydrosphingosine upon heat stress. The increased levels of sphingoid bases rapidly peak by 10–15 min and decrease to near basal levels by 30 min to 1 h of heat stress [13, 20]. Since the temperature-sensitive serine palmitoyltransferase strain lacked any sphingoid base accumulation, the observed increases are believed to be from de novo biosynthesis through activation of serine palmitoyltransferase. Concurrent with the increase in the sphingoid bases, experiments showed that dihydrosphingosine phosphate was increased around fivefold and phytosphingosine phosphate around eightfold within 10 min of heat stress. Both sphingoid base phosphates returned to basal levels by 20 min of heat stress [18]. Overall, heat stress induces a large, rapid and transient increase in both sphingoid bases and sphingoid base phosphates, and these increases may be consistent with their role as signaling molecules.

Yeast ceramide levels were also measured in response to heat stress and were determined to be increased 5-fold [21] or nearly 10-fold [13] as compared with time-matched controls. At least two distinct molecular species of ceramide were found as determined by either high-performance liquid chromatography (HPLC) or thin layer chromatography [13, 21]. The increased levels of these ceramides peaked around 1 h and were maintained at the elevated temperature for at least 2 h [21]. Significantly, the heat-induced ceramide increase peaked after the decrease of the peak in sphingoid bases, which could indicate a product/precursor relationship. In order to determine whether de novo biosynthesis was the source of the increased level of ceramides, experiments using the ceramide synthase inhibitors fumonisin B1 and australifungin were performed. Fumonisin B1 partially inhibited the heat-induced ceramide increase [13], and australifungin almost totally blocked this increase [21]. These results demonstrate that the ceramide was produced by the activity of yeast ceramide synthases. Furthermore, both groups found no changes in the levels of the inositol phosphoceramides upon heat stress, suggesting that inositol phosphoceramide breakdown to ceramide was not the source of the increased ceramide levels [13, 21]. These data indicate the increased levels of ceramide were derived from de novo biosynthesis. The possibility of signaling roles for the sphingolipids was investigated, due to the many studies which previously demonstrated the bioactivity of sphingolipids, especially ceramides.

Initial studies using the sphingolipid-deficient suppressor strain 4R3 and a wild-type strain demonstrated equal induction of the major heat shock proteins HSP-104, HSP-90s, HSP-70s and HSP-26, indicating a lack of a role for sphingolipids in this heat response. Although 4R3 yeast cells showed an increase in heat shock proteins, they accumulated little of the thermoprotectant trehalose as compared with their wild-type strain. Long-term supplementation of the 4R3 strain with D,L-erythro/threo-dihydrosphingosine in media recovered their ability to accumulate trehalose upon heat stress, thus implicating sphingolipids. A role for sphingolipids in the accumulation was further defined when exogenous treatment with 50 µM D,L-erythro-dihydrosphingosine induced production of trehalose within 3 h in wild-type yeast [20]. Intriguingly, treatment with 50 µM D,L-erythro/threo-dihydrosphingosine was found to induce a trehalose biosynthetic gene (TPS2) promoter fused to the lacZ reporter gene. This induction was proposed to be through the stress-response elements (STREs) of the TPS2 gene. However, studies using the HSP104 promoter region containing 3 STREs showed little induction by D,L-erythro/threo-dihydrosphingosine. Furthermore, heat was shown to induce both an STRE promoter construct and the HSP104 promoter region much more efficiently than exogenous treatment with D,L-erythro/threo-dihydrosphingosine. These data point to perhaps some other form of promoter specificity or additional mechanism of promoter activation. Finally, induction of the TPS2-lacZ construct demonstrated little specificity as D,L-erythro-dihydrosphingosine, phytosphingosine, the nonendogenous L-threo dihydrosphingosine and to a lesser extant C_2 ceramide induced the reporter construct at concentrations of 25–100 µM [20]. One cautionary note to these studies is that these concentrations of sphingolipids can be lethal to yeast cells in liquid cultures [G. Jenkins, unpublished data]. The aforementioned increases of sphingolipids upon heat stress and the above findings reveal an involvement for sphingolipids, particularly the sphingoid bases, in the production of trehalose upon heat stress in yeast cells.

The knowledge that heat stress causes inactivation of the uracil permease, Fur4p, and data showing exogenous

phytosphingosine treatment blocking uracil uptake, led to investigation of a function for phytosphingosine in this effect of heat stress. The Fur4 protein was found to be degraded upon heat stress of 37 °C, accounting for the inactivation of the uracil permease. This effect of heat stress was mimicked in wild-type yeast by the exogenous addition of 20 µM phytosphingosine, but not by addition of 20 µM D,L-erythro-dihydrosphingosine, keto-dihydrosphingosine, C2 phytoceramide or stearylamine. These data showed a specificity for the endogenous sphingoid base phytosphingosine in the degradation of Fur4 protein. Therefore, the in vivo role for phytosphingosine was investigated using strain lcb1-100 [22]. As noted previously, strain lcb1-100 harbors a temperature-sensitive serine palmitoyltransferase [15], and thus the de novo production of phytosphingosine is blocked upon heat stress. The heat stress degradation of the Fur4 protein was found to be abrogated in strain lcb1-100 [22]. Addition of phytosphingosine to heat-stressed lcb1-100 cells resulted in recovery of degradation of Fur4, but this is not surprising since this treatment had already been shown to degrade Fur4p in wild-type cells at 30 °C. Genetic analysis revealed that phytosphingosine was upstream of NPI1 (ubiquitin ligase) and DOA4 (deubiquitinase), both of which are required for Fur4 endocytosis and degradation. Furthermore, a strain containing a knockout of *DOA1*, a regulatory component of the 26 S proteasome degradation pathway, showed no degradation of Fur4 upon treatment with phytosphingosine. In addition, the degradation of Fur4p mediated by phytosphingosine was also shown to require the stress-responsive Lys-63 polyubiquitination residue of ubiquitin [22]. Overall, this research demonstrated the phytosphingosine-dependent activation of the 26 S proteasome pathway in degradation of the Fur4 protein upon heat stress.

The heat-stress-induced transient cell cycle arrest of yeast occurs at 1 h and is recovered by 2 h at the elevated temperature [7]. Since this is a major effect of heat stress on yeast, the temperature-sensitive serine palmitoyltransferase strain, lcb1-100, the sphingolipid deficient suppressor strain, 7R4, and their wild-type strain cell cycles were examined. Both lcb1-100 and 7R4 were found to lack the transient G0/G1 cell cycle arrest seen in wild-type yeast upon heat stress (39 °C). These data indicated a role for sphingolipids, specifically those from de novo biosynthesis, in the heat stress cell cycle arrest. Strains deleted in the dihydrosphingosine hydroxylase, sphingoid base kinases, sphingoid base phosphate phosphatase and a knockout in the fatty acyl elongation pathway, which attenuates ceramide production, displayed the arrest at 39 °C. Ergo, the epistasis analysis implicated the de novo production of the sphingoid bases in this heat-stress-induced response. Furthermore, exogenous treatment with 10 µM phytosphingosine induced a G0/G1 arrest in wild-type yeast cells within 30 min of treatment. Some specificity of this response was shown in that L-erythro-dihydrosphingosine was less potent than the endogenous form of D-erythro-dihydrosphingosine. Also, stearylamine, a structurally similar glycerolipid, did not induce any cell cycle arrest [15]. The hyperstable G1 cyclin (*CLN3-1*) had previously been demonstrated to block the heat-stress-induced cell cycle arrest [23] and was found to block the sphingoid base-induced cell cycle arrest. These data suggest a role downstream of the sphingoid bases for the G1 cyclins in the transient G0/G1 arrest, which would be consistent with their known function as mediators of cell cycle progression. However, possible upstream regulators of the G1 cyclins such as the alpha factor/MAPK pathway and ubiquitin-dependent degradation were determined not to be involved [15] in the sphingoid base-caused cell cycle arrest. In addition, a strain with both sphingoid base kinases (*LCB4, LCB5*) knocked out was unable to recover from the heat-stress-induced cell cycle arrest, indicating a function for these kinases in this physiology. The combination of genetic analysis and exogenous treatment with sphingoid bases supports a role for the de novo production of sphingoid bases in the heat-caused transient G0/G1 cell cycle arrest.

Ancillary studies using the temperature-sensitive serine palmitoyltransferase strain, lcb1-00, have shown a role for the de novo production of sphingolipids in endocytosis and demonstrated possible downstream targets for the sphingoid bases in yeast. At a restrictive temperature of 37 °C (heat stress), strain lcb1-100 was found to be defective in both the internalization step of endocytosis and in the organization of the actin cytoskeleton [14]. These findings indicate a role for sphingolipids in these physiologic processes upon heat stress. Further studies found that overexpression of *YCK2* and *PKC1*, two yeast kinases, in strain lcb1-100 suppressed the endocytic and actin defects at 37 °C, but did not suppress strain lcb1-100's temperature-sensitive growth phenotype. Thus, the action of the suppressor was specific for these effects and not the lcb1-100 strain's growth phenotype. Also, the loss of the protein phosphatase function of PP2A through inactivation or deletion of *CDC55* (the regulatory subunit of PP2A) or *PPH21/22/3* (catalytic subunits of PP2A), but not *SIT4* (a related catalytic subunit) reversed the endocytic defect of lcb1-100 at 37 °C. However, the knockouts of the kinases or the above mutations in PP2As in a wild-type strain were found not to cause an endocytic defect [24]. These data show that sphingolipids are upstream of these activities in the endocytic pathway. Subsequent research showed that overexpression of the homologs to mammalian 3-phosphoinositide-dependent-kinase-1, *PKH1* or *PKH2*, also overcame the endocytic and actin defects of strain lcb1-100 at 37 °C. Importantly, these kinases phosphorylate Pkc1p, thus indicating this kinase as a downstream effector of this signaling cascade involved in endocytosis. Phytosphingosine at 2.5–25 nM

was found to increase Pkh1p/2p activity threefold in vitro, but to inhibit it at higher concentrations (500 nM). Therefore, the model was proposed of the sphingoid bases activating Pkh1p/2p to phosphorylate Pkc1p, which subsequently controls endocytosis [25]. In conclusion, sphingoid bases are likely to have roles in the regulation of specific kinase and phosphatase reactions that may be important in both endocytosis and possibly in the yeast heat stress responses.

Role for sphingolipids in the mammalian heat shock response

The role of the sphingolipid ceramide has been implicated in a plethora of mammalian signaling pathways leading to various responses. An early study on ceramide and $\alpha\beta$-crystallin transcription showed that heat shock (42.5 °C) of 2 h in NIH WT-3T3 cells caused a twofold increase of total ceramide and that the increase was maintained for up to 6 h after the cells were returned to 37 °C. Although exogenous C2 ceramide was found to induce $\alpha\beta$-crystallin, but not the structurally similar HSP25, no link between the heat-induced ceramide increase and induction of $\alpha\beta$-crystallin was demonstrated [26]. Also, it is relevant to note that C2 ceramide has been demonstrated to have very different bioactivities than the endogenous long-chain ceramides. These experiments provided an early indication of sphingolipid changes upon heat shock of mammalian cells. Subsequent research has focused on the changes in ceramide levels, the source of these changes and on defining roles for ceramide in the mammalian heat shock response.

Recent research showed that an upshift in the growing temperature of HL-60 cells from 37 to 42.0 °C caused a nearly twofold ceramide level increase at 2 h. Furthermore, exposure of HL-60 cells for 30 min at 42 °C resulted in a sustained 1.7-fold increase of ceramide for up to 4 h after the cells were returned to 37 °C [27, 28]. A less severe heat shock of 40 °C resulted in a smaller increase of ceramide than 42 or 44 °C [28]. Kondo et al. investigated the source of the ceramide increase and discovered a 123 % increase in the in vitro activity of neutral sphingomyelinase (nSMase). Also, sphingomyelin levels were shown to be decreased after 30 min of treatment at 40, 42 and 44 °C. Two hours at 42 °C caused a drop from 43.8 to 33.4 pmol/10^6 cells, which correlated with an increase in ceramide from 11.3 to 18.1 pmol/10^6 cells [28]. The combination of the above results and the observation that fumonisin B1 did not inhibit the increase in ceramide levels led to the conclusion that sphingomyelin hydrolysis was the source of the ceramide upon heat shock [28].

In contrast to the above model, the de novo biosynthesis of sphingolipids upon heat shock was determined to be the source of ceramide increases seen in Molt-4 cells. Heat shock induced a twofold increase in total ceramide levels at 2 h and increased to 2.5-fold by 4 h. Speciation of the increased ceramides revealed C16 ceramide as the major species with a lesser amount of C22 and C24:1 ceramides. No changes were found in the dihydroceramides [29]. Myriocin, a serine palmitoyltransferase inhibitor, and fumonisin B1 were both found to inhibit the increase in total ceramide mass, which indicated that the increases were from de novo biosynthesis. Labeling with tritiated palmitate immediately before heat shock showed an accumulation of newly made ceramides, but not the sphingoid bases. In accordance with this finding, sphingoid base mass measurements showed no increases upon heat shock. Obviously, ceramide was the first sphingolipid intermediate which accumulates upon activation of de novo sphingolipid biosynthesis of Molt-4 cells by heat shock. The 1.75-fold increase of newly made ceramides within 1 min of heat shock indicates a surprisingly rapid activation of serine palmitoyltransferase. Increased production of ceramide incorporating the tritiated palmitate was observed for up to 2 h with an accompanying increase of incorporation in the complex sphingolipids of sphingomyelin, cerebrosides and gangliosides. Again, both myriocin and fumonisin B1 inhibited the heat-shock-induced increases in labeled ceramides; therefore, the ceramides produced upon heat shock are products of both serine palmitoyltransferase activity and ceramide synthase activity. The acute activation of sphingolipid de novo biosynthesis was determined to be the source of the observed twofold accumulation of total ceramide mass in Molt-4 cells [29].

The mammalian heat shock response involves an activation of sphingomyelin hydrolysis and/or an activation of the de novo biosynthesis of sphingolipids. Once the increases in ceramide mass were observed, the possible functions of these increases were investigated.

The group of Kondo et al. have shown that the ceramide produced upon heat shock in HL-60 cells has a role in causing apoptosis through activation of *c-jun/c-fos*. Increased temperature of 40, 42 and 44 °C resulted in lowering of the number of viable cells and an increase in the number of apoptotic cells correlating directly with the increase in temperature. Ceramide was implicated as a key player in the apoptosis of heat-shocked cells by use of a heat-shock-resistant subculture of HL-60 cells. These cells do not increase ceramide levels upon heat shock and undergo heat-shock-induced apoptosis at much lower rates than wild-type HL-60 cells. Addition of C2 ceramide to the resistant HL-60 cells at 42 °C resulted in their apoptosis [28]. Although promising, this result may be due strictly to the effects of C2 ceramide treatment, which was shown to induce apoptosis of HL-60 cells. Both exogenous treatment with 10 µM C2 ceramide and heat shock of 42 °C were found to induce increases in *c-jun* and *c-fos* messenger RNA (mRNA). Use of *c-fos*

and *c-jun* antisense deoxynucleotides blocked both heat shock and C2 ceramide-induced apoptosis in the HL-60 cells, leading to the conclusion that increases in *c-jun* and *c-fos* mRNA are needed for heat-shock-caused apoptosis of HL-60 cells. Finally, the use of a caspase-3 inhibitor, which did not affect the heat shock ceramide increase, was found to block both the 1.5-fold increase in caspase-3 activity caused by C2 ceramide and the nearly 2.5-fold increase of caspase-3 activity induced by heat shock. In addition, the caspase-3 inhibitor DMQD-CHO blocked the increase in *c-jun* mRNA and significantly lowered both heat shock and C2 ceramide caused apoptosis in HL-60 cells [28]. Therefore, the authors propose that heat shock induces sphingomyelin hydrolysis forming ceramide, which subsequently acts upstream of caspase-3 activation that causes an increase in the mRNA of *c-jun*, ultimately resulting in apoptosis of HL-60 cells.

The role of ceramide in the inhibition of HSP-70 production upon heat shock was also investigated. As stated previously, treatment of HL-60 cells with either heat shock or C2 ceramide results in apoptosis. However, cotreatment of HL-60 cells with both heat shock and C2 ceramide displayed a dramatic effect of increasing apoptosis as demonstrated by fluorescent-activated cell sorting (FACS), 4′-6-diamidine-2-phenyl indole (DAPI) nuclear staining, May-Giesma staining and DNA ladders. Quantitation of the aforementioned techniques (minus DNA laddering) showed that the increase in apoptosis was not additive, but synergistic [27]. As expected, heat shock induced a large increase in HSP-70 mRNA, resulting in an increased amount of cellular HSP-70 protein. This increase of HSP-70 mRNA cells was found to be inhibited by the addition of C2 ceramide at 5–10 μM. Furthermore, C2 ceramide (30 μM or higher) was found to inhibit the HSP-70 mRNA increase in heat-shocked HEK 293 cells in a time- and dose-dependent manner. The inhibition of HSP-70 mRNA by C2 ceramide addition to heat-shocked HL-60 cells was demonstrated to be specific, as HSP-60 and HSP-90 were minimally decreased at the same concentrations that fully blocked HSP-70 mRNA. C2 ceramide addition to heat-shocked HL-60 specifically inhibited HSP-70 protein levels [27], but did not block the translocation of HSF1 or HSF2. Also, the transcriptional rate of HSP-70 was not affected by cotreatment of heat shock cells with C2 ceramide. However, HSP-70 mRNA post-translational degradation was shown to be increased by C2 ceramide cotreatment in a time-dependent manner, accounting for the decrease in mRNA and subsequent inhibition of HSP-70 protein levels. Although the study demonstrates a role for exogenous addition of C2 ceramide in the suppression of HSP-70 mRNA levels post-translationally and in the synergistic activation of apoptosis, it is not clear whether the endogenous increase in ceramides also inhibits HSP-70 mRNA.

The de novo production of ceramide in Molt-4 cells was found to be upstream of the dephosphorylation of serine-rich (SR) proteins, which control gene splicing for the production of RNA. In Molt-4 cells, heat shock was demonstrated to not induce poly-(ADP-ribose) polymerase (PARP) protein cleavage or significant apoptosis for up to 4 h [29], consistent with findings using SKT6 mouse erythroleukemia cells [30]. Heat shock did induce a rapid activation of de novo biosynthesis of sphingolipids resulting in a twofold increase of ceramide mass at 2 h. The increased level of ceramide was found to correlate with the dephosphorylation of the SR proteins [29]. As expected, the use of myriocin or fumonisin B1 resulted in inhibition of dephosphorylation of the SR proteins [29]. Together, these data demonstrate an acute activation of serine palmitoyltransferase upon heat shock, resulting in the de novo accumulation of ceramide that mediates dephosphorylation of the SR proteins.

Discussion

The eukaryotic heat stress and shock response is a highly conserved physiologic response to one of nature's oldest challenges to living cells. The adaptation to or survival of an increased ambient temperature has evolved several key features, with the most prominent being the change in the pattern of gene expression resulting in induction of a subset of proteins termed heat shock proteins. Furthermore, eukaryotic cells undergo a change in their metabolism, and they also arrest at the G0/G1. Interestingly, these mechanisms are present in both unicellular and multicellular eukaryotic organisms' heat responses. The data gathered in both yeast and mammalian systems has disclosed that sphingolipids have multiple functions in the heat stress and shock responses of these organisms and that these functions appear to be mainly protective in nature. This idea is consistent with the proposal of sphingolipids, especially ceramide in mammalian cells, as stress-responsive lipids. The changes of sphingolipids and their roles are still being actively investigated in both the yeast and mammalian model systems.

Current data demonstrate that yeast and mammalian cells share common features in their response to heat stress and shock in terms of sphingolipids. The most notable is the acute activation of serine palmitoyltransferase, which results in de novo production of ceramides. In both systems serine palmitoyltransferase was activated within minutes of exposure to the elevated temperature. The activation of de novo biosynthesis in yeast cells caused a transient accumulation of sphingoid bases and sphingoid base phosphates, but the sphingoid bases were not increased in Molt-4 cells, and the sphingoid base phosphates were not measured. However, the lack of sphingoid base accumulation may be cell type spe-

cific for mammalian systems, and more research and data could clarify this issue. Despite this difference, a similar time course for significant accumulation of ceramide was observed, with yeast showing increases by 1 h and mammalian cells by 2 h. Another difference between yeast and mammalian cells was found in the hydrolysis of the membrane sphingolipids. In the mammalian studies, sphingomyelin was demonstrated to be hydrolyzed by neutral sphingomyelinase upon heat shock. However, yeast cells showed little or no changes in the inositol phosphoceramides upon heat stress, indicating a lack of hydrolysis of the complex membrane sphingolipids. Overall, both yeast and mammalian cells respond in a similar way in terms of sphingolipid enzymes and, ultimately, sphingolipids.

Research has revealed divergent functions for sphingolipids produced upon heat stress and shock in these two systems; however, these diverse functions exert a positive effect on the cells. In yeast, the sphingolipids fulfill a thermoprotective role, allowing the yeast to survive and grow at heat stress temperatures. Sphingolipid changes also serve a protective role in mammalian cells. Namely, the ceramide increase has been linked to apoptosis, which is a protective mechanism in multicellular organisms. Furthermore, ceramide changes do not necessarily lead to cell death in Molt-4 cells upon heat shock [G. M. Jenkins, et al., unpublished] and may have other thermoprotective functions in mammalian cells not yet discovered. Thus, current data demonstrate positive effects for the changes in sphingolipids, but these effects are different in terms of the bioactive lipid, mechanism and final function.

The sphingoid bases have emerged as the main bioactive species in the yeast heat stress response. Specifically, the sphingoid bases produced de novo act on trehalose levels and the G0/G1 cell cycle arrest. Also, the phytosphingosine produced de novo acts upstream of ubiquitination and protein degradation. Furthermore, the sphingoid bases have been demonstrated to have multiple kinases and phosphatases as downstream targets (summarized in fig. 2). Yeast sphingoid bases may also have a function in the production of certain yeast proteins through the STREs. In contrast, mammalian studies have not yet found a role for the sphingoid bases upon heat shock, but these may emerge as more research is performed in various mammalian systems. Despite the reported changes in sphingoid base phosphates in yeast, no downstream effect has been coupled to these changes. Ceramides are thus far the bioactive sphingolipids in the mammalian heat shock response. The increase in ceramide has been linked to *c-jun* and apoptosis, and to the dephosphorylation of the SR proteins (fig. 3). Interestingly, no biological function has yet been attached to the increased levels of ceramides found in the yeast heat stress response. However, ongoing research could elucidate further roles and functions for the sphingolipids in both yeast and mammalian systems.

The mammalian system is most interesting in that it has direct implications for the regulation and roles played by sphingolipids, especially ceramide. Ceramide has

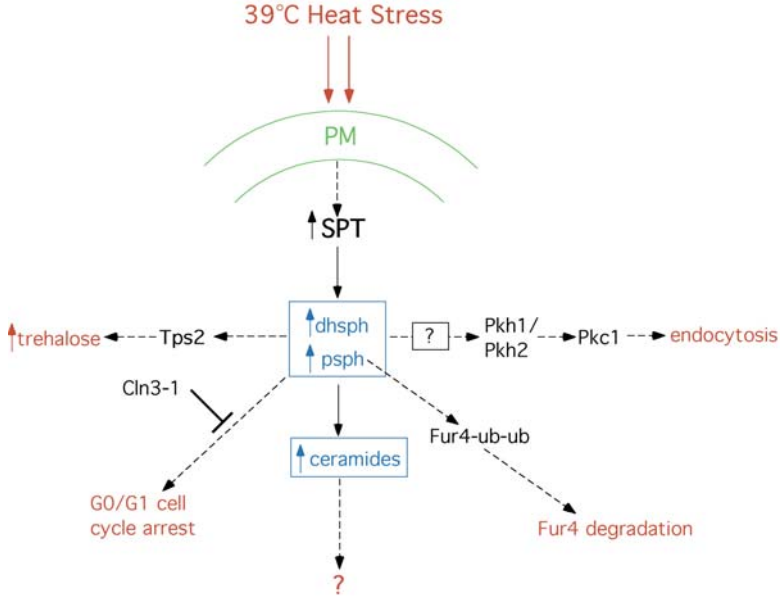

Figure 2. Summary of yeast signaling pathways in relation to increased levels of sphingoid bases upon heat stress. Sphingolipids are presented in blue, enzymes are in black, plasma membrane is green and physiologic endpoints are in red. Abbreviations used: plasma membrane (PM), serine palmitoyltransferase (SPT), dihydrosphingosine (dhsph), phytosphingosine (psph), protein kinase H1/ H2 (PKH1/2), protein kinase C (PKC), trehalose phosphate synthase (TPS2), hyperstable G1 cyclin (CLN3-1), uracil permease (Fur4) and polyubiquitination (ub-ub).

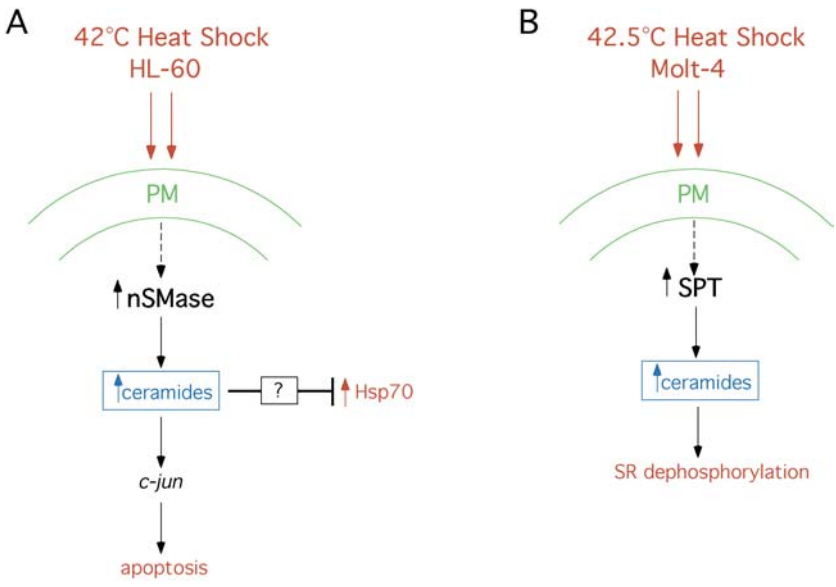

Figure 3. Summary of effects for heat shock induced ceramide increases in the mammalian heat shock response. Sphingolipids are presented in blue, enzymes are in black, plasma membrane is green and physiologic endpoints are in red. Abbreviations used: plasma membrane (PM), neutral sphingomyelinase (nSMase), heat shock protein 70 (HSP70), serine palmitoyltransferase (SPT), and serine-rich proteins (SR).

emerged as a key regulator of many mammalian cellular responses to various insults and stimuli. Because of this wealth of knowledge, the mammalian model system is attractive in terms of future research and ultimately has the advantage of tying into other sphingolipid research on mammalian cells. Yeast has the major advantage that most of the sphingolipid biosynthetic and breakdown genes have now been identified and cloned. Therefore, one can genetically dissect the roles of sphingolipids in many responses, including heat stress and heat shock. Also, the creation and study of knockouts and temperature-sensitive mutants is very much easier in yeast as compared with mammalian cells. Research in both systems will be furthered by the use of pharmacologic inhibitors of sphingolipid enzymes in both yeast and mammalian systems. Overall, future research in both yeast and mammalian systems will result in a better understanding of the conserved and divergent natures of the functions of sphingolipids in the heat shock responses of eukaryotic cells.

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1 Okazaki T., Bielawska A., Bell R. M. and Hannun Y. A. (1990) Role of ceramide as a lipid mediator of 1 alpha,25-dihydroxyvitamin D3-induced HL-60 cell differentiation. J. Biol. Chem. **265:** 15823–15831
2 Obeid L. M., Linardic C. M., Karolak L. A. and Hannun Y. A. (1993) Programmed cell death induced by ceramide. Science **259:** 1769–1771
3 Jung E. M., Griner R. D., Mann-Blakeney R. and Bollag W. B. (1998) A potential role for ceramide in the regulation of mouse epidermal keratinocyte proliferation and differentiation. J. Invest. Dermatol. **110:** 318–323
4 Hannun Y. A. and Luberto C. (2000) Ceramide in the eukaryotic stress response. Trends Cell Biol. **10:** 73–80
5 Parsell D. A., Taulien J. and Lindquist S. (1993) The role of heat-shock proteins in thermotolerance. Philos Trans. R. Soc. Lond. B. Biol. Sci. **339:** 279–85; discussion 285–286
6 Lindquist S. and Craig E. A. (1988) The heat-shock proteins. Annu. Rev. Genet. **22:** 631–677
7 Johnston G. C. and Singer R. A. (1980) Ribosomal precursor RNA metabolism and cell division in the yeast Saccharomyces cerevisiae. Mol. Gen. Genet. **178:** 357–360
8 Wiemken A. (1990) Trehalose in yeast, stress protectant rather than reserve carbohydrate. Antonie Van Leeuwenhoek. **58:** 209–217
9 De Virgilio C., Hottiger T., Dominguez J., Boller T. and Wiemken A. (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast. I. Genetic evidence that trehalose is a thermoprotectant. Eur. J. Biochem. **219:** 179–186
10 Hottiger T., De Virgilio C., Bell W., Boller T. and Wiemken A. (1992) The 70-kilodalton heat-shock proteins of the SSA subfamily negatively modulate heat-shock-induced accumulation of trehalose and promote recovery from heat stress in the yeast, *Saccharomyces cerevisiae*. Eur. J. Biochem. **210:** 125–132
11 Dickson R. C., Wells G. B., Schmidt A. and Lester R. L. (1990) Isolation of mutant *Saccharomyces cerevisiae* strains that survive without sphingolipids. Mol. Cell Biol. **10:** 2176–2181
12 Nagiec M. M., Wells G. B., Lester R. L. and Dickson R. C. (1993) A suppressor gene that enables *Saccharomyces cerevisiae* to grow without making sphingolipids encodes a protein that resembles an *Escherichia coli* fatty acyltransferase. J. Biol. Chem. **268:** 22156–22163
13 Jenkins G. M., Richards A., Wahl T., Mao C., Obeid L. and Hannun Y. (1997) Involvement of yeast sphingolipids in the heat stress response of *Saccharomyces cerevisiae*. J. Biol. Chem. **272:** 32566–32572

14 Zanolari B., Friant S., Funato K., Sutterlin C., Stevenson B. J. and Riezman H. (2000) Sphingoid base synthesis requirement for endocytosis in *Saccharomyces cerevisiae*. EMBO J. **19:** 2824–2833
15 Jenkins G. M. and Hannun Y. A. (2001) Role for de novo sphingoid base biosynthesis in the heat-induced transient cell cycle arrest of *Saccharomyces cerevisiae*. J. Biol. Chem. **276:** 8574–8581
16 Mandala S. M., Thornton R., Tu Z., Kurtz M. B., Nickels J., Broach J. et al. (1998) Sphingoid base 1-phosphate phosphatase: a key regulator of sphingolipid metabolism and stress response. Proc. Natl. Acad. Sci. USA **95:** 150–155
17 Mao C., Saba J. D. and Obeid L. M. (1999) The dihydrosphingosine-1-phosphate phosphatases of *Saccharomyces cerevisiae* are important regulators of cell proliferation and heat stress responses. Biochem. J. **342 (Pt 3):** 667–675
18 Skrzypek M. S., Nagiec M. M., Lester R. L. and Dickson R. C. (1999) Analysis of phosphorylated sphingolipid long-chain bases reveals potential roles in heat stress and growth control in *Saccharomyces*. J. Bacteriol. **181:** 1134–1140
19 Mao C., Xu R., Bielawska A., Szulc Z. M. and Obeid L. M. (2000) Cloning and characterization of a *Saccharomyces cerevisiae* alkaline ceramidase with specificity for dihydroceramide. J. Biol. Chem. **275:** 31369–31378
20 Dickson R. C., Nagiec E. E., Skrzypek M., Tillman P., Wells G. B. and Lester R. L. (1997) Sphingolipids are potential heat stress signals in *Saccharomyces*. J. Biol. Chem. **272:** 30196–30200
21 Wells G. B., Dickson R. C. and Lester R. L. (1998) Heat-induced elevation of ceramide in *Saccharomyces cerevisiae* via de novo synthesis. J. Biol. Chem. **273:** 7235–7243
22 Chung N., Jenkins G., Hannun Y. A., Heitman J. and Obeid L. M. (2000) Sphingolipids signal heat stress-induced ubiquitin-dependent proteolysis. J. Biol. Chem. **275:** 17229–17232
23 Rowley A., Johnston G. C., Butler B., Werner-Washburne M. and Singer R. A. (1993) Heat shock-mediated cell cycle blockage and G1 cyclin expression in the yeast *Saccharomyces cerevisiae*. Mol. Cell Biol. **13:** 1034–1041
24 Friant S., Zanolari B. and Riezman H. (2000) Increased protein kinase or decreased PP2A activity bypasses sphingoid base requirement in endocytosis. EMBO J. **19:** 2834–2844
25 Friant S., Lombardi R., Schmelzle T., Hall M. N. and Riezman H. (2001) Sphingoid base signaling via Pkh kinases is required for endocytosis in yeast. EMBO J. **20:** 6783–6792
26 Chang Y., Abe A. and Shayman J. A. (1995) Ceramide formation during heat shock: a potential mediator of alpha B-crystallin transcription. Proc. Natl. Acad. Sci. USA **92:** 12275–12279
27 Kondo T., Matsuda T., Tashima M., Umehara H., Domae N., Yokoyama K. et al. (2000) Suppression of heat shock protein-70 by ceramide in heat shock-induced HL-60 cell apoptosis. J. Biol. Chem. **275:** 8872–8879
28 Kondo T., Matsuda T., Kitano T., Takahashi A., Tashima M., Ishikura H. et al. (2000) Role of c-jun expression increased by heat shock- and ceramide-activated caspase-3 in HL-60 cell apoptosis. Possible involvement of ceramide in heat shock-induced apoptosis. J. Biol. Chem. **275:** 7668–7676
29 Jenkins G. M., Ashley Cowart L., Signorelli P., Pettus B., Chalfant C. and Hannun Y. (2002) Acute activation of de novo sphingolipid biosynthesis upon heat shock causes an accumulation of ceramide and subsequent dephosphorylation of SR proteins. J. Biol. Chem. **277:** 42572–42578
30 Nagata Y. and Todokoro K. (1999) Requirement of activation of JNK and p38 for environmental stress-induced erythroid differentiation and apoptosis and of inhibition of ERK for apoptosis. Blood **94:** 853–863