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Protective Effect of Ginsenoside Rg1 on H_2O_2-Induced Cell Death by the Decreased Ceramide Level in LLC-PK1 Cells

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Abstract : Ceramide has been involved in cell death and acted as a lipid mediator of stress responses. Elevation of ceramide level was reported to occur in oxidative stress and lead to cell death in many cell types. This study was undertaken to elucidate a protective role of ginsenoside Rg1 in cell death induced by oxidative stress. When LLC-PK1 cells were treated with H_2O_2 at a concentration of 400 μM for 5 hr, cell death was observed and a released LDH activity indicative of cytotoxicity was increased. H_2O_2 exposure to LLC-PK1 cells was shown to elevate the content of total ceramide by approximately 200% compared to control cells. Ceramide level was hypothesized to be a key to a reversal of cell death to survival. Ginsenoside Rg1 at the concentrations ranging from 12.5 to 250 μM protected LLC-PK1 cells from cell death induced by H_2O_2 at 400 μM for 5 hr, and decreased the ceramide level relative to H_2O_2. Ginsenoside Rg1 inhibited neutral human ceramidase by 71% of controls, while sphingomyelinase was not inhibited. These results suggest that ginsenoside Rg1 show the protection against cell death via the modulation of ceramide metabolism, and ceramide may be a promising therapeutic target for human diseases related to cell death.

Key words : *ceramide, H_2O_2, LLC-PK1 cells, ginsenoside Rg1, cell death*

INTRODUCTION

Ceramide has been involved in the regulation of cell death and is a lipid mediator of cellular stress responses.[1,2] Ceramide level in cells is upregulated by various types of stress conditions including anti-Fas antibody, ionizing radiation, serum deprivation, anti-cancer drugs, heat shock and hydrogen peroxide, and also in neurological disease conditions such as Alzheimer's disease, cerebral ischemia and epilepsy.[3,4,5,6,7,8,9,10] There are two potential pathways for intracellular ceramide formation: *de novo* synthesis via the condensation of serine and palmitoyl-CoA, and the breakdown of sphingomyelin through sphingomyelinase.[11]

Ceramide-induced cell death was dependent on generation of reactive oxygen species. Reaction with hydrogen peroxide and its products such as hydroxyl radicals and superoxide are termed as 'oxidative stress'.[12] Collectively, these species possess significant capacity for cellular damage and have been implicated in both the aging process and the pathogenesis of chronic disease, among them, atherosclerosis, cancer and diseases of the respiratory tract.[13]

Ginsenoside Rg1 is one of the active agents extracted from ginseng, and is classified as panaxatriol group.[14] Ginsenoside Rg1 has protective activity against methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced apoptosis. Ginsenoside Rg1 has potential neurotrophic and neuroprotective activities.[15] Ginsenoside Rg1 attenuates the dopamine-induced apoptosis in PC12 cells by suppressing oxidative stress.[16] Ginsenoside Rg1 pretreatment also reduced the protein level of inducible nitric oxide synthase and nitric oxide production.[16]

This study was undertaken to elucidate a protective role of ginsenoside Rg1 in cell death induced by hydrogen peroxide. Cellular ceramide level determined by ceramide metabolism was hypothesized to be a key to a reversal of cell death to survival in LLC-PK1 cells and could be a potential target for screening therapeutic drugs related to cell death.

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MATERIALS AND METHODS

Materials

Ginsenosides (>98% purity by HPLC) were gifted from KT&G Central Research Institute (Daejon, Korea). C_{17}-ceramide was from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), and fetal bovine serum (FBS) and culture medium were from Life Technologies, Inc. (Gaithersburg, MD, USA). Human neutral ceramidase was kindly gifted from professor M. Ito (Kyushu University, Japan). HPLC-grade methanol was purchased from Merck KBaA (Darmstadt, Germany), and *o*-Phthalaldehyde (OPA) was from Molecular Probes, Inc. (Eugene, OR, USA).

Cell culture

LLC-PK1 cells originated from pig kidney proximal tube (ATCC, Richmond, VA, USA) were seeded at a density of 10^5 cells in flat-bottomed 6-well plates and cultured in DMEM/F12 medium supplemented with 1.2 g/L sodium bicarbonate, 100 units/ml penicillin-streptomycin and 5% (v/v) FBS at 37°C in a humidified 5% CO_2 atmosphere. LLC-PK1 cells were pretreated with ginsenoside Rg1 at the concentrations ranging from 12.5 to 250 μM for 2 hr and incubated following an addition of H_2O_2 at 400 μM for 5 hr. Cells were harvested by scraping with rubber policeman.

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 at 340 nm using a Jasco V-530 UV-Vis spectrophotometer (Tokyo, Japan). The released LDH activity was expressed as percentage of the total cellular LDH activity.

Flow cytometry analysis

Both propidium iodide (PI) and annexin-V labeling for apoptotic or necrotic cell death detection was performed by using Annexin-V-FLOUS staining kit according to the manufacturer's instruction (Roche, Germany). Annexin-V is a protein that binds to phosphatidylserine residues, which are exposed on the surface of apoptotic cells, but not on normal cells. Briefly, LLC-PK1 cells were seeded in 6-well plates (10cm^2/well) and allowed to grow for 36 hr. Serum-deprived culture was maintained for 2 hr, and cells were treated with H_2O_2 at 400 μM for 5 hr. LLC-PK1 cells were harvested, resuspended in 400 μl binding buffer and incubated with 4 μl FITC-labeled annexin-V and 4 μl PI stock solution (50 μg/ml) for 10-15 min in the dark at room temperature. LLC-PK1 cells were analyzed by FACSCalibur (Becton & Dickinson Co., USA). After appropriate markings for negative and positive populations were set, the percentage of annexin-V-/PI- (live cells), annexin-V+/PI- (apoptotic cells) and annexin-V+/PI+ (necrotic cells) staining was determined.

Ceramide measurement

The lipids from cell pellets were extracted with chloroform:methanol (1:2, v/v) for 1 hr at 37°C. The lipid extract residue was dissolved in methanol, spotted on thin layer chromatography silica gel plates (Merck, Darmstadt, Germany) and developed in chloroform:methanol:29% NH_4OH (40:10:1, v/v) to half of the plate length. After drying, the plate was rechromatographed in heptane: diisopropylether:acetic acid (60:40:3, v/v/v). Lipids were visualized by dipping into 10% sulfuric acid and drying. The band of ceramides corresponding to the band of standard C_{17} sphingosine base-ceramide was scraped-off and eluted with methanol. Ceramidase was used to release sphingosine from ceramide by deacylation. HPLC analysis was performed using a Shimadzu Model LC-10AT pump and SIL-10AXL autoinjector. An analytical Radial-Pak cartridge (Waters Associates, Inc., Milford, MA, USA) packed with Nova-Pak C_{18} (4 μm, 10cm × 0.8cm) was equilibrated with a mobile phase (92% methanol, 0.1% triethylamine) at a flow rate of 1 ml/min. The fluorescence detector (RF-10AXL) was set at an excitation wavelength of 340 nm and emission wavelength of 455 nm.

Ceramidase assay

The assay was performed as described with modification.[17] Briefly, C_{17} sphingosine base-ceramide (10 nmol) was added as substrate for ceramidase and then resuspended in 20 μl of reaction buffer (25 mM Tris-HCl buffer, pH 7.5, 1% sodium cholate) and ceramidase (5 μunits). The enzymatic reaction was incubated at 37°C for 20 min. The reaction was stopped by adding chloroform: methanol (2:1, v/v) and the released sphingosine from ceramide was extracted with chloroform:methanol (1:2, v/v). Ceramidase activity was analyzed using HPLC with fluorescence detector.

Sphingomyelinase assay

Sphingomyelinase (EC 3.1.4.12; Sigma Chemical Co., St. Louis, MO, USA) was from *Staphylococcus aureus*. Sphingomyelin was served as substrate for sphingomyelinase, and resuspended in neutral sphingomyelinase assay buffer (40 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 0.2% Triton X-100) and sphingomyelinase (20 μunits). Reaction mixture was incubated at 37°C for 20 min. The reaction was stopped by adding chloroform:methanol (1:1, v/v), C_{17} sphingosine base-ceramide as an internal standard was added to the reaction and the released ceramide hydrolyzed from sphingomyelin was extracted with chloroform: methanol (1:2, v/v). Ceramide from sphingomyelin was deacylated by ceramidase to liberate sphingosine. Sphingomyelinase activity was analyzed using HPLC.

Statistical analysis

All values were expressed as a mean ± standard deviation (SD). Differences between treatments were analyzed statistically by unpaired Student's *t*-test. One-way analysis of variance (ANOVA) was used for multiple comparison followed by Dunnett's test. Differences with $p < 0.01$ were defined as statistically significant.

RESULTS

1. H_2O_2-induced cell death via necrotic mechanism in LLC-PK1 cells

Cells were confluenced to 60% with the typical morphology of epithelial cell type and treated with H_2O_2 at 400 μM for 5 hr. Visual observation of control cells showed characteristic dome and cobble stone-like epithelial morphology, while most of H_2O_2-treated cells were dead (Fig. 1A). Many dead cells in H_2O_2-treated culture were floating and attached cells were swelling. A double staining with annexin V-FITC and propidium iodide (PI) was performed in order to distinguish between necrotic and apoptotic cell death among dead cells (Fig. 1B). Annexin V staining can identify apoptosis at an earlier stage and PI staining was based on nuclear changes. Live cells in lower left panel, necrotic cells in upper right and apoptotic cells in lower right (Fig. 1B) indicate annexin V(-)/PI(-), annexin V(+)/PI(+) and annexin V(+)/PI(-), respectively. Hydrogen peroxide made the population of live cells move to the region of necrotic cell death. Basal level of necrotic cells in control culture was 5% of total cell population, while the level of necrotic cells in H_2O_2-treated culture was 47% of the total. Lactate dehydrogenase activity (LDH) released out of cells, the other indicator of necrotic cell death, was shown to be 75% in H_2O_2-treated culture compared to 2-3% in control culture (Fig. 1C). Thus, these results suggest that H_2O_2-induced cell death occurs via necrotic mechanism in LLC-PK1 cells.

2. Protective effect of Ginsenoside Rg1 on H_2O_2-induced cell death

Ginsenosides Rb1, Rc, Rg1 and Re, active ingredients extracted from ginseng, were tested for a protection of LLC-PK1 cells from H_2O_2-induced cell death. Ginsenos-

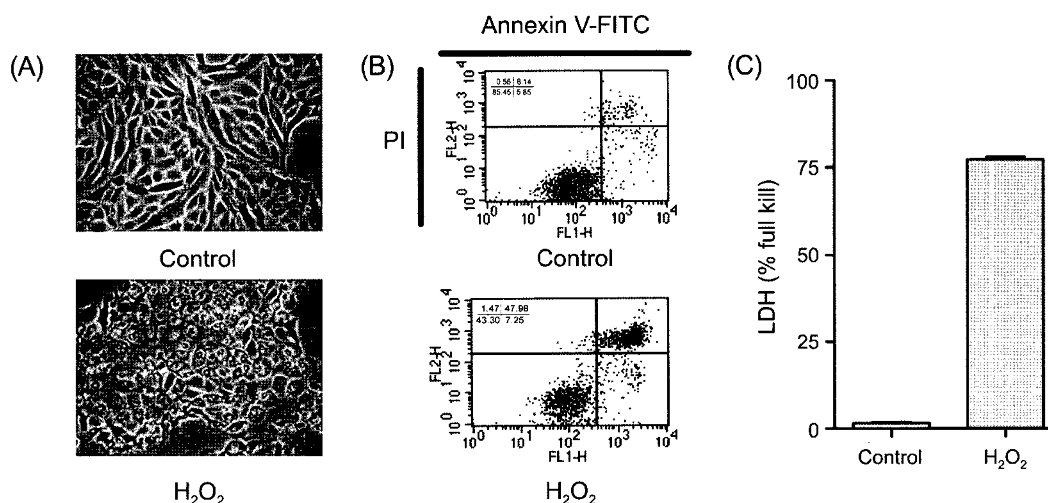

Fig. 1. H_2O_2-induced cell death in LLC-PK1 cells. Cells were seeded in 6-well plates ($10 cm^2$/well) and allowed to grow for 36 hr. Serum-deprived culture was maintained for 2 hr, and H_2O_2 at 400 μM was added and incubated for 5 hr. Visual observation (A), flow cytometry analysis (FACS) (B) and cytotoxicity from lactate dehydrogenase (LDH) assay (C) were shown. Values are means ± SD from at least three independent experiments.

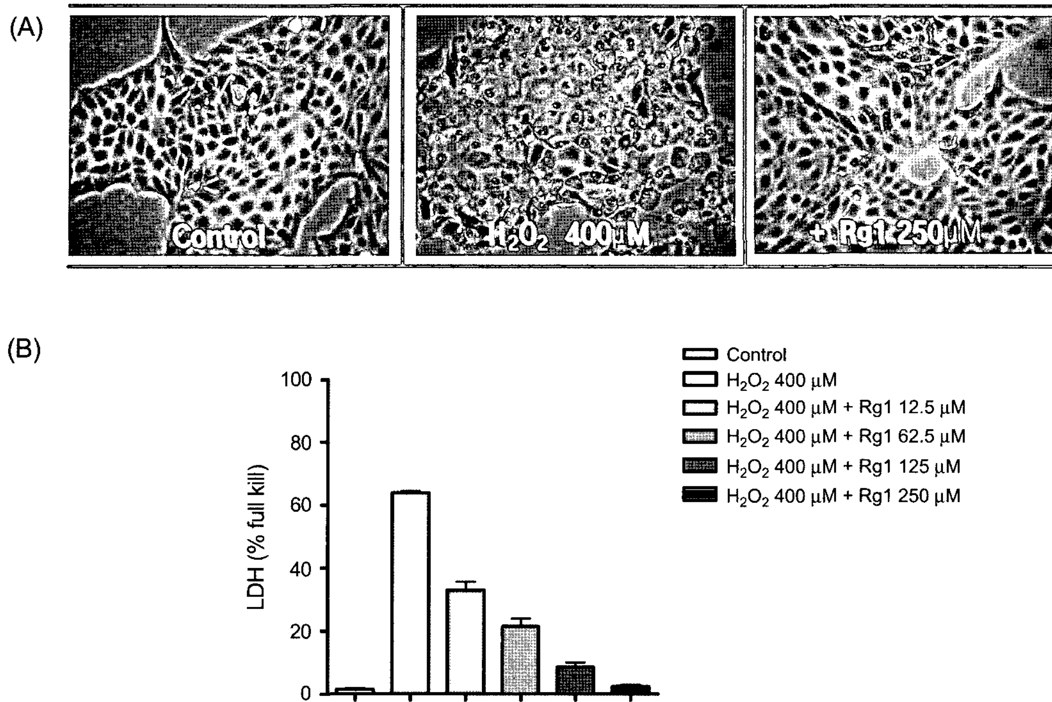

Fig. 2. Protection of cells from H_2O_2-induced cell death by ginsenoside Rg1. LLC-PK1 cells were treated with ginsenoside Rg1 at concentrations of 12.5, 62.5, 125 and 250 μM for 2 hr. Then, morphology (A) was visually observed and cytotoxicity (B) was determined. Data are means ± SD from at least three independent experiments.

ide Rg1 decreased cytotoxicity in a concentration-dependent manner at 12.5-250 μM when cell death was induced by H_2O_2. However, ginsenosides Rb1, Rc and Re at 12.5-250 μM did not show any protection of cells from cell death induced by H_2O_2 (data not shown). Ginsenosides Rb1, Rc and Re did not change the increased LDH activity induced by H_2O_2. Combination of hydrogen peroxide with ginsenoside Rb1 or Rc at 12.5-250 μM was cytotoxic to LLC-PK1 cells than H_2O_2 alone, indicating that the ginsenosides Rb1 and Rc may act as cell death inducer rather than agents for cell protection at higher concentrations. Pretreatment of LLC-PK1 cells with 250 μM ginsenoside Rg1 for 2 hr, and the combined treatment of H_2O_2 plus ginsenoside Rg1 resulted in a significant protection against H_2O_2-induced cell death compared with treatment of 400 μM H_2O_2 alone (Fig. 2A). In addition, pretreatment of LLC-PK1 cells with ginsenoside Rg1 at 12.5-250 μM decreased the released LDH activity in a concentration-dependent manner (Fig. 2B).

3. Elevation of cellular ceramide level in H_2O_2-induced cell death

Ceramide is an important second messenger in various stress responses. Elevated level of endogenous ceramide was hypothesized to contribute to H_2O_2-induced cell death in LLC-PK1 cells. The idea of determining ceramide concentration in cells came from the equivalent molecules of sphingosine released from ceramide by human ceramidase. The calculation of ceramide concentration in LLC-PK1 cells was based on the peak area of C_{17} sphingosine base-ceramide, and recovery of ceramidase as well as fluorescence sensitivity in HPLC was considered for determination of endogenous ceramide level. Cells treated with H_2O_2 at 400 μM for 5 hr in the absence of FBS were cytotoxic and increased the ceramide level by 230% as compared to control cells (Fig. 3). The ceramide concentration in control cells was approximately 0.9 nmol per mg protein, while the ceramide level in H_2O_2-treated cells was 2.1 nmol. LLC-PK1 cells were preincubated with ginsenoside Rg1 at the concentrations of 12.5 to 250 μM for 2 hr in the absence of FBS and then treated with H_2O_2 for 5 hr further. Ceramide levels in H_2O_2-treated cells were elevated to 2.1 nmol per mg protein from 0.9 nmol in control cells (Figs. 3). Ginsenoside Rg1 ranging from 12.5 to 250 μM was shown to decrease concentration-dependently the ceramide level elevated by H_2O_2 (Fig. 3) in LLC-PK1 cells. These results indicated that H_2O_2-induced cell death may occur by the elevation of cellular

ceramide in LLC-PK1 cells.

4. Alteration of ceramide metabolism by ginsenoside Rg1

Increased ceramide level leading to cell death comes from either *de novo* synthesis due to stimulation of serine palmitoyl transferase and/or dihydroceramide synthase, or by degradation of sphingomyelin via sphingomyelinase, or by inhibiting ceramidase activity and glucosylceramide synthase. Cellular ceramide level appeared to be modulated by ginsenoside Rg1, and was correlated with cell death. Ceramide level in LLC-PK1 cells was regulated by the activities of sphingomyelinase, ceramidase, glucosylceramide synthase, ceramide synthase, ceramide kinase and sphingomyelin synthase. Enzyme activity of purified human neutral ceramidase was measured with an *in vitro* assay, and ginsenoside Rg1 inhibited ceramidase activity (Fig. 4). Inhibition of ceramidase activity at 400 μM H_2O_2 was 63% compared to control value. Ginsenoside Rg1 at a concentration of 250 μM inhibited ceramidase activity by 71% of control. However, H_2O_2 and ginsenoside Rg1 did not show any sphingomyelinase activity (Fig. 5). These results indicate that the decrease in ceramide level elevated by H_2O_2 may be due to the altered enzyme activity in ceramide metabolism. Ginsenoside Rg1 alone at much higher concentration than 250 μM may cause cell death by inhibiting ceramidase activity and elevating cellular ceramide level.

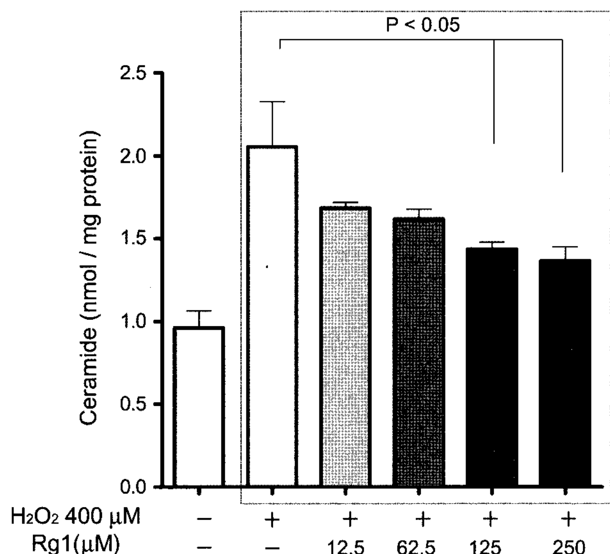

Fig. 3. Decrease in H_2O_2-induced elevation of ceramide level by ginsenoside Rg1. LLC-PK1 cells were treated with ginsenoside Rg1 (12.5, 62.5, 125 and 250 μM) for 2 hr, and then treated with H_2O_2 at 400 μM for 5 hr. Ceramide concentration was determined by HPLC following lipid extraction and deacylation by ceramidase. Data are means ± SD from at least three independent experiments.

DISCUSSION

Ceramide has been implicated in the regulation of cell death via signaling pathway.[18] Recent studies have shown that the increased cellular ceramide, exogenous sphingomyelinase or pharmacologic agents that interfere with enzymes in ceramide metabolism pathway can induce cell death. Hydrogen peroxide turned out to induce ceramide-

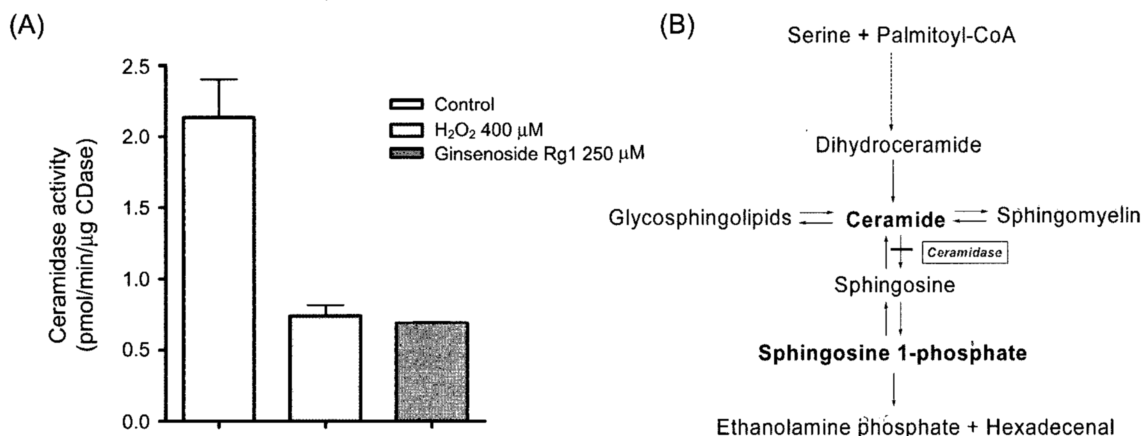

Fig. 4. Effect of ginsenoside Rg1 on ceramidase activity *in vitro*. Purified ceramidase was used for enzyme assay, C_{17} sphingosine base-ceramide was added as an internal standard and a sphingosine, a product of ceramidase reaction, was analyzed by HPLC. Ceramidase activity of H_2O_2 (400 μM), ginsenoside Rg1 (250 μM) was measured by incubating the enzyme reaction at 37°C for 30 min (A). Ceramidase converts ceramide into sphingosine by deacylation reaction (B). Data are means ± SD from at least three independent experiments.

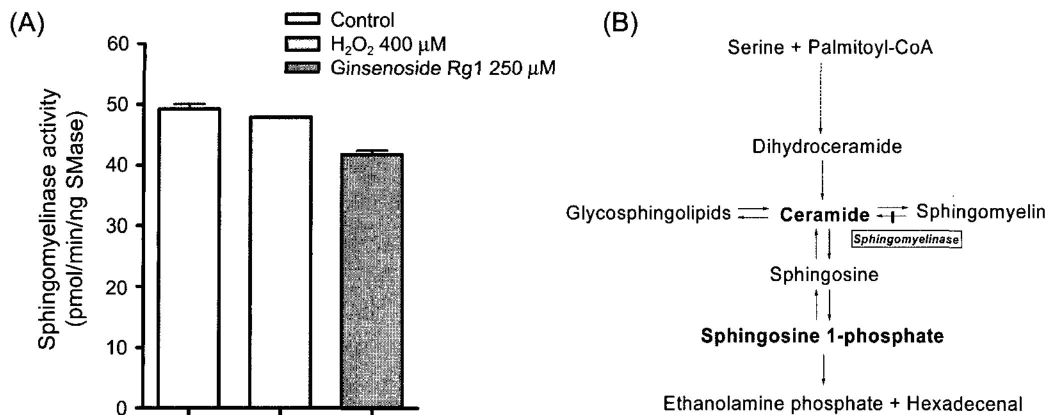

Fig. 5. Effect of ginsenoside Rgl on sphingomyelinase activity *in vitro*. Purified human neutral sphingomyelinase was used for enzyme assay. C_{17} sphingosine base-ceramide was added as an internal standard and a ceramide, a product of sphingomyelinase reaction, was deacylated by ceramidase and then analyzed by HPLC. Sphingomyelinase activity of H_2O_2 (400 μM) and ginsenoside Rgl (250 μM) was measured by incubating the enzyme reaction at 37°C for 30 min (A). Sphingomyelinase converts sphingomyelin into ceramide by deacylation reaction (B). Data are means ± SD from at least three independent experiments.

associated necrotic cell death in LLC-PK1 cells (Fig. 3), suggesting that cells insulted by oxidative stress may lead to the alteration of enzymes related to ceramide metabolism and then elevate ceramide level which in turn accelerates cell death by stimulating death signals. Hydrogen peroxide itself was shown to be ceramidase inhibitor (Fig. 4). Therefore, the mechanism of H_2O_2-induced cell death appeared to be involved in the inhibition of ceramide breakdown pathway which resulted in accumulation of cellular ceramide.

Ginsenoside Rgl turned out to be the most effective in protection of cells from hydrogen peroxide-induced cell death. However, ginsenosides Rb1, Rc and Re did not protected LLC-PK1 cells from H_2O_2 exposure. Ceramide level elevated by H_2O_2 was effectively decreased by ginsenoside Rgl (Fig. 3). Therefore, it could be presumed that hydrogen peroxide-induced cell death is strongly related to the level of signal under oxidative stress. Many evidences that ceramide plays an essential role in oxidative stress-related cell death need to be accumulated. Ginsenoside Rgl acts as ceramidase inhibitor (Fig. 4). However, the decreased cellular ceramide level by ginsenoside Rgl after H_2O_2 exposure indicates that the ceramide level may be due to the concerted modulation of enzymes in ceramide metabolism. Ginsenoside Rgl has been reported to show potential neurotrophic and neuroprotective effects.[15] Protective mechanism of ginsenoside Rgl against cell death occurred by inhibiting the expression of Bax, inducible nitric oxide synthase and caspase-3.[16]

Several enzyme targets of sphingolipid metabolism for modulating ceramide level include ceramidase, sphingomyelinase, ceramide kinase, sphingomyelin synthase, glucosyl ceramide synthase and *de novo* pathway. Ceramide can be formed from the hydrolysis of sphingomyelin by sphingomyelinase. It can be phosphorylated by ceramide kinase to ceramide 1-phosphate, or utilized for the synthesis of sphingomyelin or glycosphingolipids. Ceramide kinase cloning has helped to uncover new physiological functions of ceramide 1-phosphate.[19]

Oxidative stress has been reported to induce cell death in many cell types by activating either ceramide synthase or sphingomyelinase existed in cell membrane.[20,21] Paradoxically, ginsenoside Rgl in this study inhibited human ceramidase, while there was no effect on sphingomyelinase activity although it protected cells from H_2O_2 exposure by decreasing the ceramide level. These results indicate that other enzyme activities in sphingolipid metabolism rather than either ceramidase or sphingomyelinase may act as major contributor to the protection of LLC-PK1 cells by ginsensoside Rg1. Collectively, ginsenoside Rgl showed cytoprotective effects from necrotic cell death and inhibited the elevation of ceramide level by H_2O_2 in LLC-PK1 cells. Protection of cells by ginsenoside Rgl occurs by the decreased ceramide level and ceramide metabolism may be a promising therapeutic target for human diseases related to cell death.

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