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Protection of LLC-PK1 Cells Against Hydrogen Peroxide-Induced Cell Death by Modulation of Ceramide Level

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Oxidative stress has been reported to elevate ceramide level during cell death. The purpose of the present study was to modulate cell death in relation to cellular glutathione (GSH) level and GST (glutathione *S*-transferase) expression by regulating the sphingolipid metabolism. LLC-PK1 cells were treated with H_2O_2 in the absence of serum to induce cell death. Subsequent to exposure to H_2O_2, LLC-PK1 cells were treated with desipramine, sphingomyelinase inhibitor, and *N*-acetylcysteine (NAC), GSH substrate. Based on comparative visual observation with H_2O_2-treated control cells, it was observed that 0.5 μM of desipramine and 25 mM of NAC exhibited about 90 and 95% of cytoprotection, respectively, against H_2O_2-induced cell death. Desipramine and NAC lowered the release of LDH activity by 36 and 3%, respectively, when compared to 71% in H_2O_2-exposed cells. Cellular glutathione level in 500 μM H_2O_2-treated cells was reduced to 890 pmol as compared to control level of 1198 pmol per mg protein. GST P1-1 expression was decreased in H_2O_2-treated cells compared to healthy normal cells. In conclusion, it has been inferred that H_2O_2-induced cell death is closely related to cellular GSH level and GST P1-1 expression in LLC-PK1 cells and occurs *via* ceramide elevation by sphingomyelinase activation.

Key words: LLC-PK1 cells, Ceramide, GST, Glutathione, Cell death

INTRODUCTION

Ceramides have gained importance in determining cell death responses induced by conditions of stress. Cellular ceramide can be produced by the activation of either sphingomyelinase or through *de novo* sphingolipid pathway. Neutral sphingomyelinase (nSMase) and acid sphingomyelinase (aSMase) are rapidly activated by diverse stimuli resulting in elevated ceramide levels (Hannun *et al.*, 2001; Mathias *et al.*, 1998). Recent evidences support the contention that aSMase can be translocated to the outer cell membrane and can be activated by various cell surface receptors, such as CD95 (Fas) and CD40 resulting in the hydrolysis of membrane sphingomyelin into ceramide (Grassme *et al.*, 2002). Ionizing radiation-induced apoptosis is dependent on increase in ceramide levels (Jaffrezou *et al.*, 2001). *De novo* ceramide biosynthesis can be stimulated by drugs and ionizing radiation, and can lead to a prolonged ceramide elevation. Ceramide-activated protein kinase and ceramide-activated protein phosphatase have been identified as downstream target proteins for ceramide signaling pathway (Mathias *et al.*, 1991; Dobrowsky *et al.*, 1993), however, the molecular mechanism of ceramide-induced cell death are yet to be understood. Recently, ceramide-1-phosphate has been implicated in bringing about membrane fusion of brain synaptic vesicles and in formation of neutrophil phagolysosome, and the allied ceramide kinase has been characterized and cloned (Sugiura *et al.*, 2002).

Glutathione *S*-transferases (GSTs) are detoxification enzymes that catalyze the conjugation of electrophilic compounds to glutathione (GSH) and are found in all mammalian cells (Salinas and Wong, 1999). Cytosolic and microsomal forms of GSTs have been identified (Papp *et al.*, 1995; Pickett and Lu, 1989). Mammalian GSTs have been classified into five different gene

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families: alpha (α), mu (μ), pi (π), sigma (σ) and theta (δ). In the cells exposed to oxidative stress, expression of phase II detoxifying enzymes is primarily regulated by the transcription factors, including the NF-E2-related factor (Nrf) family (Wasserman and Fahl, 1997). It has been reported that GSTs played a significant role in protecting rat testis against oxidative stress and were related to germ cell apoptosis resulting from lipid peroxidation products, when their activity was inhibited (Rao and Shaha, 2000). Overexpression of GST A2-2 (GSTα class) in K562 cells attenuated the H_2O_2-induced cytotoxicity and apoptosis by blocking caspase-3 activation (Yang *et al.*, 2001). GST P1-1 has been shown to be involved in inhibiting apoptosis in hematopoietic cells (Cumming *et al.*, 2001) and to protect against H_2O_2-induced cell death (Yin *et al.*, 2000). However, the gene expression of inflammatory and immune responses was increased under oxidative stress (Schreck *et al.*, 1991; Mihm *et al.*, 1991; Schulze-Osthoff *et al.*, 1993).

Recently, several evidences related to human diseases such as Alzheimer's disease, ischemia and atopic dermatitis have been reported. Alzheimers disease is an age-related disorder characterized by amyloid β-peptide accumulation and neuron degeneration in brain. Aggregated amyloid β-peptide was suggested to induce oxidative stress and ceramide elevation, resulting in neuronal cell death (Cutler, 2004). A recent study showed that myocardial elevation of ceramide during preconditioning and ischemia/reperfusion-mediated cardiac dysfunction was partially restored with desipramine, sphingomyelinase inhibitor (Cui *et al.*, 2004). Ceramides have amphiphilic structure with extremely long-chains, and are essential for barrier function of stratum corneum in skin. Impaired sphingomyelinase activity resulting in reduced ceramide content in skin appeared to be involved in the defective barrier function found in atopic dermatitis (Jensen *et al.*, 2004).

Serum deprivation and exposure to H_2O_2 are involved in sphingolipid biosynthesis. Fumonisin B1 and ISP-1, inhibitors of sphingolipid biosynthesis, blocked cell death and decreased the elevated level of sphingolipid contents induced by serum deprivation in LLC-PK1 cells (Yu *et al.*, 2004). Ceramide production has been found to be modulated by H_2O_2-mediated oxidative stress in lung epithelial cells (Goldkorn *et al.*, 2005). To understand the relationship between GSH contents/GST P1-1 expression and cellular ceramide level in H_2O_2-induced cell death, we have measured the cell death following H_2O_2 exposure in LLC-PK1 cells, modulated the cellular ceramide level by the inhibition of either sphingomyelinase or *de novo* sphingolipid biosynthesis, and determined GSH content and GST P1-1 expression level.

MATERIALS AND METHODS

Reagents

ISP-1, D-*erythro*-sphingosine and D-*erythro*-sphinganine were purchased from Biomol Research, Inc. (Plymouth Meeting, PA, USA). Desipramine and *o*-phthalaldehyde (OPA) were obtained from Sigma (St. Louis, MO, USA). Serum and culture medium were obtained from Gibco, Inc. (Gaithersburg, MD, USA). HPLC-grade methanol, acetonitrile were purchased from Merck KGaA (Darmstadt, Germany). 1,2-sn-diacyl-sn-glycerol kinase (DAGK) and TLC, was obtained from Calbiochem Corp. (La Jolla, CA, USA) and [γ-^{32}P]ATP from Amersham Biosciences (Piscataway, NJ, USA). All other organic solvents and chemicals were of analytical grade.

Cell culture

LLC-PK1 cells originated from pig kidney proximal tubule (CL-101) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were grown and maintained in 75 cm^2 culture flasks containing Dulbecco's modified Eagle medium/Ham's F12 with 5-10% fetal bovine serum, 50 units/mL penicillin, 50 μg/mL streptomycin in a CO_2 incubator at 37°C. For all experiments, cells were seeded on 6-well plate (10 cm^2/well) and 100 mm dish at a density of 1×10^5 cells. The medium was changed 36 h after seeding, and then the cells were pretreated with desipramine, *N*-acetylcysteine and ISP-1 for 1 h, and were then treated with H_2O_2 for 3 h. The cultured cells were harvested with either 0.25 M trypsin-EDTA treatment or rubber scraper followed by a brief centrifugation, and cell pellets were saved for further experiments.

Lactate dehydrogenase assay

Cytotoxicity was determined by measuring the released activity of lactate dehydrogenase (LDH) from total LDH activity. Culture medium was transferred to a glass tube for the determination of released LDH activity. Total LDH was liberated out of cells by the brief incubation at 37°C followed by the addition of 0.1% Triton X-100. Stock solutions of sodium pyruvate (1%, w/v) and β-NADH (0.75%, w/v) were added to both medium and cell lysate. Then, LDH activity was measured using a Jasco V-530 UV-Vis spectrophotometer (Tokyo, Japan). The released LDH activity was expressed as percentage of the total cellular LDH activity.

Measurement of cellular ceramide levels

Cell lysate equivalent to 100 μg total protein was aliquoted and cellular lipids were extracted by chloroform/ethyl alcohol/1*N* HCl (100:100:1 v/v/v), buffered saline solution consisting of 10 mM HEPES pH 7.2, 135 mM

NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, and 100 mM EDTA. Lipid extract was present in the chloroform phase and cellular ceramide content was estimated by the diacylglycerol kinase (DAGK) enzymatic method which includes *in vitro* conversion of ceramide and 1-[γ-^{32}P]-ATP to 1-[γ-^{32}P]-ceramide. The cellular lipid extract after DAGK reaction was separated by TLC. Lipid bands for all samples were detected by iodine vapor. The bands containing 1-[γ-^{32}P]-ceramide were scraped-off and transferred to a scintillation vial for measurement of the radioactivity by scintillation counting.

Western blot analysis

Cell lysate equivalent to 10 μg total protein was resolved by SDS-PAGE in a 12.5% acrylamide using the Mini Gel Protein system (Bio-Rad Co., Hercules, CA, USA). Proteins on acrylamide gel were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Co., Bedford, MA, USA) at 350 mA with a transfer buffer containing 25 mM Tris-HCl, 192 mM glycine and 20% methanol (pH 8.4). PVDF membrane was blocked with 5% bovine serum albumin at room temperature for 1 h (BSA; Sigma Co., St. Louis, MO, USA) in PBS-T. The membrane was washed, incubated with anti-glutathione S-transferase P1-1 antibody (Calbiochem Co., La Jolla, CA, USA) overnight at 4°C, and with anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (Cell signaling Co., Beverly, MA, USA) for 90 min at room temperature. After washing, the membrane was visualized by chemiluminescent reaction using WestZol kit (Intron Co., Daejeon, Korea) followed by exposure of the membrane to X-ray Agfa film (Agfa Gevaert Co., Kontich, Belgium). Band intensity was quantified by a free version of Scion Corporation imaging system (Frederick, MD, USA).

Cellular glutathione assay

Cellular GSH level was analyzed by using a glutathione assay kit (Calbiochem Co., La Jolla, CA, USA). Cell pellet was lysed by the treatment with 0.2*N* NaOH. Cell lysate equivalent to 100 μg total protein was added to GSH assay kit and the reaction was continued for 10 min at 25°C under darkness. Cellular GSH content was measured by Molecular Devices ELISA reader (Sunnyvale, CA, USA) at 380 nm based on the GSH standard curve.

BCA protein assay

The content of total cellular proteins was determined (Smith *et al.*, 1985) for both the normalization of results and the estimation of cell growth. Cell lysate from the solubilized pellet was mixed with BCA reagent and incubated for 30 min. The protein content was quantitated with Molecular Devices ELISA reader (Sunnyvale, CA) at 562 nm based on the standard curve.

Statistics

All values were expressed as a mean ± SD. Differences between treatments were analyzed statistically by unpaired Student's *t*-test. Differences with $p < 0.01$ were defined as statistically significant.

RESULTS

Sphingomyelinase inhibitor protected cells against hydrogen peroxide-induced cell death

Cells were confluenced to 50% with the typical morphology of epithelial cell type. After 2 h of the addition of 500 μM H_2O_2, cells began to die and were apparently swollen and were floating in the medium. Cell confluency following H_2O_2 exposure was visually estimated by approximately 50% of normal cell population. Released

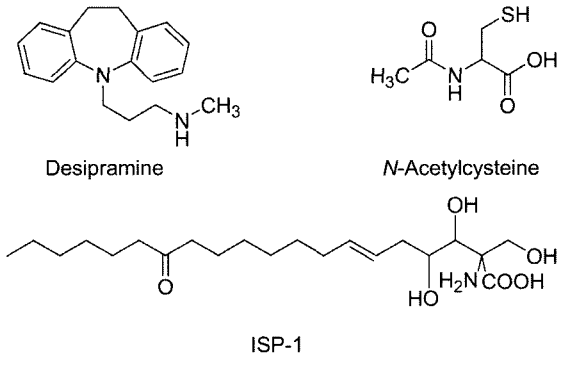

Fig. 1. Structures of desipramine, *N*-acetylcysteine and ISP-1 (myriocin). Desipramine is a tricyclic antidepressant and *N*-acetylcysteine is glutathione substrate. ISP-1 was isolated from fungi *Isaria sinclairii* (ATCC 24400) and is an analogue of sphingosine.

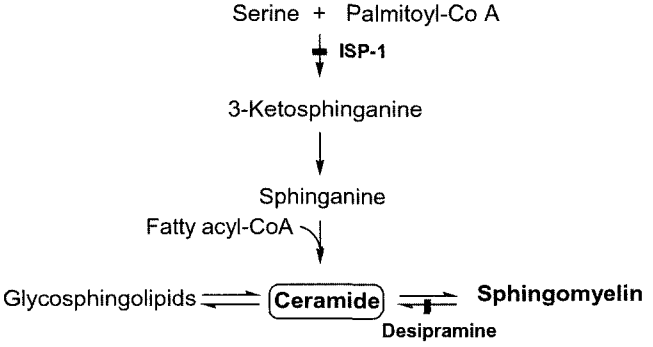

Fig. 2. Sphingolipid metabolic pathway and enzymatic steps inhibited by ISP-1 and desipramine. ISP-1 is a specific inhibitor of serine palmitoyltransferase, the enzyme that is responsible for the condensation of serine and palmitoyl CoA in *de novo* sphingolipid biosynthesis. Desipramine is an inhibitor of sphingomyelinase, the enzyme that catalyzes sphingomyelin hydrolysis resulting in ceramide formation.

LDH activity, an indicator of cytotoxicity, at the different concentrations of H_2O_2 (0, 10, 50, 100, 200, 500, and 1000 μM) and for the given time periods (0, 1, 2, 3, 4, 5, 6 h) of H_2O_2 exposure is shown in a concentration-dependent manner (Fig. 3A), and time-dependent manner (Fig. 3B). Cell death was apparent at H_2O_2 concentration greater than 200 μM and was abruptly increased at 500 and 1000 μM H_2O_2 upon incubation for 3 h. Cells appeared to be resistant to H_2O_2 exposure after 6 h incubation with H_2O_2 (data not shown), indicating that catalase activity in LLC-PK1 cells may become activated upon H_2O_2 exposure. ISP-1, a specific inhibitor of serine palmitoyltransferase in *de novo* sphingolipid biosynthesis, at 0.5 μM concentration was shown to slightly protect LLC-PK1 cells against H_2O_2 exposure (Fig. 4C), however, desipramine, a sphingomyelinase inhibitor, at 0.5 μM concentration rescued cells from H_2O_2 exposure for 6 h (Fig. 4D), indicating that sphingomyelinase activation may be a major contributor to H_2O_2 induced-cell death and it may block the elevation of cellular ceramide level. Sphingomyelinase catalyzes sphingomyelin hydrolysis to produce ceramide. *De novo* sphingolipid biosynthesis appears to protect cells minimally from H_2O_2 induced-cell death. LLC-PK1 cells were pretreated with ISP-1 and desipramine for 1 h and were then exposed to H_2O_2 for 3 h. However, simultaneous treatment of LLC-PK1 cells with desipramine and H_2O_2 did not show any cytoprotection from H_2O_2 exposure-induced cell death (unknown data). The pretreatment with desipramine and ISP-1 appears to be important in the protection of cells from H_2O_2 induced cellular death. The combined treatment of ISP-1 with desipramine (Fig. 4E) to H_2O_2-exposed LLC-PK1 cells exhibited better cytoprotection with much healthier cells than desipramine, indicating that *de novo* sphingolipid biosynthesis may be involved in H_2O_2-induced cytotoxicity even though the protective effect may be minor. Cell death induced by H_2O_2 treatment was inhibited completely in the presence of 25 mM NAC, a GSH substrate (Fig. 4F). NAC alone at a concentration of 25 mM did not show any cytotoxicity in LLC-PK1 cells. These results suggest that H_2O_2-induced cell death appears to be involved in both sphingolipid metabolism and cellular GSH level. Hydrogen peroxide at 500 μM concentration in the absence of FBS for 3 h increased the released LDH activity by 71.86 ± 3.72% when compared to 5.37 ± 0.85% of control cells (Fig. 5A). However, desipramine and NAC reduced the released LDH activity to 36.25 ± 1.74 and 3.26 ± 0.49%, respectively, from 71.86% in H_2O_2-exposed control cells. After exposure to H_2O_2, ceramide content in LLC-PK1 cells was elevated to 3.27 ± 0.08-fold as compared to control cells (Fig. 5B). Desipramine and NAC inhibited the elevation of ceramide to the similar levels (1.31 ± 0.05, 1.20 ± 0.05, respectively) on the basis of control cells, suggesting that the NAC protection of cells from H_2O_2 exposure appears to occur *via* the reduction of cellular ceramide level.

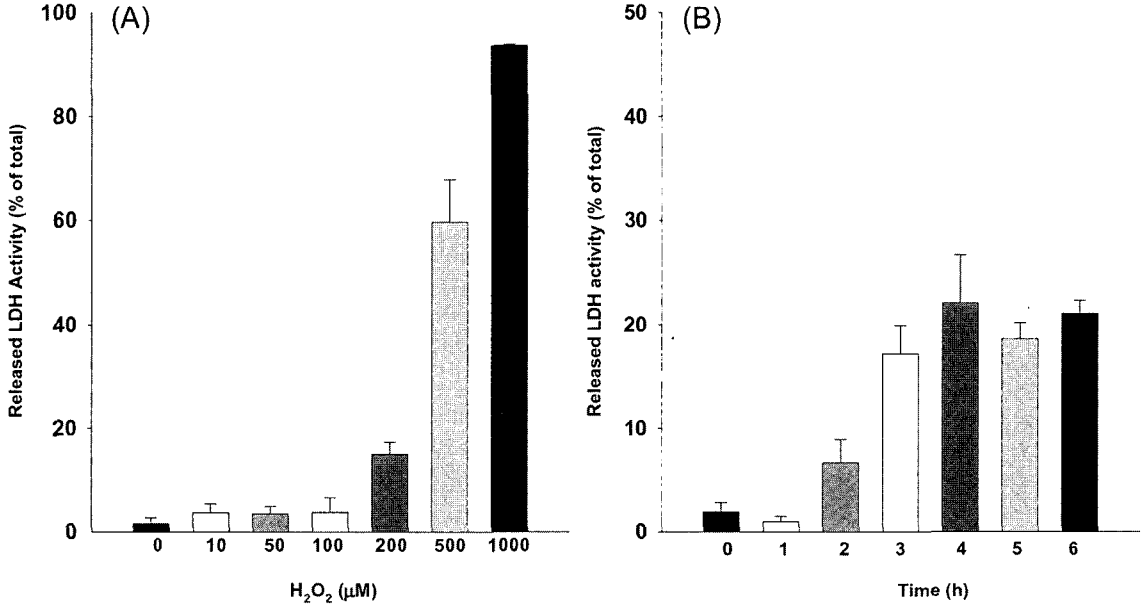

Fig. 3. Released LDH activity of cells following H_2O_2 exposure. LLC-PK_1 cells were grown on 6-well plate (10 cm^2/well) until 50% confluency was achieved and were treated with H_2O_2 at the indicated concentrations (0, 10, 50, 100, 200, 500, 1000 μM) for 6 h (A) and for the given time periods (1, 2, 3, 4, 5 and 6 h) at a concentration of 200 μM (B). Basal level of released LDH activity in cells grown in 10% FBS was approximately 1.3%. Culture cells were assayed for released LDH activity. Y axes in panels A and B are ranged as 100 and 50%, respectively. Data are means ± SD from at least three independent experiments.

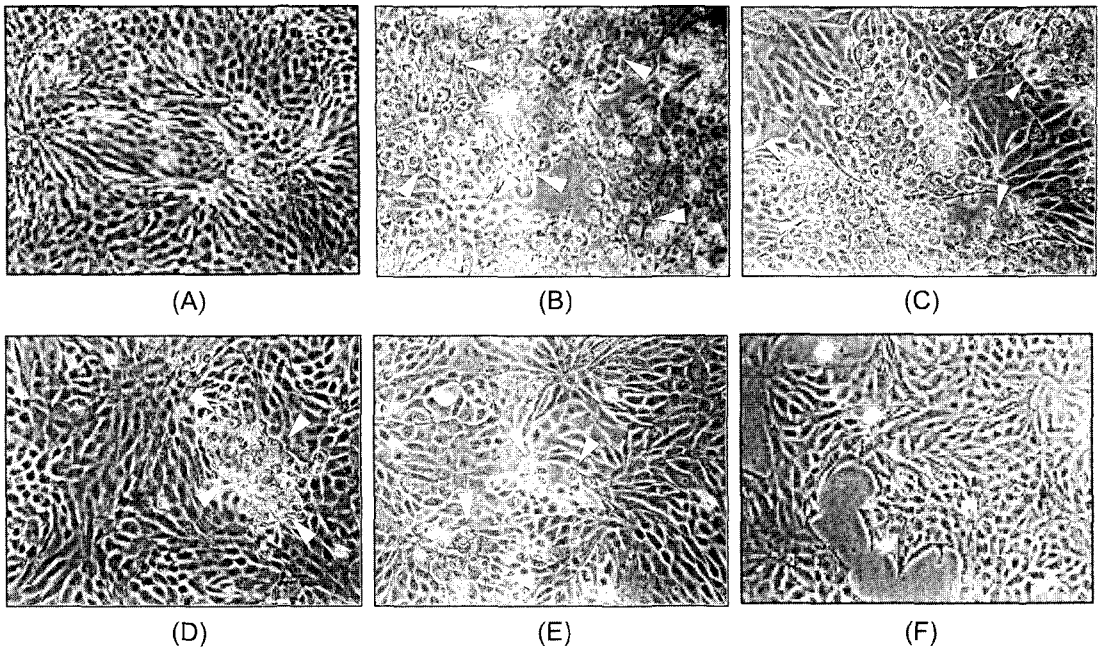

Fig. 4. Cytoprotection by sphingolipid metabolism inhibitors against H_2O_2-induced cell death. Pig proximal tuble cells were grown on 100 mm dish for 36 h. Cells were pretreated with 0.5 μM ISP-1, 0.5 μM desipramine and 25 mM NAC for 1 h in the presence of FBS, and 500 μM H_2O_2 was added to cells and incubated for further 6 h. Cell morphology was observed under light microscopy (100× magnification). White arrow heads indicate the area of cell death. All the pictures include control cells (A), H_2O_2-treated cells (B), cells pretreated with ISP-1 (C), desipramine (D), ISP-1 plus desipramine (E) and NAC (F) for 1 h followed by H_2O_2 exposure.

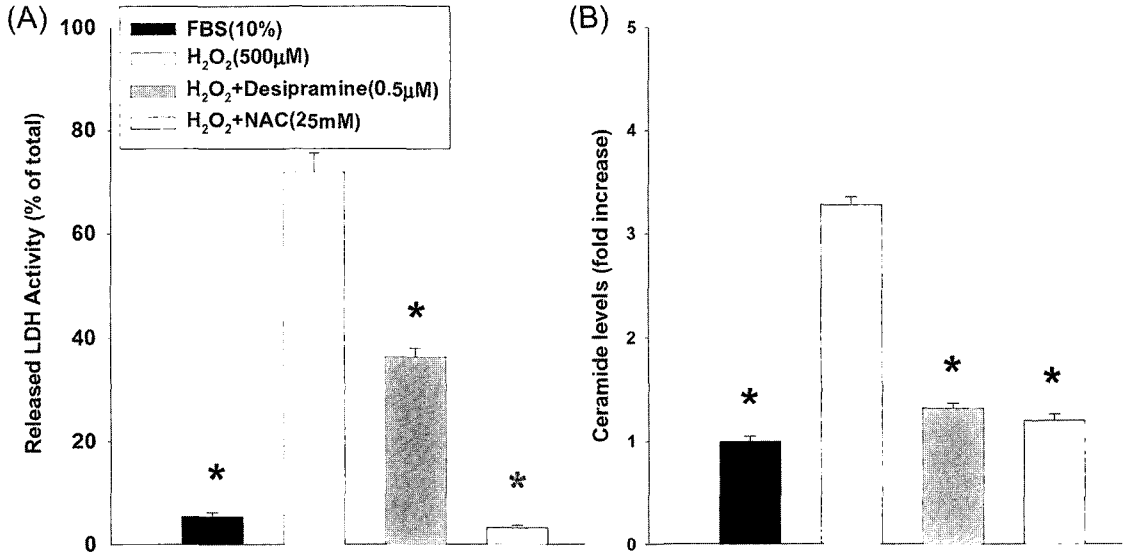

Fig. 5. The relationship between ceramide levels and H_2O_2-induced cell death. Pig kidney proximal tuble cells were grown to 50% confluency, pretreated with 0.5 μM desipramine and 25 mM NAC in the presence of 10% FBS for 1 h, and were then treated with 500 μM H_2O_2 for further 6 h. Released LDH assay (A) was measured and ceramide levels (B) were analyzed. Values are means ± SD from at least three independent experiments. Asterisk (*) indicates other treatments which were significantly different ($p < 0.01$) from the H_2O_2-treated cells.

Modulators of endogenous ceramide metabolism increased cellular GSH level and GST expression in hydrogen peroxide-induced cell death

Sphingolipid metabolism in LLC-PK1 cells was modulated by ISP-1, desipramine and NAC, resulting in lowered elevation of ceramide, when cells were exposed to H_2O_2. LLC-PK1 cells had enhanced expression of GST P1-1 protein under serum-enriched growth conditions. However, cells treated with 500 μM H_2O_2 for 6 h had decreased expression of GST P1-1 (ratio of GST/β-actin)

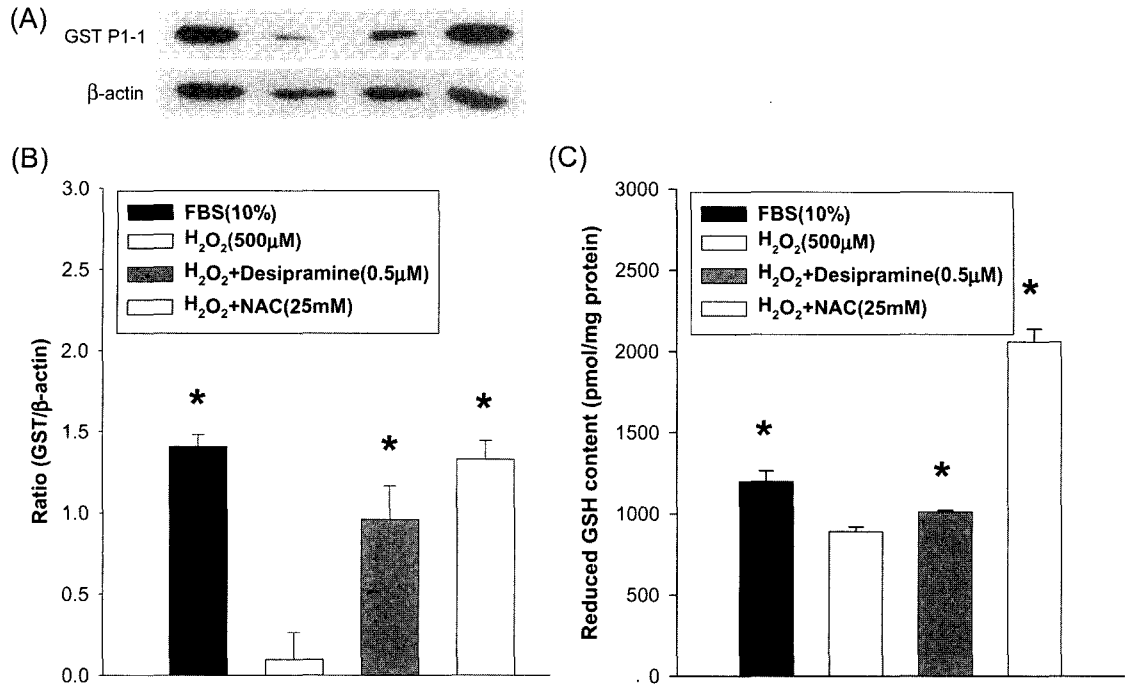

Fig. 6. Cellular GSH contents and GST levels following H_2O_2-exposure. Pig kidney proximal tuble cells were grown to 50% confluency, pretreated with 0.5 μM desipramine and 25 mM NAC in the presence of 10% FBS for 1 h, and were then treated with 500 μM H_2O_2 for further 6 h. Cells were harvested, and GST P1-1 protein was analyzed by Western blot. The band intensities (A) were quantified by densitometric imaging (B), and cellular GSH contents were measured (C). Des is abbreviated as desipramine (A). Values are means ± SD. Asterisk (*) indicates other treatments which were significantly different ($p < 0.01$) from the H_2O_2-treated cells.

with a value of 0.09 ± 0.16 when compared to the healthy normal cells grown in 10% FBS (1.40 ± 0.07) (Fig. 6A and B). The cellular GSH level was reduced to 890 ± 29 from 1198 ± 65 pmol per mg protein as in control cells (Fig. 6C). These results suggest that GST P1-1 expression and cellular GSH level in LLC-PK1 cells may be a key factor for H_2O_2-induced cell death under the conditions of serum deprivation. Upon exposure to 6% H_2O_2 and on subsequent treatment with desipramine and NAC, GST P1-1 expression was enormously increased to 67 and 95%, when compared to H_2O_2 exposed control cells. In cells treated with H_2O_2, cellular GSH concentration was 890 pmol, and 1011 ± 9 and 2060 ± 79 pmol per mg protein, respectively, in desipramine and NAC-treated cells, thus showing a 2.3-fold increase. There was a significant difference between H_2O_2 and H_2O_2 plus desipramine with a p value of 0.002. These results indicate that the changes in H_2O_2-induced GSH level and GST expression may be related to a decreased level of total endogenous ceramide in the H_2O_2 plus NAC and H_2O_2 plus desipramine-treated cells, which leads to cytoprotection against a diversity of stress conditions.

DISCUSSION

Ceramides are known to be lipid signaling molecules of cell death, which occurs in response to several extracellular stress signals such as heat shock, ionizing and ultraviolet radiation, growth factor withdrawal, injury, infections and cytotoxic agents (Hannun and Luberto, 2000). Oxidative stress induced by amyloid-β peptide (Aβ) 1-42 and 4-hydroxynonenal (HNE) have been reported to accumulate cellular ceramide in hippocampal neurons (Dbaibo *et al.*, 1998), and ionizing radiation-induced apoptosis has been shown to be dependent on increase in ceramide levels (Santana *et al.*, 1996). Ceramide can be generated by the activation of sphingomyelinases (SMase) or through *de novo* sphingolipid biosynthesis (Fig. 1). Neutral SMase and acid SMase have been shown to be rapidly activated by stress, thus leading to abrupt increase in cellular ceramide levels (Hannun *et al.*, 2001). Aβ-induced cell death was mediated via SMase activation resulting in an increased cellular ceramide level (Lee *et al.*, 2004). *De novo* ceramide synthetic pathway (Yu *et al.*, 2004) and SMase activation (data not shown) in LLC-PK1 cells were stimulated by serum deprivation. Cells grown in serum-deficient culture medium had 3-fold elevated levels of total complex sphingolipid than the basal level in control cells, and an elevation of total complex sphingolipid was inhibited by both ISP-1, a specific inhibitor of serine palmitoyltransferase in *de novo* pathway, and desipramine, a SMase inhibitor, indicating that stress

in the form of serum deprivation can bring about the activation of *de novo* sphingolipid synthesis and sphingomyelinase in LLC-PK1 cells. However, the degree of either *de novo* sphingolipid biosynthesis or SMase activation in cells exposed to extracellular stress may vary with the type and condition of stress. Exposure of LLC-PK1 cells to hydrogen peroxide induced an elevated cellular ceramide level by 3.2-fold when compared to control cells (Fig. 5B). Desipramine blocked the H_2O_2-induced ceramide elevation, however, ISP-1 barely inhibited the elevation (Fig. 5B). These results suggested that H_2O_2-induced ceramide elevation might be contributed by SMase activation rather than by *de novo* sphingolipid biosynthesis. Therefore, stress appeared to be closely related to sphingolipid metabolism and modulated sphingolipid metabolism may be a potential target for stress-induced human disease such as Alzheimer's disease.

Cell death was observed under oxidative stress while cellular ceramide level was elevated. Cell death upon exposure to H_2O_2 appeared to occur at the same time when total ceramide level was elevated (data not shown), suggesting that ceramide may act as late phase of cell death process even though the mechanism of ceramide-related cell death has not been clearly understood. Elevated cellular ceramide has been identified as a key regulatory step in signaling pathway leading to apoptosis. Ceramide may induce a release of cytochrome *c* from mitochondria leading to activation of caspase-3 and then apoptosis in TNF-α-treated PC-12 cells.

H_2O_2-induced cytotoxicity in LLC-PK1 cells was inhibited by desipramine and ISP-1 (Fig. 4C and D, Fig. 5A). Thus, sphingolipid metabolism in LLC-PK1 cells plays a key role in the process of H_2O_2-induced cell death. When LLC-PK1 cells were grown in the absence of serum for 48 h, cell death occurred and total sphingolipid content as well as ceramide level were increased (Yu *et al.*, 2004, data not shown). Desipramine and ISP-1 imparted lessened degree of cytoprotection to LLC-PK1 cells against 3 h of H_2O_2 exposure (Fig. 4C, D, E), while cell death by serum deprivation for 48 h was inhibited by both desipramine and ISP-1 to the similar extent. The difference in levels of cytoprotection of desipramine and ISP-1 between serum deprivation and H_2O_2 induced cell deaths may depend on the exposure time of stress inducers, as cell proliferation requires *de novo* sphingolipid biosynthesis. It can be postulated that SMase activation may be turned on by acute stress signal.

Glutathione *S*-transferases (GSTs) play an important role in the protection of cells against oxidative stress. GST P1-1 expression was increased in many tumors and was involved in the development of antineoplastic drug resistance (Morceau *et al.*, 2004). GSH depletion was suggested to be a link between oxidative stress and ceramide-mediated apoptosis in the lung epithelial cells (Lavrentiadou *et al.*, 2001). GST P1-1 expression level was reduced upon exposure to H_2O_2 (Fig. 6A and B), and cellular GSH level in H_2O_2-exposed cells was simultaneously decreased. However, desipramine (Fig. 1, Fig. 2), sphingomyelinase inhibitor, restored GST P1-1 expression and GSH level to the level of normal LLC-PK1 cells (Fig. 6). These results suggest that sphingolipid metabolism may be involved in H_2O_2-induced cell death via GSH and GST expression. However, there was no change in GST P1-1 expression and cellular GST level between LLC-PK1 cells grown in serum-sufficient and serum-deficient culture media, even though total sphingolipid content was upregulated by serum deprivation (data not shown). NAC, in H_2O_2 exposure increased GST expression from the basal level and also elevated cellular GSH level when compared to the control cells (Fig. 6B and C). NAC in H_2O_2 exposed cells inhibited the elevation of cellular ceramide level (Fig. 5B), which may be due to the consequences of blocking cellular GSH depletion, completely removing the cellular H_2O_2 and then inhibiting cell death. The result indicated that ceramide elevation may be a prerequisite for oxidative stress-induced cell death. Therefore, the modulation of sphingolipid metabolism may be a potential cellular target for cell death. In conclusion, activation of either SMase or *de novo* sphingolipid pathway appeared to act as a contributing factor to H_2O_2-induced cell death *via* cellular GSH and GST expression in LLC-PK1 cells.

The abbreviations used are: GSH, glutathione; GST, glutathione *S*-transferase; HPLC, high performance liquid chromatography; LDH, lactate dehydrogenase; NAC, *N*-acetylcysteine; OPA, *o*-phthalaldehyde

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