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Dihydroceramide Desaturase Activity is Modulated by Oxidative Stress

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Abstract

Oxidative stress was previously implicated in regulation of ceramide metabolism. Here, its effects on dihydroceramide desaturase were investigated. To stimulate oxidative stress HEK293, MCF7, A549, and SMS-KCNR cells were treated with hydrogen peroxide, menadione, or *tert*-butylhydroperoxide. In all cell lines, an increase in dihydroceramide was observed upon oxidative stress as measured by LC/MS. In contrast, total ceramide levels were relatively unchanged. Mechanistically, dihydroceramide desaturase activity was measured by an *in-situ* assay and was decreased in a time- and dose-dependent fashion. Interestingly, no detectable changes in the protein levels were observed, suggesting that oxidative stress does not induce degradation of dihydroceramide desaturase. In summary, oxidative stress leads to potent inhibition of dihydroceramide desaturase resulting in significant elevation in dihydroceramide levels *in vivo*.

Keywords

oxidative stress; ceramide; sphingolipids

INTRODUCTION

Sphingolipids comprise a group of cellular lipids with important regulatory functions [1]. Different species of sphingolipids have been implicated in a variety of cellular processes. Two well known bioactive sphingolipids, ceramide and sphingosine 1-phosphate have been shown to be involved in regulation of proliferation, differentiation, angiogenesis, senescence, and apoptosis [2, 3]. Generation of different bioactive sphingolipids results from either *de-novo* synthesis or break down of complex sphingolipids [4]. Due to their signaling properties, maintaining cellular homeostasis requires tight control of sphingolipid levels in the cell.

Dihydroceramide desaturase facilitates the last step of *de-novo* ceramide synthesis, i.e., addition of the 4,5-trans-double bond to the sphingoid backbone of dihydroceramide. Enzymatic activity of dihydroceramide desaturase has been described using rat liver

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microsomes [5, 6], and the gene encoding the enzyme (DEGS-1) was identified by Ternes et al [7]. Dihydroceramide desaturase belongs to the desaturase/hydroxylase family of enzymes characterized by the presence of conserved histidine motifs in the active site [8, 9] and appears to be the main ceramide desaturase in human cells [10].

Dihydroceramide is thought to be an inactive precursor of the well-known bioactive sphingolipid ceramide, especially in the regulation of apoptosis. Nevertheless, it was shown recently that accumulation of dihydroceramide after downregulation of DEGS-1 leads to cell cycle arrest [10]. Furthermore, dihydroceramide inhibits ceramide channel formation [11] and induces autophagy [12, 13]. In addition, γ-Tocopherol (vitamin E available from diet) was shown to inhibit prostate and lung cancer cells proliferation, through mechanism involving dihydroceramide and dihydrosphingosine accumulation [14]. Among chemopreventive agents, recent data described that the synthetic retinoid fenretinide *N*-(4-hydroxyphenyl)-retinamide (4-HPR) induces accumulation of dihydroceramide in cells [10]. Furthermore, 4-HPR has been shown to induce dose/time dependent cell cycle arrest, autophagy and/or apoptotic cell death in several cancer cell lines. In addition, reactive oxygen species (ROS) have been suggested to be involved in 4-HPR-mediated effects [15–19]. ROS (or redox environment) have also been suggested to play a “second messenger” role in cell cycle regulation [20–22]. These results suggested the possibility that ROS may modulate the activity of dihydroceramide desaturase.

Since dihydroceramide desaturase is a key enzyme regulating ceramide *vs*. dihydroceramide availability in the cell, understanding how its activity can be regulated is of high importance. Here we show that hydrogen peroxide and *tert*-butylhydroperoxide, as well as the intracellular ROS-inducer, menadione (2-methyl-1,4-naphtoquinone), were able to effectively inhibit dihydroceramide desaturase activity.

MATERIALS AND METHODS

Materials

Minimal Essential Medium (MEM), RPMI 1640, and Dulbecco’s Modified Eagle Medium (DMEM) were from GIBCO/Invitrogen (Carlsbad, CA). Red phenol free RPMI 1640 (L-Glutamine free) and L-Glutamine were from Sigma (St. Louis, MO). The HEK293, MCF-7, and A549 cell lines were purchased from American Tissue Culture Collection (Manassas, VA). The SMS-KCNR cell line was obtained from Dr. C. Pat Reynolds (Texas Tech University, Amarilo, TX). D-*erythro*-2-*N*-[12′-(1″-Pyridinium)dodecanoyl]-sphingosine bromide (D-*erythro*-C12-ceramide; C12-CCPS) and D-*erythro*-2-*N*-[12′-(1″-pyridinium)dodecanoyl]-4,5-dihydrosphingosine bromide (D-*erythro*-C12-dihydroceramide; C12-dhCCPS) were synthesized and kindly provided by the Lipidomics Core Facility of the Medical University of South Carolina [23]. Anti-DEGS-1 antibody (MLD 3906) was a generous gift from Dr. Gordon N. Gill (University of California, San Diego). CM-H_2DCFDA (#C6827) and CellTracker Green CMFDA (#C7025) were from Molecular Probes (Eugene, OR). Menadione, hydrogen peroxide, *tert*-butylhydroperoxide, and all other chemicals were from Sigma (St. Louis, MO). The radio-labeled substrate, N-Octanoyl-[4,5-3H]-D-erythro-dihydrosphingosine, was purchased from American radiolabeled chemicals (St. Louis, MO). The unlabeled substrate, *N*-Octanoyl-*D-erythro*-dihydrosphingosine and Cyclopropenylceramide (C8-CPPC) was purchased from Matreya, LLC (Pleasant Gap, PA)

Cell Culture

HEK293 (Human Embryonic Kidney) cells were maintained in MEM supplemented with 10% (v/v) fetal bovine serum (FBS) in a 5% CO_2 incubator at 37 C. Cells were passaged

every 3–4 days to maintain logarithmic growth. MCF-7 (Human Breast Adenocarcinoma) and SMS-KCNR (Human Neuroblastoma) cells were maintained in 1640 RPMI, while A549 (Human Lung Carcinoma) cells were maintained in DMEM supplemented with 10% (v/v) FBS and cultured as above.

Treatments

All solutions were freshly prepared for each experiment. Hydrogen peroxide and *tert*-butylhydroperoxide stock solutions were diluted in culture medium whereas menadione was first dissolved in ethanol and then in culture medium

In Situ Dihydroceramide Desaturase Assay

This assay has been described previously. Briefly, cells were incubated with 500nM C12-dhCCPS for 1h followed by treatment with menadione, hydrogen peroxide or *tert*-butylhydroperoxide for the indicated times. Cells were collected on ice, washed with PBS, lipids were extracted, and levels of C12-dhCCPS and its product (C12-CCPS) were detected by LC/MS as described previously [10].

In Vitro Dihydroceramide Desaturase Assay

Assay was performed as described by Rahmaniyan et al. (manuscript in preparation). Briefly, SMS-KCNR cells were grown in T-150 flasks with a density of 3 millions/flask. Experiments were performed at about 85% confluence. Cells were treated with 200 M of peroxide for 1 hour and 2.5 M of C8CPPC for 4hrs. The medium was removed and flasks were washed twice with ice-cold PBS. Cells were scraped and centrifuged at 1000×g. Total cell homogenate was prepared from the pellet as described [24]. Briefly the pellet was resuspended in homogenization buffer (50mM sucrose, 5mM HEPES, pH 7.4) and kept on ice for 10 minutes. Then, the suspension was homogenized employing a 1ml insulin syringe using 10 strokes. Cell homogenates were spun at 250g for 5min at 4°C to remove unbroken cells.

All reactions were performed using 400μg of total cell homogenate and 20 minutes incubation time at 37°C unless stated otherwise. 2nM of labeled substrate (equal to 0.125μCi, and about 100.000dpm) and 500 nM unlabeled substrate were used in all reactions. The assay was performed as described [6] [25]. In this procedure the enzyme activity is determined by formation of tritiated water that accompanies the 4,5-double bond formation if the substrate is labeled appropriately. N-Octanoyl-[4,5-3H]-D-erythro-dihydrosphingosine was the substrate with its correspondent unlabeled substrate, and NADH was used as a co-factor.

For the direct *in vitro* assay 400μg of total cell homogenate or 100μg of rat liver microsomes (RLM) have been used. RLM have been prepared as described [26]. The direct *in vitro* assay was performed as described above except that peroxide (250μM, 1mM, and 5mM) was added directly to the assay tube with a total volume of 1ml. In calculating the results, the amount of radioactivity was divided by 2 to take into account only the amount of radioactivity that is relevant to the enzyme activity. This is important due to the action of the enzyme in abstracting only one of the two hydrogen atoms, only one of which is ‘randomly’ labeled with tritium.

Measurements of endogenous ceramide and dihydroceramide levels

After treatment, cells were harvested on ice, lipids were extracted, and levels of endogenous sphingolipids were measured by LC/MS analysis at the Lipidomics Core Facility of the Medical University of South Carolina. LC/MS results were normalized to phosphates.

Detection of DEGS-1 by Western blotting

Protein samples were boiled for 10min in reducing SDS-sample buffer and separated by 10% polyacrylamide gels. Proteins were transferred to PVDF membranes, blocked with PBS, 0.1% Tween 20 containing 5% nonfat dried milk, washed with PBS-Tween, and incubated for 1h at room temperature with rabbit polyclonal anti-DEGS-1 (1:1000) in PBS/0.1% Tween 20 containing 5% nonfat dried milk. The blots were washed with PBS-Tween and incubated with secondary antibody conjugated with horseradish peroxidase in PBS-Tween containing 5% nonfat dried milk. Detection was performed using enhanced chemiluminescence reagent (Pierce; Rockford, IL). Loading was normalized to β-actin using mouse monoclonal anti-β-actin antibody (SIGMA, St. Louis, MO).

Reactive Oxygen Species

For ROS detection in SMS-KCNR cells, 50000cells/well (50 l central drop) were seeded in a 24-well plate and experiments performed after cell attachment (about 5h after seeding). Cells were loaded for 30min (5% CO_2 incubator at 37 C) with freshly prepared 10M CM-H_2DCFDA (10mM stock in DMSO; final concentration in PBS). Probe containing PBS was replaced by red-phenol free culture medium and cells incubated for another 15min. Basal fluorescence intensity was measured (ex.485nm/em.530nm, Fluoroskan Ascent plate reader (Labsystems)) before adding hydrogen peroxide, menadione or *tert*-butylhydroperoxide. ROS production for each time point was calculated as % of fluorescence of each well related to corresponding basal values. Probe-free cells were used as internal autofluorescence control. A modified protocol was used for HEK293 cells; 24-well plates were polylysine-coated (0.01%, 1h at 37°C), washed 3 times with H_2O, and dried. 50000cells/well (50 l central drop) were seeded and 450 l red-phenol free culture medium was added after 1h. Cells were incubated for minimum 2 days. Fresh medium was added before the experiment. Red-phenol free RPMI 1640 was used for CM-H_2DCFDA loading; treatment and fluorescence measurement performed as mentioned for SMS-KCNR cells.

Cellular Thiol Content

SMS-KCNR cells 50000cells/well (50 l central drop) were seeded in a 24-well plate. After attachment, cells were treated for 30min and treatment containing culture medium was replaced by freshly prepared 10 M CellTracker Green CMFDA (10mM stock in DMSO; final concentration in red-phenol free RPMI 1640. After 30min (5% CO_2 incubator at 37 C), probe containing RPMI 1640 was replaced by red-phenol free culture medium for another 30min and by PBS prior measuring fluorescence (ex.485nm/em.530nm, Fluoroskan Ascent plate reader (Labsystems)). Thiol content was calculated as % of fluorescence related to untreated control cells.

Statistical Analysis

Statistical significance was determined using an unpaired two-tailed *t* test and analysis of variance (ANOVA) with Bonferroni post-test to correct for multiple comparisons (GraphPad Prism, version 4). *p* values of <0.05 were considered to be statistically significant.

RESULTS

Hydrogen peroxide leads to an increase in dihydroceramide without significant effects on ceramide levels in several cell lines

Hydrogen peroxide (H_2O_2) was implicated in modulating cellular levels of sphingolipids by increasing ceramide [27–29], but there was no distinction between ceramide and dihydroceramide in those studies. In order to determine which cellular species are increased upon H_2O_2 treatment, LC/MS analyses were performed. Four different cell lines (HEK293,

MCF-7, SMS-KCNR, and A549) were used for these studies. Upon treatment with H_2O_2 for 1 hour, a significant increase in dihydroceramide levels was observed in all cell lines (Fig. 1A, B, C, D). Importantly, the increase was observed for all the dihydroceramide species that were identified (Table 1). In contrast, no increase in ceramide levels was observed after the treatment. These results demonstrate that, unlike previously concluded, H_2O_2 leads to a selective increase in dihydroceramide and not ceramide. It should be also noted that in 3 of these cell lines total ceramide levels were 7–20 fold higher than the levels of dihydroceramide. In addition, lack of increase in dihydrosphinogosine levels (Table 1) suggested that the peroxide has no significant effect on *de novo* ceramide synthesis.

Hydrogen peroxide inhibits dihydroceramide desaturase activity in a time and dose dependent manner without affecting cellular levels of DEGS-1

To determine if the observed increase in dihydroceramide results from inhibition of dihydroceramide desaturase, an *in situ* desaturase assay was employed. In this assay, cells are labeled with C12-dhCCPS prior to peroxide treatment, and conversion of C12-dhCCPS to the desaturated C12-CCPS is measured by mass spectrometry, thus providing a quantitation of cellular activity of the desaturase. Using this assay, it was observed that in HEK293 cells (Fig. 2A, left panel), the degree of conversion was decreased with concentrations of H_2O_2 as low as 25μM and dropped significantly with 100μM H_2O_2. In SMS-KCNR cells, H_2O_2 also induced a dose-dependent decrease on C12-CCPS production (Fig. 2A, right panel). Data from both cell lines implicate strong effect of hydrogen peroxide on dihydroceramide desaturase activity. As additional evidence, cellular levels of endogenous dihydroceramide were measured in the same samples and a comparable dose dependent increase in dihydroceramide levels was observed (Fig. 2B).

Next, the effect of H_2O_2 over time was examined. Treatment of HEK293 and SMS-KCNR cells with 200μM H_2O_2 inhibited conversion of C12-dhCCPS into C12-CCPS as rapidly as at 5 minutes in both cell lines (Fig. 2C). Furthermore, increases in endogenous dihydroceramide in HEK293 and SMS-KCNR cells were also observed after 5 minutes of 200μM hydrogen peroxide and followed a clear time dependent pattern (Fig. 2D).

In order to establish if H_2O_2 decreases desaturase activity due to degradation of dihydroceramide desaturase, DEGS-1 protein levels were measured by western blot in cells after treatment with various concentrations of hydrogen peroxide. Treatment as high as 400μM did not affect DEGS-1 levels in HEK293 or SMS-KCNR cells, indicating that there is no degradation of DEGS-1 upon H_2O_2 treatment (Fig. 3). Taken together, these results demonstrate that H_2O_2 has strong inhibitory effects on dihydroceramide desaturase activity.

Hydrogen peroxide inhibits dihydroceramide desaturase activity *in situ* but not *in vitro*

The above results on inhibition of cellular activity without a change in protein levels raised the possibility that H_2O_2 may cause an inactivation of the desaturase. First, the direct effect of H_2O_2 on desaturase activity was determined. The direct in-vitro assay at 20min (a time at which the assay is in the linear range) showed that peroxide inhibited the desaturase activity in RLM by only 7% at concentration of 250μM, 14% at 1mM and 29% at 5mM. By contrast, a known dihydroceramide desaturase inhibitor (C8CPPC) resulted in strong inhibition of the enzyme (Table 2). Using total cell homogenate, the activity was inhibited by 27% at a concentration of 5mM (Table 2). Moreover, a time course with 250μM peroxide did not show strongly significant inhibition even at 2h (Table 3). These results implied lack of profound direct enzyme inhibition by peroxide. Taken together, the above results show that H_2O_2 inhibited the activity of the desaturase in cells but not directly *in vivo*.

In order to determine if H_2O_2 induced indirect inactivation of the desaturase, cells were first treated with H_2O_2 and then dihydroceramide desaturase activity was measured *in vitro* in cell lysates. For the *in vitro* assay, labeled dh-Cer (0.125 Ci=100,000dpm) (2nM final concentration) was used. Treating the cells with hydrogen peroxide inhibited the desaturase activity by 40% after 1hr of treatment at a concentration of 200 M (Table 4). To validate the assay, desaturase inhibitor, C8CPPC, was used as a positive control, and this compound showed approximately 88% inhibition of desaturase activity after 4 hours treatment at a concentration of 2.5 M (Table 4). Thus, H_2O_2 appears to induce indirect inactivation of the desaturase in cells.

Oxidative stress leads to inhibition of dihydroceramide desaturase

To establish if other ROS-inducing agents can also inhibit dihydroceramide desaturase, the effects of *tert*-butylhydroperoxide and menadione were examined. As 4-HPR was shown to increase intracellular ROS [18, 19] and affect dihydroceramide desaturase activity [10] in SMS-KCNR cells, this cell lines was used for further studies. Initially, the levels of ROS production in the cells upon treatment were determined. All compounds tested induced a time dependent increase of ROS; (Fig. 4). Similar to the effect of peroxide on dihydroceramide desaturase inhibition and dihydroceramide accumulation (Fig. 2C,D), H_2O_2 induced a time and dose dependent increase in intracellular ROS production. ROS increase in HEK293 cells could be detected as early as within 5minutes with H_2O_2 200 M (Fig. 4A, right panel). In SMS-KCNR cells, as low as 25 M were enough to increase ROS production within 5minutes (Fig. 4A). In addition, *tert*-butylhydroperoxide and menadione led to increase in ROS in SMS-KCNR cells at concentrations used (Fig. 4B,C). Of note, different reagents (H_2O_2, *tert*-butylhydroperoxide and menadione) showed different capacity to induce CM-H_2DCFDA-reacting ROS species. Additionally, intracellular thiol levels were measured in SMS-KCNR cells. After 30 minutes of treatment, H_2O_2 induced a clear dose-dependent decrease with concentrations 50 M; values with tested *tert*-butylhydroperoxide and menadione concentrations were similar to those with 200–400 M H_2O_2 (Fig. 4D). Next, the effects of *tert*-butylhydroperoxide and menadione on endogenous ceramide and dihydroceramide levels were determined. Similarly to H_2O_2, both t-butyl hydroperoxide and menadione led to a several fold increase of dihydroceramide in SMS-KCNR cells (Fig. 5A) with no significant changes in ceramide levels observed (Fig. 5B).

Subsequently, *in situ* desaturase assays were performed. Both oxidants strongly inhibited the conversion of C12-dhCCPS into C12-CCPS in magnitudes comparable to 200 μM H_2O_2 over a 1 hour treatment (Fig. 5C). Similar experiments were performed with addition of C12-dhCCPS together with inhibitors or for 1 hour after pretreatment with inhibitors (Fig. 5D). Inhibition of desaturase was even more pronounced under these assay conditions. As with H_2O_2, *tert*-butylhydroperoxide did not have significant effect on DEGS-1 protein level (Fig. 5E). Menadione only slightly reduced DEGS-1 level, but this decrease does not fully account for the potent enzyme inhibition provoked by this compound. Overall, these results show that oxidative stress effectively inhibits dihydroceramide desaturase activity.

DISCUSSION

Dihydroceramide desaturase is an important enzyme that has the potential to modulate dihydroceramide vs. ceramide levels in the cell. Despite its key role in ceramide synthesis, processes that regulate dihydroceramide desaturase activity have not been well defined. Data presented herein demonstrate that dihydroceramide desaturase activity can be modulated by oxidative stress. The treatment of cells with H_2O_2, menadione, and *tert*-butylhydroperoxide led to potent inhibition of desaturase activity and accumulation of dihydroceramide in several cell lines.

Previous studies showing formation of ceramide in cells treated with H_2O_2 were based on the DAG kinase assay [27–29]. Although a well established technique that allows rapid measurement of ceramide formation, this assay is limited in that it does not readily differentiate between ceramide and its precursor, dihydroceramide. Work presented here employed LC-MS technology that allows precise identification of ceramide species generated upon oxidative stress. Treatment with H_2O_2 led to accumulation of dihydroceramide in cells without significant changes in ceramide levels. Notably, all detectable species of dihydroceramide were increased upon peroxide treatment (Table 1). Similar results were obtained using *tert*-butylhydroperoxide as well as menadione, a synthetic derivative of vitamin K3 with antitumor properties [30], which was previously shown to inhibit desaturase activity in rat liver microsomes [6]. Taken together, this indicates that oxidative stress leads to accumulation of dihydroceramide and not ceramide in different cell lines.

Importantly, the results point to indirect inhibition and inactivation of the desaturase by oxidative stress. Thus, in addition to detecting changes in endogenous sphingolipids, an *in situ* desaturase assay was performed in cells treated with ROS-inducing agents. In all instances, cellular conversion of C12-dhCCPS into C12-CCPS was strongly inhibited reflecting a decrease in desaturase activity. This was confirmed by *in vitro* assays performed on cell lysates from cells treated with peroxide which showed that the enzyme became inactivated following treatment of cells with peroxide. However, the fact that the direct in vitro assay using RLM or cell lysate did not show significant direct effects of peroxide on desaturase activity suggests that the inhibition of the enzyme is indirect. Dihydroceramide desaturase, like other desaturase systems is thought to involve a series of coupled reactions that transport electrons from NAD(P)H to a terminal desaturase that reduces oxygen [6, 25]. NAD(P)H however is added to reaction and should be present in both RLM and cell homogenates. Therefore, the results suggest that the inhibition of the dihydroceramide desaturase activity is mediated by secondary metabolites in the cell upon peroxide treatment.

Along these lines and since oxidative stress inducers employed by this work have been previously shown to regulate glutathione levels [31–33], it was important to define the relationship between dihydroceramide desaturase activity and cellular thiol levels. Glutathione is the most abundant thiol species in the cell and it plays a particularly important role in the regulation of the cellular redox status. Moreover, the glutathione/glutathione disulfide (GSH/GSSG) ratio has been described as an important redox indicator and disruption of its level was shown to be involved in many pathological processes [34]. In our hands, all treatments affecting dihydroceramide desaturase activity led to depletion of cellular thiol levels. Interestingly, treatments inducing similar dihydroceramide desaturase inhibition levels showed comparable thiol depletion capacity despite differences in the range of ROS production. Of note, it was recently shown that resveratrol, a polyphenol with antioxidant properties, can also cause inhibition of dihydroceramide desaturase [12]. Similarly, affecting cellular levels of thiols was shown to inhibit dihydroceramide desaturase activity [5]. This implies that not only ROS induction, but any changes in the cellular redox state can modulate dihydroceramide desaturase activity.

It is interesting that the levels of ceramide showed either no significant changes or modest decreases. Two factors may contribute to the selective accumulation of dihydroceramide without significant drops in the total levels of ceramide. First, the levels of ceramide in most cells are several fold higher than those of dihydroceramide and thus over the time course of these studies, dihydroceramide may accumulate at the expense of minimal changes in ceramide (less than 10%). The only exception in the current studies was the A549 cell line which contains relatively high basal levels of dihydroceramide. The second reason for the apparent lack of changes in ceramide levels relates to the dynamic nature of sphingolipid

metabolism and the fact that the current analysis reveals only snapshots of the levels of total lipid mass and not fluxes in those levels. Thus, additional mechanisms such as attenuation of incorporation of ceramide into complex sphingolipids and/or increased turnover of complex sphingolipids could serve to further attenuate changes in total ceramide mass. Moreover, oxidative stress may have additional effects on sphingolipid metabolism. For example, previous work has shown that peroxide also increases neutral sphingomyelinase (N-SMase) activity [28, 35] and depletion of cellular glutathione can activate N-SMases [36]. Consistent with this, in our hands, 1h peroxide treatment induced an approximately 30% increase in N-SMase activity (*in vitro*) in A549 cells (data not shown). However, unlike previous studies, no detectable increases in ceramide levels by H_2O_2 were observed. Although it is possible that increases in ceramide due to N-SMase activation could be masked by decreases in ceramide that would result from inhibition of desaturase activity, we also observed no significant effect of H_2O_2 on sphingomyelin levels in A549 cells (data not shown). Nevertheless, our data do not exclude the possibility that there is some degree of N-SMase activation with peroxide treatment.

This work addressed the role of oxidative stress in ceramide metabolism. The desaturation reaction has been shown to be NAD(P)H-dependent [5, 6], thus cellular redox state is important for this process. Disturbing cellular redox balance among other effects would profoundly influence dihydroceramide *vs*. ceramide levels in the cell.

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Abbreviations

4-HPR	*N*-(4-hydroxyphenyl)-retinamide
ROS	reactive oxygen species
C12-CCPS	D-*erythro*-2-*N*-[12′-(1″-Pyridinium)dodecanoyl]-sphingosine bromide (D-*erythro*-C12-ceramide)
C12-dhCCPS	D-*erythro*-2-*N*-[12′-(1″-pyridinium)dodecanoyl]-4,5-dihydrosphingosine bromide (D-*erythro*-C12-dihydroceramide
NADH	nicotinamide adenine dinucleotide
RLM	rat liver microsomes
SDS	sodium dodecyl sulfate
PVDF	polyvinylidene fluoride
DMSO	Dimethyl sulfoxide
H_2O_2	hydrogen peroxide
DAG	diacylglycerol
GSH	glutathione
GSSH	glutathione disulfate

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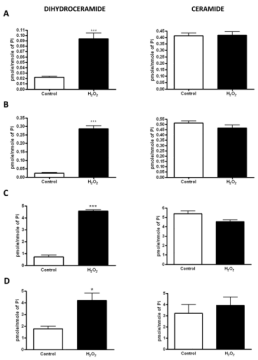

Figure 1. Effects of hydrogen peroxide on dihydroceramide and ceramide levels

(A) HEK293, **(B)** MCF-7, **(C)** SMS-KCNR, and **(D)** A549 cells were treated with 200μM hydrogen peroxide for 1 hour. Bars represent levels of total dihydroceramide (left) and total ceramide (right) measured by LC/MS. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

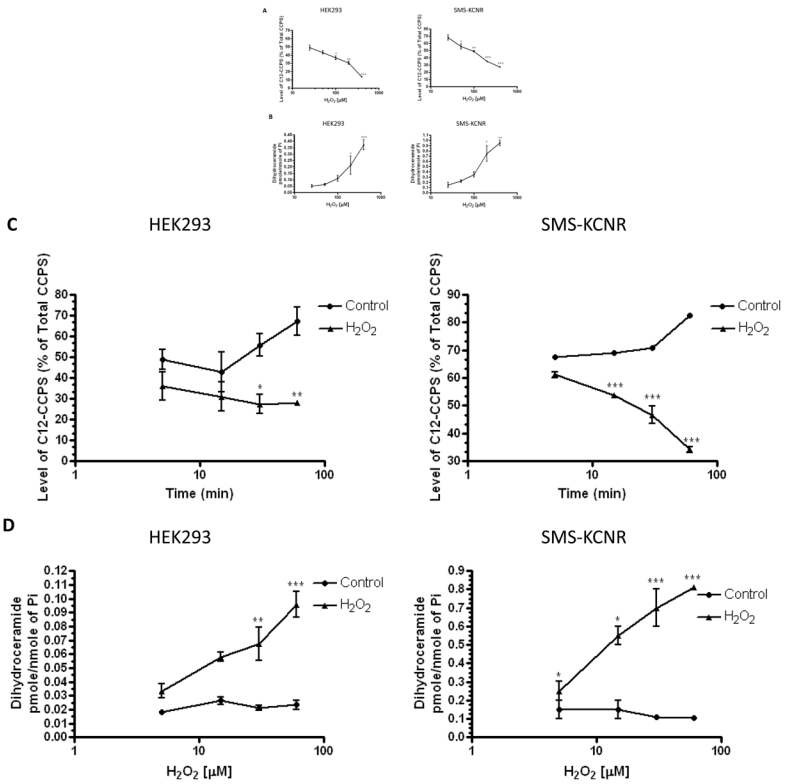

Figure 2. Effects of hydrogen peroxide on *in situ* dihydroceramide desaturase activity

HEK293 and SMS-KCNR cells were labeled with C12-dhCCPS for 1 hour and then treated with the indicated concentration of hydrogen peroxide for 1h **(A,B)** or with 200μM hydrogen peroxide for the indicated time **(C,D)**. Percent of conversion of C12-dhCCPS into C12-CCPS **(A,C)** as well as levels of endogenous dihydroceramide **(B,D)** were measured by LC/MS. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

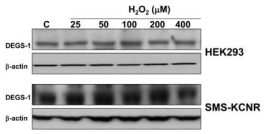

Figure 3. Effects of hydrogen peroxide on DEGS-1 protein levels

HEK293 and SMS-KCNR cells were treated with the indicated concentrations of hydrogen peroxide for 1h. After treatment, cells were harvested and lysed. Levels of endogenous DEGS-1 were determined by Western blot. Blots were stripped and reprobed for β-actin to normalize for loading. Insets are representative of two independent experiments.

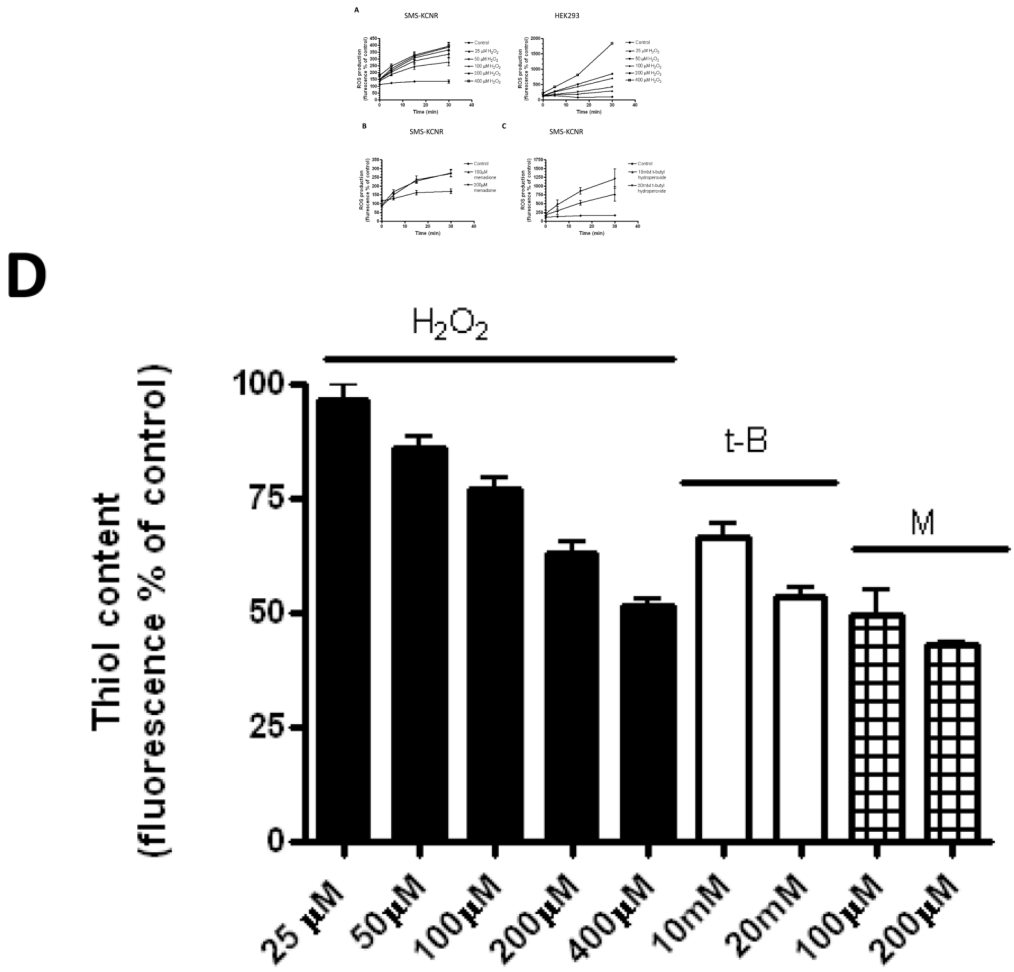

Figure 4. Effects of hydrogen peroxide, menadione and *tert*-butylhydroperoxide on ROS and thiol levels

For ROS measurement **(A,B,C)** HEK293 and SMS-KCNR cells were loaded with CM-H_2DCFDA and treated as indicated. Fluorescence was measured at the indicated time points, and ROS production was calculated as % of fluorescence related to the corresponding basal value. Data are shown as averages of at least 3 independent experiments performed in triplicate for SMS-KCNR cells and as a representative experiment performed in triplicate for HEK293 cells. For thiol measurements **(D)** SMS-KCNR cells were treated as indicated for 30min prior to loading with thiol-reacting probe (CMFDA). Data shown is average of at least 3 independent experiments performed in triplicate.

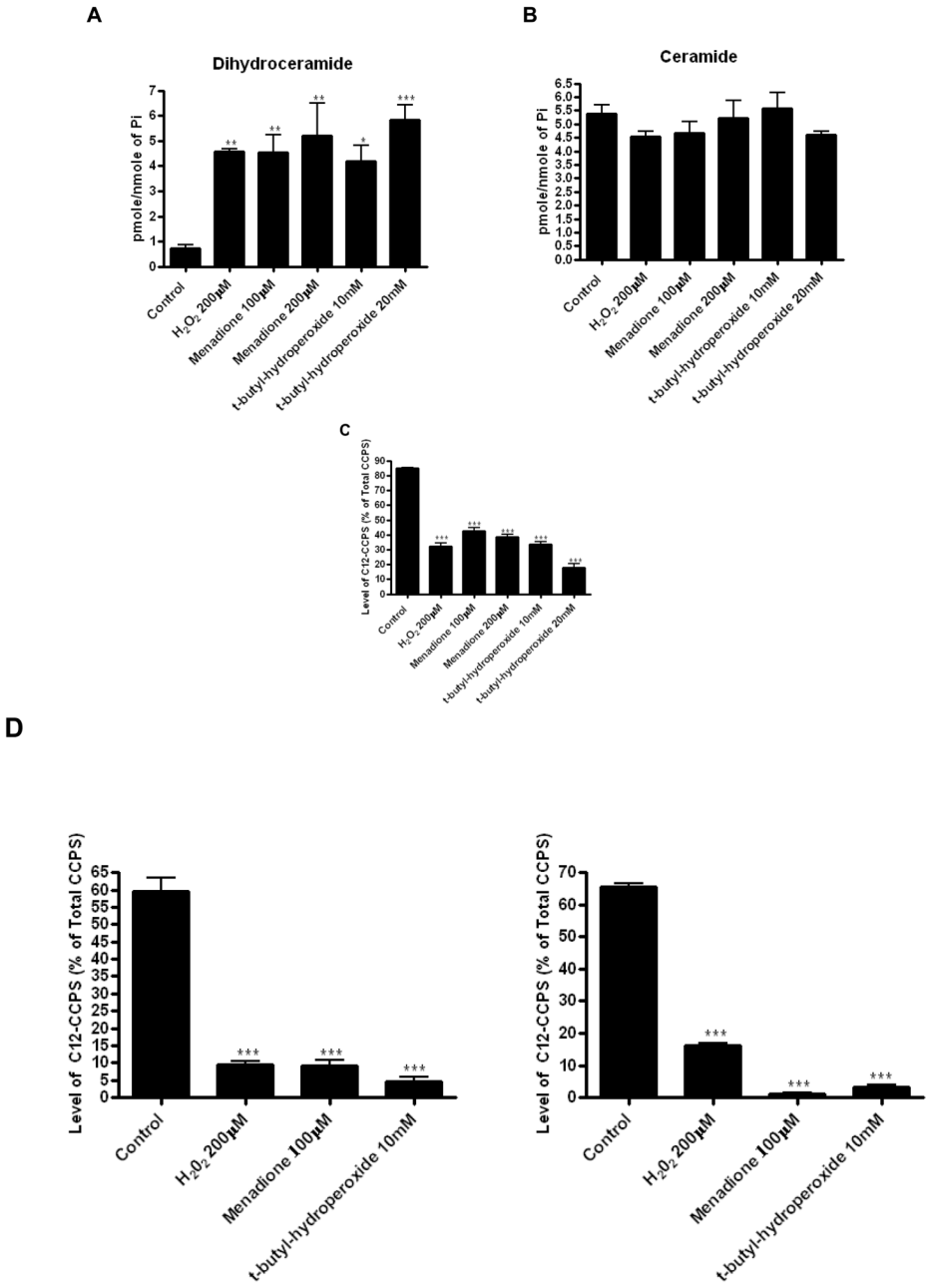

Figure 5. Effects of menadione and *tert*-butylhydroperoxide on dihydroceramide desaturase activity

SMS-KCNR cells were treated with indicated concentrations of hydrogen peroxide, menadione and *tert*-butylhydroperoxide for 1h. Levels of endogenous ceramide and dihydroceramide **(A,B)** as well as dihydroceramide desaturase activity **(C)** were measured by LC/MS. Average of 2 independent experiments performed in duplicates. ***p<0.001. **(D)** In situ assay with addition of substrate (C12-dhCCPS) together with inhibitors (left panel) or for 1h after pretreatment with inhibitors (right panel). **(E)** After treatment with menadione and *tert*-butylhydroperoxide levels of endogenous DEGS-1 were determined by Western blot. Blots were stripped and re-probed for β-actin to normalize for loading. Insets are representative of two independent experiments.

Table 1

Effect of hydrogen peroxide on ceramide and dihydroceramide species in HEK293 cells treated with 200μM H_2O_2 for 1h.

	dhC14-Cer	dhC16-Cer	dhC18-Cer	dhC20-Cer	dhC22-Cer	dhC22:1-Cer	dhC24-Cer	dhC24:1-Cer	dhC26-Cer	dhC26:1-Cer	DHSph	Sph
Control	0.00072±0.00007	0.01106±0.002	0.00103±0.0003	0.00344±0.0007	0.00077±0.00009	0.00182±0.00023	0.00160±0.0002	0.00131±0.00014	0.00037±0.00014	0.00005±0.00002	0.000812±0.00015	0.00479±0.0004
H_2O_2	0.00290*±0.00154	0.05528**±0.01802	0.00216**±0.00052	0.00440±0.00047	0.00386**±0.00129	0.00197±0.00024	0.01288***±0.00165	0.00974**±0.00352	0.00043±0.00042	0.00030**±0.00011	0.00095±0.00025	0.00387 ±00038
	C14-Cer	**C16-Cer**	**C18-Cer**	**C18:1-Cer**	**C20-Cer**	**C20:1-Cer**	**C22-Cer**	**C22:1-Cer**	**C24-Cer**	**C24:1-Cer**	**C26-Cer**	**C26:1-Cer**
Control	0.00168±0.00026	0.14530±0.02186	0.01659±0.00267	0.00382±0.00063	0.02039±0.00229	0.00234±0.00036	0.03420±0.00546	0.00301±0.00007	0.10791±0.01305	0.07417±0.00624	0.00187±0.00016	0.00169±0.00062
H_2O_2	0.00207±0.00038	0.14767±0.03153	0.01984±0.00087	0.00480±0.00056	0.02172±0.00281	0.00301±0.00066	0.03843±0.00480	0.00329±0.00029	0.10328±0.00891	0.06998±0.00517	0.00216*±0.00011	0.00169 ±0.00025

Values are shown as pmoles/nmole of Pi and are mean ±S.D. of three independent experiments done in duplicate.

* $p<0.05$,

** $p<0.01$,

*** $p<0.001$.

Table 2

Direct effect of hydrogen peroxide on desaturase activity *in vitro*.

Rat liver microsomes	**nmol/min/g**
Control	0.43±0.04
250μM H_2O_2	0.4±0.052
1mM H_2O_2	0.37±0.032
5mM H_2O_2	0.31±0.019
Rat liver microsomes	nmol/min/g
Control	0.588±0.045
500nM C8CPPC	0.37±0.044**
750nM C8CPPC	0.243±0.025***
1μM C8CPPC	0.201±0.023***
Cell Lysate	pmol/min/g
Control	161.23±40.03
5mM H_2O_2	118.06±31.45

Values are mean ±S.D. of two separate experiments done in triplicate.

* $p<0.05$,

** $p<0.01$,

*** $p<0.001$.

Table 3

Time course of direct effect of hydrogen peroxide (250μM) on desaturase activity *in vitro* measured in rat liver microsomes.

	Control	**H_2O_2**
20min	0.221±0.049	0.232±0.05
40min	0.24±0.031	0.203±0.015
60min	0.217±0.023	0.181±0.014*
90min	0.205±0.023	0.192±0.023
120min	0.159±0.016	0.16±0.016

Values are shown as nmol/min/g and are mean ±S.D. of two separate experiments.

* $p<0.05$.

Table 4

Effect of hydrogen peroxide and C8CPPC on desaturase activity *in vitro* in SMS-KCNR cells.

	H_2O_2 (200μM, 1h)		**C8CPPC (2.5 μM, 4h)**
control	100 ±5.15	control	100 ±9.06
H_2O_2	59.44 ±5.47**	C8CPPC	11.37 ±1.44***

Values are shown as pmol/min/g and are mean ±S.D. of two separate experiments done in triplicate.

** $p<0.01$,

*** $p<0.001$.