Identification of C_{18:1}-Phytoceramide as the Candidate Lipid Mediator for Hydroxyurea Resistance in Yeast*

Received for publication, December 17, 2012, and in revised form, April 23, 2013 Published, JBC Papers in Press, April 25, 2013, DOI 10.1074/jbc.M112.444802

Nabil Matmati¹, Alessandra Metelli², Kaushlendra Tripathi², Shuqi Yan¹, Bidyut K. Mohanty², and Yusuf A. Hannun¹,²

From the ¹Stony Brook Cancer Center and the Department of Medicine, Stony Brook University, Stony Brook, New York 11794-8155 and the ²Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29403

Background: Deletion of the sphingolipid enzyme Isc1 in yeast makes cells sensitive to hydroxyurea.

Results: Phytoceramides are the main sphingolipid involved in yeast cell response to hydroxyurea toxicity.

Conclusion: C_{18:1}-phytoceramides are identified as the specific ceramide that provide protection from hydroxyurea in a Cdc55-dependent manner.

Significance: This is the first specific sphingolipid species identified to play a role in the genotoxic response pathway.

Recent studies showed that deletion of ISC1, the yeast homologue of the mammalian neutral sphingomyelinase, resulted in an increased sensitivity to hydroxyurea (HU). This raised an intriguing question as to whether sphingolipids are involved in pathways initiated by HU. In this study, we show that HU treatment led to a significant increase in Isc1 activity. Analysis of sphingolipid deletion mutants and pharmacological analysis pointed to a role for ceramide in mediating HU resistance. Lipid analysis revealed that HU induced increases in phytoceramides in WT cells but not in isc1Δ cells. To probe functions of specific ceramides, we developed an approach to supplement the medium with fatty acids. Oleate (C_{18:1}) was the only fatty acid protecting isc1Δ cells from HU toxicity in a ceramide-dependent manner. Because phytoceramide activates protein phosphatases in yeast, we evaluated the role of CDC55, the regulatory subunit of ceramide-activated protein phosphatase PP2A. Overexpression of CDC55 overcame the sensitivity to HU in isc1Δ cells. However, addition of oleate did not protect the isc1Δ,cdc55Δ double mutant from HU toxicity. These results demonstrate that HU induces a lipid pathway mediated by a specific sphingolipid, C_{18:1}-phytoceramide, produced by Isc1, which provides protection from HU by modulating Swe1 levels through the PP2A subunit Cdc55.

Bioactive sphingolipids constitute an important group of regulatory molecules in the eukaryotic cell, and they have been implicated in a multitude of cellular functions ranging from regulation of cell growth to angiogenesis, inflammation, and responses to stress stimuli. The group of bioactive sphingolipids includes ceramide, sphingosine 1-phosphate, sphingosine, and ceramide 1-phosphate. Overall, ceramide has been appreciated as a transducer of stress stimuli; however, recent biochemical and analytical studies have revealed significant complexity and diversity in ceramide structure, and we have recently proposed that ceramides are actually a family of closely related molecules that may mediate distinct functions.

The yeast Saccharomyces cerevisiae has emerged as a powerful model in the study of sphingolipid metabolism and function. Indeed, most of the key enzymes of metabolism of bioactive sphingolipids were initially identified in yeast (1). Also, stress stimuli in yeast, such as heat stress, have been shown to regulate the acute production of bioactive sphingolipids, which in turn have been implicated in mediating many key responses such as cell cycle arrest, regulation of translation, and regulation of nutrient permeases (2, 3).

The sphingolipid enzyme Isc1, the yeast homologue of mammalian neutral sphingomyelinases, catalyzes the hydrolysis of complex sphingolipids, inositol phosphorylceramide (IPC), mannosylinositol phosphorylceramide (MIPC), and mannosyl-diinositol phosphorylceramide (M(IP)₂C), to dihydro- and phytoceramides (4–6). Deletion of the ISC1 gene has revealed that this enzyme is important for many biological processes, such as growth on nonfermentable carbon sources, gene expression, cell cycle arrest, regulation of translation, and regulation of nutrient permeases (7–9). The response to stress conditions such as high salt, heat stress, and oxidative stress (10–12).

In previous studies, we described a role for ISC1 in the genotoxic stress response to both the ribonucleotide reductase inhibitor HU and the alkylating agent methyl methanesulfonate (13). Isc1 has also been found to be involved in cellular morphogenesis under genotoxic stress (14). Deletion of the ISC1 gene conferred sensitivity to HU, mediated by the activation of a latent G₁/M checkpoint. Mechanistically, the results implicated persistent phosphorylation on tyrosine 19 of the cyclin-dependent kinase Clb2-Cdc28, a known activator of the G₁/M checkpoint (13, 15). In turn, this was traced to persistent activation of the Swe1 kinase responsible for phosphorylating...
Phytoceramide Provides Hydroxyurea Resistance

CB2-Cdc28. Either mutation of the phosphorylation site tyrosine 19 of CB2-Cdc28 or the deletion of SWE1 was able to rescue the growth defect of isc1Δ on HU. These results indicated that Isc1 regulates phosphorylation of CB2-Cdc28 and the G2/M checkpoint activation mediated by controlling Swe1 activity. This unexpected involvement of an enzyme of lipid metabolism in mediating the response to HU raised a number of important questions. Here, we address the question of whether Isc1 mediates response to HU action as a downstream effector. We also endeavor to determine the specific lipid mediator of Isc1 in response to HU and the downstream targets of the lipid mediator involved in Swe1 level regulation.

To address these questions, we employed biochemical and genetic approaches. The results demonstrated activation of Isc1 in response to HU. Genetic analysis suggested that phytoceramides were the likely mediators. Because yeast contain many species of ceramides, distinguished by the length of the fatty acyl group, α-hydroxylation of the fatty acid, and the hydroxylation of the sphingoid base, we employed a lipidomic approach. These results suggested more specifically a role for phytoceramide, but not dihydroceramides or α-hydroxyceramides, in the HU response. We next developed an approach to selectively enrich yeast cells in specific ceramides by providing selected fatty acid precursors. These results specifically supported a role for C18:1-phytoceramide in mediating the HU response. Our data define a highly specific pathway of sphingolipid metabolism in the response to HU and implicate Cdc55, the regulatory subunit of phosphatase P2A, as the downstream target of C18:1-phytoceramide. The implications of these results for the delineation of ceramide-specific pathways are also discussed.

EXPERIMENTAL PROCEDURES

Strains and Media—Strains used in this study were as follows: JYK-3d (MATα trpl1 leu2-3 his4 ura3 ade2 rme1), JYK-3d, isc1Δ (MATα trpl1 leu2-3 his4 ura3 ade2 rme1 isc1::KanMX) from our laboratory (4), and JMY 1469 (MATα SWE1::HIS2 ade his2 leu2-3, 112 trp1-1 ura3Δns) a gift from D. Lew (Duke University). ISC1 was deleted in JMY 1469 strains to obtain JMY 1469,isc1Δ (13).

BY4741 (MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), deletion library mutant strains were also used. The isogenic BY4741 deletion mutants were used as follows: isc1Δ, surΔ1, surΔ2, surΔ4, csg2Δ, fen1Δ, ssc7Δ, ipt1Δ, lag1Δ, lac1Δ, ypc1Δ, and ydc1Δ.

Deletion of CDC55 was done using the genomic DNA extracted from Y300-cdc55Δ (MATα ura3-1 his3-11,15 leu2-3,112 trp1-1 ade2-1 can1-100 cdc55::HIS3), a gift from Y. Wang (Florida State University) (16). PCR was performed using forward primer 5′-TAGGGCAATGGCCCGGTATATAA-3′ and reverse primer 5′-GGGAGATATGGGTAATTATAAGGCG-3′ from Operon. The PCR product was used to transform BY4741 and BY4741-isc1Δ strains. Colonies growing on histidine minus (HIS−) agar plates were checked for the deletion of CDC55 by extracting DNA and using the same primers to perform PCR as follows: W303-1A (MATα can1-100 ade2-1 his3-11,15 leu2-3 112 trp1-1 ura3-1); lac1Δ,lag1Δ, same as W303-1A, but lac1Δ:LEU2 lag1Δ::TRP1, a gift from M. Jazwin-
ski (University of Louisiana).

Standard yeast media, yeast extract, peptone, dextrose, adenine (YPD), Synthetic Complete (SC) plus SC-inositol, and SD URA+ dropout media were used in this study. Minimal SD base with galactose and raffinose URA− dropout media was used to overexpress genes under GAL promotor, purchased from Clontech, catalog no. 630420. HU was purchased from US Biological and was employed at 7.5 and 10 mg/ml in liquid and solid SC and YPD media, respectively. Fumonisin B1 was purchased from ENZO Life Sciences and was used at a concentration of 400 μM. Fatty acids were purchased from Sigma; myristate, palmitate, stearate, oleate, arachidate, and hexacosanate were dissolved in methanol then added to SC medium or SC URA− with or without agar, at a concentration of 0.003%.

Spot tests were performed as described previously (13); briefly, cells were grown to stationary phase overnight, then diluted to an A600 of 0.1, and then grown to an A600 of 0.8. The cultures were diluted to an A600 of 0.3 for the first spot on the left. 1:10 dilutions were done for the consecutive spots on the plates. Plates were incubated at 30 °C for 3 days.

Plasmids—pYES2 containing a galactose-inducible promoter was from Invitrogen. ISCI ORF containing a FLAG tag was cloned into pYES2 using restriction enzymes KpnI and XbaI (4). ISCI-D163A and ISCI-K168A were generated in our laboratory (17). YPC1 and YDC1 plasmid construction was done as follows; BY4741 genomic DNA was prepared using yeast DNA extraction kit from thermo scientific catalog no. 78870.

The YPC1 and YDC1 genes were cloned using the following primers: YPC1forward, 5′-CGTAAAGCTTTAATGGAAACCAAAT-3′, and reverse, 5′-CTTTTGATTCGGTCAATTAAATAGAACA-3′; YDC1forward, 5′-TCCCATATTGTGGATTGAA-3′, and reverse, 5′-TTGGTCAATTGCAGTACA-3′.

PCR was carried out using the following programs: YPC1, 94 °C for 1 min, 49 °C for 1 min, 72 °C for 2 min, and 72 °C for 10 min; YDC1, 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min, and 72 °C for 10 min, 30 cycles. The PCR product was then cloned using a PCR 2.1-TOPO vector from Invitrogen catalog no. K4560-40; TOP10 competent bacteria cells purchased from Invitrogen were transformed with the made plasmid. Bacterial colonies grown on plates containing 50 μg/ml carbenicillin plates were screened for positive clones containing the YPC1 and the YDC1 inserts by digesting plasmid recovered by a standard Qiagen miniprep using BamHI and XhoI restriction enzymes and BamHI and NotI, respectively. The positive clones were extracted from the gel and cloned into YEPl24-PL (YEPl24) (18).

Bacterial transformation was then performed, and positive clones were identified and verified if they contained the YEPl24 containing YPC1 or YDC1 by restriction digestion with BamHI and XhoI or BamHI and NotI.

Enzymatic Activity Assay—The assay for Isc1 activity was conducted as described previously (4). Briefly, sphingomyelin labeled with 14C on the choline moiety was used as a substrate, and the hydrolysis of choline-methyl-14C-sphingomyelin was determined by liquid scintillation counting.
Phytoceramide Provides Hydroxyurea Resistance

Western Blot Analysis—Western blots were performed as described previously (13). An anti-Myc tag was used to detect Swel-Myc and also an anti-Swe1 from Santa Cruz Biotechnology catalog no. sc7171. Antibody against PSTAIRE (PSTAIRE domain of Cdc2p34) from Santa Cruz Biotechnology, catalog no. sc-53, was used as a loading control.

Lipid Analyses by HPLC-MS/MS—Levels of dihydroceramide, phytosphingosine, phytosphingosine 1-phosphate, dihydrophosphoglycerine, and dihydrophosphoglycerine 1-phosphate were measured by the high performance liquid chromatography/mass spectrometry (LC-MS/MS) methodology as described previously (19). Analytical results of lipids were expressed as lipid level/total cell number.

Lipid Determination—Cells were grown overnight and then resuspended in the morning to an absorbance of A600 of 0.15 in 50 ml of medium. Cells were grown to reach an A600 of 0.7 and then were either treated with HU or vehicle. Cells were harvested after 3 and 20 h of treatment with HU. Absorbance at A600 was measured; all the cultures were diluted to A600 of 0.7. Cells were then centrifuged at 3000 × g, and the pellets were resuspended in 1 ml of a lipid extraction solvent containing isopropyl alcohol (50%), diethyl ether (10%), pyridine (2%), ammonia (25%), and water (15%). Acid-washed glass beads (425–600 μm) were added to a 200-μl volume. Tubes were placed on a Destroyer vortex for five repeats of a 3-min on, 3-min off regiment. The cells were then transferred into 15-ml tubes by pouring, and the Eppendorf tubes were washed with 1 ml of extraction solvent and poured into 15-ml tubes, the tubes contained ~2 ml of solvent, and cells and glass beads were sent to the Lipidomic Core at the Medical University of South Carolina for lipid analysis.

Thin Layer Chromatography (TLC)—Overnight cultures were diluted to an A600 of 0.1 and then grown to an A600 of 0.6 in SC medium. Cells were then centrifuged and washed twice with SC-inositol medium (YNB-inositol catalog no. 1533-050 from Sunrise Science Products) and inoculated into 20 ml of SC-inositol containing 4 μCi/ml [3H]myoinositol for 3 h. The resulting labeled lipids were extracted and separated by TLC and developed for visualization by autoradiography. All experiments have been repeated three independent times.

Statistics—All data represent the median of at least three independent experiments unless otherwise stated on the figure legend. GraphPad Prism software was used in all illustrations.

RESULTS

HU Treatment Leads to an Increase of ISC1 Activity—To determine whether ISC1 is involved in a regulatory pathway downstream of HU, we investigated the effects of HU on Iscl activity. An in vitro enzymatic assay was performed in WT cells overexpressing ISC1, and the results showed a net increase in Isc1 activity in response to HU starting at 90 min, and the activity remained elevated throughout the time course that lasted 20 h as shown (Fig. 1A). To investigate if the Isc1 enzymatic activity is required for its role in protecting cells from HU toxicity, catalytically inactive mutants of Isc1 were expressed in an ISC1diptheria toxin::URA3 strain transformed with a pYES2 vector containing wild type ISC1 and the two inactive mutants, ISC1-G163A and ISC1-K168A. Neither catalytically inactive mutant was able to protect cells from HU toxicity (Fig. 1B). In contrast, wild type ISC1 allowed cells to resume growth on HU. The level of IPC, MIPC, and M(IP)2C decreased significantly when WT cells were treated with HU after 3 h (Fig. 1C). This change in complex sphingolipid due to HU treatment corresponds to the increase of Isc1 activity after HU. These results indicate that HU treatment increases activity of Isc1 and that this activity is needed to protect cells from HU toxicity.
Phytoceramide Provides Hydroxyurea Resistance

Identifying the Lipid Species That Are Regulated by HU—Because HU activated Isc1, it became important to determine the candidate lipid mediators of Isc1 action. This is a particularly difficult issue with bioactive sphingolipids because these molecules are interconvertible in cells. For example, the production of ceramide by Isc1 may result in further metabolism to sphingoid bases or more complex sphingolipid species. The results also rule out a specific role for α-hydroxyceramide because SCS7 deletion mutant showed no response to HU treatment.

To confirm that ceramides are important for protecting cells from HU toxicity, a pharmacological approach was additionally employed using fumonisin B1, an inhibitor of ceramide synthases. The results (Fig. 2E) showed that fumonisin B1-treated WT cells became sensitive to HU. Taken together, these results suggest that one or more ceramides play critical roles in cell survival in response to HU.

Interestingly, deletion of the two ceramidases YPC1 and YDC1 rendered cells more resistant to HU. This was observed during the initial screening of sphingolipid deletion mutants (Fig. 2B) and was confirmed with additional spot tests illustrated in Fig. 2F. These results clearly support a role for ceramide and not further downstream metabolites (such as sphingoid bases or their phosphates) in mediating the resistance to HU. These are also corroborated by the studies with fumonisin B1.

These results in turn suggested that YPC1 and/or YDC1 overexpression would make cells more sensitive because of the increased Ypc1 and/or Ydc1 activity and the consequent decrease of phytoceramide and/or dihydroceramide levels. When wild type cells overexpressed YPC1, they became more sensitive to HU (Fig. 2G). Thus, these results again support a role for ceramide and not further downstream products.

Very interestingly, and in contrast to the results with YPC1, overexpression of YDC1 had no effect on the sensitivity of wild type cells to HU (Fig. 2G). These results begin to distinguish among “ceramide” species in the HU response by providing evidence that phytoceramides (the preferred substrates of YPC1) and not dihydroceramides (the preferred substrates of YDC1) (23) are the candidate mediators of the action of Isc1 in response to HU. A map illustrating the yeast sphingolipid pathway indicates the responses to the sphingolipid deletion mutants (sensitivity or resistance) to HU (Fig. 3).

Based on the above results, it became important to determine whether a subset of ceramide molecular species was involved in the HU response. In yeast, ceramides can exist in at least 40 distinct molecular species based on the length of the fatty acyl chain, hydroxylation of the sphingoid base (phyto series), and hydroxylation of the fatty acyl group (α-hydroxy series) (24). Therefore, the sphingolipid response to HU was analyzed in the wild type and in isc1Δ cells, using LC-tandem mass spectrometry.

Several ceramide species showed some changes in the various conditions; therefore, we elected to focus on specific cera-
Phytoceramide Provides HydroxyureaResistance

mides that would show the following patterns: 1) increases in response to HU, and 2) loss of this increase in \( \text{isc1} \). These criteria define the subset of ceramides that are directly produced in response to HU in an \( \text{ISC1} \)-dependent manner. To make sure that WT and \( \text{isc1}/\text{H9004} \) cells would not lose viability after 20 h of HU treatment, a colony-forming unit assay was done. The results showed that 68% of WT cells were viable, and 57% of \( \text{isc1}/\text{H9004} \) cells were viable (data not shown). Thus, there was only a modest 11% increase in cell death in \( \text{isc1}/\text{H9004} \), but this difference was not statistically significant.

After 20 h of HU, many phytoceramide species increased in WT but failed to do so in \( \text{isc1}/\text{H9004} \) cells. These changes were not seen after 3 h of HU treatment (Fig. 4, A and B). The most prominent increases were in \( C_{18}, C_{18:1}, C_{20}, \) and \( C_{26} \)-phytoceramides after 20 h of treatment (Fig. 4, C and D). These results pointed toward the phytoceramide subspecies most likely involved in the protection against HU. In contrast, analysis of dihydroceramides (Fig. 5, A–D) did not reveal major changes in response to HU in their levels after 3 and 20 h in WT and \( \text{isc1}/\text{H9004} \) cells. No changes were detected in the levels of \( \text{C26} \)-hydroxyphytoceramides. In fact, deletion of \( \text{SCS7} \), the \( \text{C26} \)-hydroxyylase that transforms phytoceramide into \( \text{C26} \)-hydroxyceramide, did not show sensitivity to HU, indicating that these species do not play a major role in protecting cells from the genotoxic effect of HU (data not shown). Importantly, overexpression of \( \text{YPC1} \) in WT cells abolished the increases in phytoceramides in response to HU, and more so the \( C_{16}, C_{18}, C_{18:1}, C_{20}, \) and \( C_{26:1} \) species (Fig. 6, A and B). These results therefore point to a subset of phytoceramides as candidate regulators of the HU response.

**Specific Role for** \( C_{18:1} \)-**Phytoceramide in Mediating Protection from HU**—Because the above results suggested that one or more phytoceramides may be specifically involved in the HU response, it became important to determine whether generating phytoceramides was sufficient to protect \( \text{isc1}/\text{H9004} \) cells from the HU effects and if indeed a specific phytoceramide was needed. Given the very hydrophobic nature of long chain and very long chain ceramides, it was not possible to directly administer these lipids to yeast culture. Therefore, we sought a different approach to selectively enrich yeast in specific ceramides. To this end, specific fatty acid precursors, myristate (\( C_{14} \)), palmitate (\( C_{16} \)), stearate (\( C_{18} \)), oleate (\( C_{18:1} \)), arachidate (\( C_{20} \)), and hexacosanoic acid (\( C_{26} \)), were separately added to the medium to determine whether \( \text{isc1}/\text{H9004} \) cell growth on HU can be rescued.

Initially, the effects of these fatty acids on ceramide composition was determined to establish whether addition of fatty acids could influence the increase in specific ceramides with the corresponding fatty acid. Therefore, the lipid levels were analyzed in WT cells growing on a medium supplemented individually with \( C_{14}, C_{16}, C_{18}, C_{18:1}, \) and \( C_{20} \) fatty acids. \( C_{26} \) was not developed further due to very poor solubility in culture medium. When myristate was added to the medium for 3 h, the levels of \( C_{14} \)-phytoceramides increased significantly compared with all other species (Fig. 7A). Addition of palmitate resulted in increased levels of \( C_{14}, C_{16}, C_{18}, \) and \( C_{26:1} \) with \( C_{16} \) being the highest (Fig. 7B). Stearate addition increased \( C_{18} \)-phytoceramide but not \( C_{18:1} \)-phytoceramide significantly compared with other species. However, other chain lengths such as \( C_{14}, C_{16}, C_{22:1}, \) and \( C_{24:1} \) increased but not to the extent of the \( C_{18} \) increase (Fig. 7C). Conversely, oleate increased the levels of \( C_{14}, C_{16}, C_{18}, C_{18:1}, \) and \( C_{24:1} \) increased but not to the extent of the \( C_{18} \) increase (Fig. 7D). Arachidate increased the levels of \( C_{14} \)- and \( C_{24:1} \)-phytoceramide (Fig. 7E). No increase of \( C_{20} \) was detected. The poor response to arachidate in increasing the specific chain length

---

**FIGURE 3.** Basic scheme of the sphingolipid pathway in *S. cerevisiae* with enzymes color-tagged for sensitivity or resistance to HU, green (sensitive), magenta (resistant), not colored (no sensitivity or resistance was observed or not tested); black arrows represent de novo pathway, red arrows represent the salvage pathway.
FIGURE 4. Analysis of ceramide species following HU treatment in WT and isc1Δ cells by mass spectrometry. Phytoceramide levels were determined after 3 and 20 h of treatment with HU. To determine the fold changes of phytoceramide species due to HU, the values obtained from the treated samples were divided by the untreated ones. Fold changes in phytoceramides are represented in A for WT, in B for isc1Δ after 3 h, in C for WT, and in D, for isc1Δ after 20 h. All measurements represent the mean of two independent experiments.

FIGURE 5. Dihydroceramide species after 3 h of HU in WT cells (A) compared with the dihydroceramide fold changes in isc1Δ (B). C and D are fold changes of dihydroceramides after 20 h in WT and isc1Δ, respectively. All measurements represent the mean of two independent experiments.
Phytoceramide Provides Hydroxyurea Resistance

FIGURE 6. Changes in phytoceramide levels upon overexpression of YPC1. A, fold changes in phytoceramides in WT cells transformed with vector control YEp24 compared with WT cells overexpressing YPC1 in YEp24 plasmid after 20 h HU treatment (B). All measurements represent the mean of three independent experiments.

FIGURE 7. Effect of addition of fatty acid on phytoceramide species levels increases after 3 h of HU treatment. Effect when adding myristate (A), palmitate (B), stearate (C), oleate (D), and arachidate (E). The values are represented as a fold change of treated with fatty acid compared with untreated. The results are the mean of three independent experiments.
was probably due to the poor solubility of this fatty acid when added to the medium. Dihydroceramide species seemed less responsive to the addition of fatty acids, as we noticed no specific chain length increases corresponding to the respective fatty acid added (Fig. 8, A–E). Taken together, these results show that besides arachidate, C14, C16, C18, and C18:1 fatty acids selectively increased the levels of their respective phytoceramides with some effects for some of the fatty acids on other ceramides. This then allowed us to explore the ability of these fatty acids to rescue the growth defect of *isc1/H9004* in response to HU.

The results showed that oleate (C18:1) was the only fatty acid that when added to the medium rescued the growth of *isc1Δ* in the presence of HU (Fig. 9A). To verify that oleate did not interfere with HU toxicity in a nonspecific manner, we evaluated the KAR3 deletion strain known to be sensitive to HU (25); Kar3 is a microtubule motor protein that functions in mitosis and meiosis and has no known relationship to sphingolipids. Adding oleate to the medium containing HU had no effect on HU toxicity (data not shown).

To determine whether the effects of oleate were due to incorporation into phytoceramide, *isc1Δ* cells were treated with fumonisin B1 to inhibit the synthesis of phytoceramide in response to oleate. The results showed that fumonisin B1 prevented the ability of oleate to reverse HU sensitivity (Fig. 9B), indicating that the protective effects of oleate were due to incorporation of the C18:1 fatty acids into phytoceramide. Moreover, oleate was also able to rescue the increased sensitivity to HU of cells overexpressing *YPC1* (Fig. 9C).

Taken together, the above results pointed to C18:1 phytoceramide as the candidate. However, due to the overlap in effects of some of the fatty acids on ceramide species, we resorted to examining which lipids showed the most specific relationship to the protection from HU. Table 1 summarizes the increase of...
Phytoceramide Provides Hydroxyurea Resistance

FIGURE 9. Evaluation of the ability of specific fatty acids to rescue cells from HU toxicity. A, spot tests were performed on WT and isc1Δ on SC medium supplemented with different chain length fatty acids (myristate, palmitate, stearate, oleate, and arachidate) and the same fatty acids with HU. This experiment was independently repeated three times. B, effects of fumonisin B1 on isc1Δ cells’ HU sensitivity rescue by oleate. C, effect of oleate on the HU sensitivity of WT cells overexpressing the YPC1 gene. These experiments were repeated three times.

at least 3-fold registered in each chain length species of phytoceramides and dihydroceramides in WT and isc1Δ treated with HU and the increase due to the addition of oleate or other fatty acids. The only species that increased in WT with HU, failed to do so in isc1Δ with HU and increased when oleate was added but did not increase when other fatty acids were provided was C18:1-phytoceramide. Thus, taken together, these results show that phytoceramides are sufficient to mediate the protective effects of ISC1 in response to HU, and the results also suggest that C18:1-phytoceramide is the primary ceramide species involved in this process.

Oleate Decreases Swe1 Kinase Levels in isc1Δ in the Presence of HU—In a previous study, we showed that HU sensitivity of isc1Δ cells was due to Swe1 kinase stabilization, leading to increased phosphorylation of the cyclin kinase Cbl2-Cdc28 resulting in a G2/M checkpoint activation. Those results revealed a necessary role for sphingolipids in regulating the Swe1/Cdc28 checkpoint. The above results with oleate allowed us to investigate whether phytoceramides are sufficient to regulate the stabilization of Swe1 levels. Degradation of Swe1 has been found to be regulated by the regulatory subunit of protein phosphatase 2A (PP2A), CDC55 (26). Deletion of CDC55 renders cells sensitive to the drug (Fig. 10A), consistent with previous results (16). Interestingly overexpression of CDC55 was able to protect isc1Δ cells from the HU toxicity (Fig. 10B); thus, Cdc55 functions downstream of Isc1 in the pathway leading to HU. Importantly, addition of oleate failed to protect the single deletion cdc55Δ strain and the double deletion isc1Δ,cdc55Δ from HU toxicity (Fig. 10A, right panel) suggesting that oleate acts upstream of Cdc55.

Next, we evaluated the levels of Swe1 kinase in the presence of oleate and HU in isc1Δ and in isc1Δ,cdc55Δ double deletion strain. The results in Fig. 11A show that oleate was sufficient to induce loss of Swe1 in isc1Δ treated with HU, thus overcoming

### TABLE 1
Comparison between the effect of oleate and other fatty acids on phytoceramide and dihydroceramide levels in WT and isc1Δ strains

| Phytoceramides | Increased in WT with HU | Increased in isc1Δ with HU | Increased by oleate | Increased by other fatty acids |
|----------------|-------------------------|---------------------------|---------------------|-------------------------------|
| PHS           | –                       | –                         | +                   | –                             |
| PHS 1-phosphate | –                     | –                         | +                   | –                             |
| C14           | +                       | +                         | +                   | +                             |
| C16           | +                       | +                         | +                   | –                             |
| C18           | +                       | +                         | +                   | –                             |
| C20           | +                       | +                         | +                   | –                             |
| C22           | +                       | +                         | +                   | –                             |
| C24           | +                       | +                         | +                   | –                             |
| C26           | +                       | +                         | +                   | –                             |

| Dihydroceramides | Increased in WT with HU | Increased in isc1Δ with HU | Increased by oleate | Increased by other fatty acids |
|-------------------|-------------------------|---------------------------|---------------------|-------------------------------|
| DHS               | –                       | –                         | –                   | –                             |
| DHS 1-phosphate   | –                       | –                         | +                   | –                             |
| C12              | +                       | +                         | +                   | –                             |
| C14              | +                       | +                         | +                   | –                             |
| C18              | +                       | +                         | +                   | –                             |
| C20              | +                       | +                         | +                   | –                             |
| C22              | +                       | +                         | +                   | –                             |
| C24              | +                       | +                         | +                   | –                             |
| C26              | +                       | +                         | +                   | –                             |

FIGURE 10. Role of Cdc55 in protecting isc1Δ from HU. A, deletion of CDC55 renders cells more sensitive to HU, and addition of oleate does not protect cdc55Δ from HU toxicity. Double deletion mutant isc1Δ,cdc55Δ is sensitive to HU in the presence and absence of oleate. B, overexpression of CDC55 protects isc1Δ cells from HU toxicity. These experiments were repeated three times.
the defect observed in the iscΔ cells. However, oleate had no effect on the stabilization levels of Swe1 in the double deletion iscΔ/cdc55Δ strain (Fig. 11B). This indicated that Cdc55 deletion in the iscΔ strain was sufficient to abrogate the protective effect of oleate.

**DISCUSSION**

The goal of this study was to define the regulation of bioactive sphingolipids by Isc1 in response to HU and to determine whether a specific sphingolipid species is required for cell protection against HU by regulating downstream target Swe1 kinase. The results initially demonstrated a requirement for enzymatic activity of Isc1 in protecting cells from HU, and the results subsequently pointed to phytoceramides as the main sphingolipids responsible for protecting cells from HU genotoxic effects. Further analysis highly implicated C18:1-phytoceramide as the main mediator of the Isc1 response.

The initial results clearly implicate Isc1 and its product phytoceramide in the HU genotoxic effect. Treating cells with HU induced an increase of Isc1 enzymatic activity, and the overexpressing inactive form, G167A and K168A, failed to protect the cells from the HU toxicity. Furthermore, HU treatment resulted in a significant increase in ceramides as shown by mass spectrometry. Also, we found that phytoceramides are the only sphingolipid species that increased with HU but failed to increase in ISCI-deleted cells.

To confirm the involvement of ceramides, we evaluated the sensitivity to HU of deletions of each nonessential sphingolipid gene. This analysis indicated that in addition to ISCI, deletion of SLIR4, DPL1, and double deletion of LAG1 and LAC1 increased the sensitivity to HU. With the exception of DPL1, these genes are involved in the generation of ceramide by the de novo (Sur4, Lag1, and Lac1) or salvage pathway (Isc1). Furthermore, deletion of YPC1 or YDC1 provided protection from HU toxicity, thus ruling out a key role for sphingoid bases. Moreover, the overexpression of YPC1 but not YDC1 made the WT cells more sensitive to HU. In contrast, double deletion of LCB4 and LCB5, known to cause accumulation of LCBs, showed no sensitivity to HU, indicating that LCBs have no role in HU protection. In addition, no changes in LCB levels were detected when lipid levels were checked after HU treatment. Together, these results clearly implicate ceramides as the major players in regulating the sensitivity to HU. It is important to note that deletion of SLIR2, which produces mainly phytoceramides, showed no sensitivity to HU. This result may suggest that phytoceramides have no role in protecting cells from HU. However, genetic, biochemical, and pharmacological methods indicated clearly that the main species are phytoceramides. It is therefore possible that the observation that sur2Δ is not sensitive could be due to the large accumulation of dihydroceramides and dihydrosphingosines in this mutant, probably making dihydroceramide’s IPC that would be used as a substrate for activated...
Phytoceramide Provides Hydroxyurea Resistance

Isc1 that may suffice to replace the need for phytoceramides and provide protection from HU toxicity.

At this point, we cannot explain the role of DPL1 in sensitivity to HU. Deletion of DPL1 induces accumulation of the phosphorylated LCB. Accumulation of phosphorylated LCB has been shown to cause slow growth and even a lethal phenotype in DPL1 and YSR2 double mutant (27). Thus, dpl1Δ is sensitive to HU either because of accumulation of phosphorylated LCB or because of decrease of long chain aldehyde and/or ethanolamine.

Sphingolipidomic analysis indicated that not one but several phytoceramide subspecies increased in WT after HU treatment. One challenging experimental goal was identifying the specific phytoceramide subspecies distinguished by the fatty acyl group length that specifically protects the cells from HU. Addition of different chain length fatty acids in the medium has been used previously to increase lipid species (28). Among the fatty acids we used, oleate was the only one that provides HU protection to isc1Δ cells and to WT cells overexpressing YPC1. Phytoceramide C18:1 was the main species to increase following oleate addition. Importantly, C18:1-phytoceramide was the only species that satisfied the following characteristics: 1) increased in WT after HU treatment; 2) did not increase in isc1Δ deletion; 3) decreased with overexpression of YPC1; 4) increased when oleate was added to the medium, and 5) did not increase when other fatty acids were added to the medium. Thus, taken together, these results clearly implicate C18:1-phytoceramide as the most likely candidate. Our data suggest that both sphingoid chain, double bonds, and the 1-head group.

Mechanistically, the results place C18:1-phytoceramide downstream of Isc1 (overcomes defect of ISC1 deletion) and as a regulator upstream of Swe1 and CDC55. In fact, the levels of Swe1 in isc1Δ in the presence of HU were lowered to WT levels when oleate was added to the medium.

These findings raised important questions on how the Isc1/ceramide pathway mediates this response to HU; our results show that the downstream pathway involves protein phosphatases, and especially the CDC55 regulatory subunit of the ceramide-activated protein phosphatase PP2A. As suggested in a previous study (26), Swe1 levels are controlled by Cdc55, a regulatory subunit of the p subunit of ceramide-activated protein phosphatase, the eukaryotic class of serine/threonine protein phosphatases (29, 30). In yeast it has been shown that C2-ceramide, a cell-permeable analogue of ceramide, caused a dose-dependent inhibition of growth and that yeast cells exhibited a ceramide-activated phosphatase activity (29). A G1 arrest in response to ceramide was shown to be mediated by the regulatory subunit Cdc55 and by the Sit4 catalytic subunit, and deletion of CDC55 protected the cells from C2-ceramide sensitivity (31). Furthermore, CDC55 was also found to protect the amphipysin-like mutant Rvs161 and that overexpression of CDC55 suppresses multiple growth defects in this mutant (32). In this study, overexpression of CDC55 rescued isc1Δ viability in HU, whereas the double mutant isc1Δ,cdc55Δ was not rescued from HU by oleate. Furthermore, deletion of CDC55 prevented oleate from correcting the levels of Swe1. Thus, these results implicate Cdc55 downstream of Isc1/C18:1-phytoceramides.

These current results implicate for the first time a bioactive lipid as a key mediator of DNA damage response in yeast. Although our previous study implicated Isc1 in HU response, those results, based on genetic analysis, could not determine whether Isc1 was a component of a signaling pathway initiated by HU/DNA damage or whether it affected those responses in an indirect manner. These current results show that Isc1 is a regulated component of those pathways. Thus, HU activates isc1 and increases ceramides. In turn, this regulates the response to HU, and it specifically mediates the response of SWE1 through the CDC55 phosphatase pathway (Fig. 11C).

Although the activation mechanisms of Isc1 by HU remain unknown, we speculate based on preliminary data that it may be related to the regulation of Isc1 stability.

Another major implication of these studies pertains to defining a specific and distinct role for subclasses and specific species of ceramide. Emerging evidence is already pointing to the complexity of ceramide, both structurally and functionally. Thus, we have estimated that mammals may harbor at least 200–300 distinct ceramides that are distinguished by their fatty acyl chains, hydroxylations on different carbons, length of the sphingoid chain, double bonds, and the 1-head group. Importantly, these structurally distinct ceramides are products of a combinatorial pathway of synthesis and modification that allow creation of many more ceramides from a limited set of precursors.

These considerations have led us to propose the “Many Ceramides” hypothesis whereby distinct ceramides are regulated by distinct pathways and participate in specific and distinct responses (24). Although there have been strong hints for these diverse functions in mammalian studies, the current results provide a solid genetic, biochemical and pharmacological, and mechanistic basis for defining a very specific pathway of C18:1-phytoceramide in the HU response. In mammalian studies, C18:1-ceramide, generated by human ceramide synthase 1, was found to be the candidate species that inhibited the activity of human telomerase reverse transcriptase via deacetylation of the Sp3 transcription factor in A549 human lung adenocarcinoma cells (33), although those studies did not rule out other downstream metabolites of ceramide. Other studies showed an increase of predominantly C16-ceramides and C24-ceramides after 3 and 24 h, respectively, after B-cell apoptotic stimulation with the B-cell receptor (34) with distinct roles proposed for each.

A distinct involvement for C18-ceramide in cell death induction was also described in head and neck squamous cell carcinoma. In these cells C18-ceramide production increased due to elevated ceramide synthase 1 (LASS1) enzymatic activity following gemcitabine/doxorubicin treatment (35).

In this study, we also provide a rather simple approach for probing lipid-specific functions using supplementation of fatty

17282 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 288 • NUMBER 24 • JUNE 14, 2013
acids along with lipidomic analysis and specific pharmacological and genetic manipulations.

In conclusion, the results identified a specific sphingolipid species, C\textsubscript{18:1}-phytoceramide, as the lipid mediator for protection from genotoxic effects of HU. We also provided mechanistic evidence that phytoceramide C\textsubscript{18:1} can regulate the Swe1 kinase levels in the isc1A cells in a Cdc55-dependent manner. The results in this study establish a strong platform for future studies to dissect the molecular interface between a sphingolipid species and a phosphatase. This study also reveals a new mechanism characterized by the activation of a sphingolipid pathway in a HU genotoxic dependent manner.

Acknowledgments—We are grateful Dr. Cungui Mao for revising the manuscript and to Lina Obeid, Chiara Luberto, and the Hannun group members for helpful discussions. We thank the Lipidomics Center at the Medical University of South Carolina for all lipid analyses performed in this study. Research at the Lipidomics Shared Resource, Hollings Cancer Center, Medical University of South Carolina, was supported in part by National Institutes of Health Grant P20 CA138313 and at the Lipidomics Core in the SC Lipidomics and Pathobiology COBRE, Department Biochemistry, Medical University of South Carolina, by National Institutes of Health Grant P30 RR017677.

REFERENCES

1. Dickson, R. C. (1998) Sphingolipid functions in Saccharomyces cerevisiae: comparison with mammals. Annu. Rev. Biochem. 67, 27–48
2. 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
3. Cowart, L. A., and Hannun, Y. A. (2007) Selective substrate supply in the regulation of yeast de novo sphingolipid synthesis. J. Biol. Chem. 282, 12330–12340
4. Sawai, H., Okamoto, Y., Luberto, C., Mao, C., Bielawska, A., Domaz, N., and Hannun, Y. A. (2000) Identification of ISC1 (YER019w) as inositol phosphophingolipid phospholipase C in Saccharomyces cerevisiae. J. Biol. Chem. 275, 39793–39798
5. Matmati, N., and Hannun, Y. A. (2008) Thematic review series: sphingolipids. ISC1 (inositol phosphophingolipid-phospholipase C), the yeast homologue of neutral sphingomyelinases. J. Lipid Res. 49, 922–928
6. Clarke, C. J., Snook, C. F., Tani, M., Matmati, N., Marchesini, N., and Hannun, Y. A. (2006) The extended family of neutral sphingomyelinases. Biochemistry 45, 11247–11256
7. Sawai, H., and Hannun, Y. A. (1999) Ceramide and sphingomyelinases in the regulation of stress responses. Chem. Phys. Lipids 102, 141–147
8. Vaena de Avalos, S., Okamoto, Y., and Hannun, Y. A. (2004) Activation and localization of inositol phosphophingolipid phospholipase C, Isclp, to the mitochondria during growth of Saccharomyces cerevisiae. J. Biol. Chem. 279, 11537–11545
9. Kitagaki, H., Cowart, L. A., Matmati, N., Montefusco, D., Gandy, J., de Avalos, S. V., Novgorodov, S. A., Zheng, J., Obeid, L. M., and Hannun, Y. A. (2009) ISC1-dependent metabolic adaptation reveals an indispensable role for mitochondria in induction of nuclear genes during the diauxic shift in Saccharomyces cerevisiae. J. Biol. Chem. 284, 10818–10830
10. Almeida, T., Marques, M., Mojzita, D., Amorim, M. A., Silva, R. D., Almeida, B., Rodrigues, P., Ludovico, P., Hohmann, S., Moradas-Ferreira, P., Correia-Real, M., and Costa, V. (2008) Isclp plays a key role in hydrogen peroxide resistance and chronological lifespan through modulation of iron levels and apoptosis. Mol. Biol. Cell 19, 865–876
11. Cowart, L. A., and Hannun, Y. A. (2005) Using genomic and lipidomic strategies to investigate sphingolipid function in the yeast heat-stress response. Biochem. Soc. Trans. 33, 1166–1169
12. Betz, C., Zajonc, D., Moll, M., and Schweizer, E. (2002) ISC1-encoded inositol phosphophingolipid phospholipase C is involved in Na*/Li* halotolerance of Saccharomyces cerevisiae. Eur. J. Biochem. 269, 4033–4039
13. Matmati, N., Kitagaki, H., Montefusco, D., Mohanty, B. K., and Hannun, Y. A. (2009) Hydroxyurea sensitivity reveals a role for ISC1 in the regulation of G2/M. J. Biol. Chem. 284, 8241–8246
14. Tripathi, K., Matmati, N., Zheng, W. J., Hannun, Y. A., and Mohanty, B. K. (2011) Cellular morphogenesis under stress is influenced by the sphingolipid pathway gene ISC1 and DNA integrity checkpoint genes in Saccharomyces cerevisiae. Genetics 189, 533–547
15. McMillan, J. N., Longtine, M. S., Sia, R. A., Theesfeld, C. L., Bardes, E. S., Pringle, J. R., and Lew, D. J. (1999) The morphogenesis checkpoint in Saccharomyces cerevisiae: cell cycle control of Swi1p degradation by Hsl1p and Hul7p. Mol. Cell. Biol. 19, 6929–6939
16. Liu, H., and Wang, Y. (2006) The function and regulation of budding yeast Swi1 in response to interrupted DNA synthesis. Mol. Biol. Cell 17, 11247–11256
17. Okamoto, Y., Vaena de Avalos, S., and Hannun, Y. A. (2003) Functional analysis of ISC1 by site-directed mutagenesis. Biochemistry 42, 7855–7862
18. Reid, R. J., Kauh, E. A., and Bjorntsi, M. A. (1997) Camptothecin sensitivity is mediated by the pleiotropic drug resistance network in yeast. J. Biol. Chem. 272, 12091–12099
19. Bielawski, J., Pierce, J. S., Snider, J., Rembisia, B., Szulc, Z. M., and Bielawski, A. (2010) Sphingolipid analysis by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Adv. Exp. Med. Biol. 688, 46–59
20. Saba, J. D., Naru, F., Bielawski, A., Garrett, S., and Hannun, Y. A. (1997) The BST1 gene of Saccharomyces cerevisiae is the sphingosine 1-phosphate lyase. J. Biol. Chem. 272, 26087–26090
21. Gottlieb, D., Heideman, W., and Saba, J. D. (1999) The DPL1 gene is involved in mediating the response to nutrient deprivation in Saccharomyces cerevisiae. Mol. Cell. Biol. Res. Commun. 1, 66–71
22. Oh, C. S., Toke, D. A., Mandala, S., and Martin, C. E. (1997) ELO2 and ELO3, homologues of the Saccharomyces cerevisiae ELO1 gene, function in fatty acid elongation and are required for sphingolipid formation. J. Biol. Chem. 272, 17376–17384
23. Mao, C., Xu, P., Bielawski, 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
24. Hannun, Y. A., and Obeid, L. M. (2011) Many ceramides. J. Biol. Chem. 286, 27855–27862
25. Liu, H., Jin, F., Liang, F., Tian, X., and Wang, Y. (2011) The Ckl1/Kar3 motor complex is required for the proper kinetochore-microtubule interaction after stressful DNA replication. Genetics 187, 397–407
26. Yang, H., Jiang, W., Gentry, M., and Hallberg, R. L. (2000) Loss of a protein phosphatase 2A regulatory subunit (Cdc55p) elicits improper regulation of SWE1 degradation. Mol. Cell. Biol. 20, 8143–8156
27. Kim, S., Fyrst, H., and Saba, J. (2000) Accumulation of phosphorylated sphingoid long chain bases results in cell growth inhibition in Saccharomyces cerevisiae. Genetics 156, 1519–1529
28. Al-Feel, W., DeMar, J. C., and Waki, S. J. (2003) A Saccharomyces cerevisiae mutant strain defective in acetyl-CoA carboxylase arrests at the G1/M phase of the cell cycle. Proc. Natl. Acad. Sci. U.S.A. 100, 3095–3100
29. Fishbein, J. D., Dobrowsky, R. T., Bielawski, A., Garrett, S., and Hannun, Y. A. (1993) Ceramide-mediated growth inhibition and CAPP are conserved in Saccharomyces cerevisiae. J. Biol. Chem. 268, 9255–9261
30. Dobrowsky, R. T., Kamibayashi, C., Mummy, M. C., and Hannun, Y. A. (1993) Ceramide activates heterotritmeric protein phosphatase 2A. J. Biol. Chem. 268, 15523–15530
31. Nickels, J. T., and Broach, J. R. (1996) A ceramide-activated protein phosphatase mediates ceramide-induced G1 arrest of Saccharomyces cerevisiae. Genes Dev. 10, 382–394
32. McCourt, P. C., Morgan, J. M., and Nickels, J. T., Jr. (2009) Stress-induced ceramide-activated protein phosphatase can compensate for loss of amphiysin-like activity in Saccharomyces cerevisiae and functions to reini-
Phytoceramide Provides Hydroxyurea Resistance

33. Wooten-Blanks, L. G., Song, P., Senkal, C. E., and Ogretmen, B. (2007) Mechanisms of ceramide-mediated repression of the human telomerase reverse transcriptase promoter via deacetylation of Sp3 by histone deacetylase 1. *FASEB J.* **21**, 3386–3397

34. Kroesen, B. J., Jacobs, S., Pettus, B. J., Sietsma, H., Kok, J. W., Hannun, Y. A., and de Leij, L. F. (2003) BcR-induced apoptosis involves differential regulation of C16 and C24-ceramide formation and sphingolipid-dependent activation of the proteasome. *J. Biol. Chem.* **278**, 14723–14731

35. Senkal, C. E., Ponnusamy, S., Rossi, M. J., Bialewski, J., Sinha, D., Jiang, J. C., Jazwinski, S. M., Hannun, Y. A., and Ogretmen, B. (2007) Role of human longevity assurance gene 1 and C18-ceramide in chemotherapy-induced cell death in human head and neck squamous cell carcinomas. *Mol. Cancer Ther.* **6**, 712–722