Anti-apoptotic and Beneficial Metabolic Activities of Resveratrol in Type II Gaucher Disease

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Received October 7, 2014; accepted March 7, 2015

Gaucher disease (GD) is one of the most common lysosomal storage disorders and is caused by an inherited deficiency in glucocerebrosidase. Resveratrol is a phytoalexin that has many beneficial activities, including anti-oxidant, anti-apoptotic, and neuroprotective effects. The aim of this study was to determine if resveratrol has a therapeutic effect on primary fibroblast cells derived from a patient with type II GD. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyloxazole-tetrazolium (MTT) assays were performed to determine the effect of resveratrol on cell viability. The expression patterns of apoptosis-inducing factor (AIF), Bel-2-associated X protein (Bax), caspase-3, acetyl-coenzyme A acetyltransferase 1 (ACAT1), E3-binding protein (E3BP), and citrate synthase (CS) were evaluated by Western blotting to characterize the effect of resveratrol treatment on GD cells. TLC was performed to determine glucosylceramide levels in resveratrol-treated GD cells. Resveratrol increased GD cell viability compared to untreated control cells. Further, resveratrol treatment dose-dependently decreased the apoptotic factors AIF, Bax, and cleaved caspase-3 levels, whereas ACAT1, E3BP, and CS expression dose-dependently increased. TLC analysis showed reduced levels of intracellular glucosylceramides in resveratrol-treated GD cells. These findings demonstrate that resveratrol can reduce cellular stress resulting from glucosylceramide accumulation, and suggest that resveratrol should be studied further as a novel therapeutic agent for GD.

Key words resveratrol; phytoalexin; polyphenol; Gaucher disease; lysosomal storage disease; therapeutics

Gaucher disease (GD) is a multi-systemic lipidosis caused by an inherited deficiency in glucocerebrosidase (GCase). GD is the most common sphingolipid storage disease, characterized by intracellular accumulation of glucosylceramide (GluCer) in tissues, resulting in various pathological conditions, including hepatosplenomegaly, bone lesions, blood disorders (anemia, leukopenia, and thrombocytopenia), and involvement of other organs, such as the brain and lungs in some GD variants. The disease is classified into 3 subtypes based on the clinical symptoms present and the extent of central nervous system involvement. Type I GD is a chronic non-neuronopathic disease. In contrast, type II GD, which is the infantile or acute neuronopathic form, is characterized by rapid neurodegeneration and death within the first 2 years. Type III GD is a subacute neuronopathic form of intermediate severity, with patients surviving into their teens or adulthood. Defective folding of mutant GCase results in the accumulation of this protein in the endoplasmic reticulum (ER). This accumulation and impaired trafficking of GCase from the ER to lysosomes can induce ER stress. Prolonged ER stress results in apoptosis via activation of apoptotic enzymes, such as Bel-2 family members and caspases. Metabolic disorders, including insulin resistance and abnormal lipid metabolism, are also reported in GD.

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a polyphenol produced by various plants, such as grapes and mulberries, that has been shown to possess many beneficial properties, including anti-inflammatory, anti-oxidant, anti-apoptotic, anti-tumor, and neuroprotective activities. In addition, resveratrol can regulate carbohydrate and lipid metabolism. Current GD therapies have a limited ability to alleviate disease symptoms, in particular neurological symptoms, because of the inability to cross the blood–brain barrier or limited efficacy. There is a significant need to develop novel GD therapies that are more effective. We investigated the therapeutic potential of resveratrol on cells derived from a patient with type II GD by evaluating cell survival, biomarkers involved in metabolic and apoptotic pathways, and changes in GluCer concentrations.

MATERIALS AND METHODS

Cell Line and Cell Culture Type II GD fibroblasts were obtained from the Coriell Institute for Medical Research (GM# 08760; homozygous for the L444P mutation, Coriell, Camden, NJ, U.S.A.). Normal fibroblasts were obtained from the Korean Cell Line Bank (KCLB# 21947, CCD-986sk; Seoul, South Korea). Cells were grown in 100 mm dishes in Dulbecco’s modified Eagle’s medium (DMEM; Mediatech Inc., Herndon, VA, U.S.A.) containing 20% fetal bovine serum (Thermo Scientific, South Logan, UT, U.S.A.), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Invitrogen, Carlsbad, CA, U.S.A.). Cultures were maintained at 37°C in a 5% CO₂ humidified incubator.

Cell Viability Assay The GD fibroblasts and normal fibroblasts were seeded in 96-well plates at a density of 10⁴ cells/200 µL. Cells were then cultured in DMEM containing 0.1 µM, 1 µM, or 10 µM resveratrol (prepared in dimethyl sulfoxide (DMSO); Sigma, Saint Louis, MO, U.S.A.) for 24 h at 37°C in a 5% CO₂ incubator. Both type II GD fibroblasts and normal fibroblasts were treated with 0.1% DMSO as a vehicle control. Ten microliters of EZ-cytox 3-(4,5-dimethylthiazol-
2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay solution (Daeiellab Service, Seoul, South Korea) was added per well and incubated for 2 h at 37°C. After incubation, MTT reduction was determined by measuring the absorbance at 450 nm using a microplate spectrophotometer (Bio-Rad, Hercules, CA, U.S.A.). Cell viability was expressed as a percentage of the cell viability of the control group.

**Western Blot Procedure** Approximately 1×10⁶ GD and normal fibroblasts were cultured in 100 mm dishes in culture medium containing resveratrol (0.1 μM, 1 μM, or 10 μM) at 37°C. GD fibroblasts and normal fibroblasts were also cultured in 0.1% DMSO (vehicle control). After incubation for 24 h, cells were washed with cold phosphate buffered saline and lysed in whole cell lysis buffer (20 mM 2-[4-(2-hydroxyethyl)-piperazin-1-yl]ethanesulfonic acid (HEPES) pH 7.5, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM ethylene glycol-bis(2-aminoethylether)N,N,N′,N′-tetraacetic acid (EGTA), 350 mM NaCl, 10 mM NaF, 1 mM MgCl₂, and 1% Triton X-100 (Sigma) with a protease inhibitor cocktail (Roche, Penzberg, Germany). Protein concentration was determined with the Coomassie Plus protein assay reagent (Pierce, Rockford, IL, U.S.A.). Thirty micrograms of protein were collected from each sample, run on electrophoresis gels, and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, U.S.A.). Membranes were incubated with antibodies against apoptosis-inducing factor (AIF; Abcam, Cambridge, MA, U.S.A.), Bel-2-associated X protein (Bax; Cell Signaling, Beverly, U.S.A.), cleaved caspase-3 (Cell Signaling), E3-binding protein (E3BP; Abcam), citrate synthase (CS; Abcam), acetyl-coenzyme A acetyltransferase 1 (ACA T1; Abcam), and β-actin (Sigma). Membranes were then incubated with antimouse (Abcam) and anti-rabbit (Abcam) secondary antibodies. Bands were visualized with an enhanced chemiluminescence system (SuperSignal West Pico Luminol/Enhancer Solution, Pierce).

**Lipid Analysis and TLC** Quantitative changes in GluCer concentrations in cells treated with resveratrol or DMSO (vehicle control) were evaluated by TLC. Lipid extraction and GluCer separation were performed as described in a previous article. Densitometric analysis was carried out to measure GluCer levels with TINA 2.0 software (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

**Statistical Analysis** Each experiment was performed independently, from three to six times. Student’s t-test was performed to confirm the presence of significant differences. A p value <0.05 was considered statistically significant.

**RESULTS**

**Analysis of Cell Viability in Response to Resveratrol Treatment** We evaluated the viability of type II GD fibroblasts after the challenge with increasing concentrations of resveratrol (0.1 μM, 1 μM, and 10 μM) by MTT assay. Resveratrol treatment at 0.1 μM, 1 μM, and 10 μM dose-dependently increased the viability of GD fibroblasts 27–49%, compared to DMSO-treated GD fibroblasts (p<0.05, Fig. 1). To characterize the molecular effects of resveratrol, the expression levels of AIF, Bax, and caspase-3 were determined. Resveratrol treatment at 0.1 μM, 1 μM, and 10 μM resulted in dose-dependent reductions in the expression of AIF (38–62%; p<0.05), Bax (26–41%; p<0.05), and cleaved caspase-3 (53–75%; p<0.05), compared to DMSO-treated GD fibroblasts (Fig. 2).

**Western Blot Analysis of ACAT1, E3BP, and CS Protein Expression** After 24 h treatment with different concentrations of resveratrol, the expression level of ACAT1 and E3BP dose-dependently increased (13–66% and 11–57%, respectively), compared to the vehicle control (p<0.05). In addition, the expression level of CS increased by 10–48% in a dose-dependent manner (p<0.05, Fig. 3) in the presence of resveratrol.

**Measurement of GluCer Concentrations** Quantitative changes in GluCer levels in type II GD cells were determined by TLC. Increased levels of GluCer were observed in GD cells treated with DMSO, compared to those in the normal cells. Treating GD cells with increasing concentrations of resveratrol dose-dependently decreased GluCer accumulation in the treated cells (11–37% reduction by relative density scan, p<0.05, Fig. 4).

**DISCUSSION**

The ER is the site of protein production, processing, and maturation. Defective processing of mutant GCase results in the accumulation of this protein in the ER lumen, which can cause ER stress. Chronic ER stress can activate the intrinsic apoptotic pathway, which ultimately activates effector caspases, such as caspase-3, leading to cell death. In this study, we demonstrated the novel finding that resveratrol dose-dependently decreases the expression level of AIF, Bax,
and cleaved caspase-3 in type II GD cells, while increasing cell viability. AIF mediates a caspase-independent apoptotic pathway. Therefore, this finding indicates that resveratrol has a broad spectrum of mechanisms of action affecting both caspase-dependent and caspase-independent cell death pathways. In addition, because insulin resistance and impaired lipid metabolism were reported in GD, and resveratrol was documented to improve carbohydrate and lipid metabolism, we evaluated the effect of resveratrol on enzyme markers associated with these metabolic pathways in type II GD cells.

Fig. 2. Western Blot and Densitometric Analyses of AIF, Bax, and Caspase-3 Proteins
(A) Resveratrol dose-dependently reduced expression levels of AIF, Bax, and cleaved caspase-3 in type II GD cells. (B) Densitometric analysis. β-Actin served as a loading control. Vehicle represents type II GD cells treated with DMSO, which was defined as 100% relative density. Normal-vehicle, normal cells treated with DMSO; GD II, type II GD cells. *p<0.05 vs. vehicle by Student’s t-test.
GD cells treated with resveratrol showed a dose-dependent reduction in the concentration of GluCer. Further these cells displayed a dose-dependent increase in the expression of E3BP and CS, enzymes involved in carbohydrate metabolism and the citric acid cycle, and an increase in ACAT1, which is an enzyme involved in lipid metabolism (Fig. 3). GluCer is normally degraded into glucose and ceramide in the lysosome, and the glucose produced by this process can be used as an energy source via the citric acid cycle. Our findings suggest that resveratrol reduces intracellular stress by decreas-

Fig. 3. Western Blot and Densitometric Analyses of ACAT1, E3BP, and CS Proteins
(A) Resveratrol dose-dependently increased expression levels of ACAT1, E3BP, and CS in type II GD cells. (B) Densitometric analysis. β-Actin served as a loading control. Vehicle represents type II GD cells treated with DMSO, which was defined as 100% relative density. Normal-vehicle, normal cells treated with DMSO; GD II, type II GD cells. *p<0.05 vs vehicle by Student’s t-test.
ing GluCer accumulation in GD cells. Cells then recover from this pathological condition by increasing the activity of metabolic pathways with increased acetyl-CoA requirements. The enzyme ACAT1 catalyzes the formation of acetoacetyl-CoA from two molecules of acetyl-CoA, which serves as the basis for the biosynthesis of factors required for various cellular processes, including growth and differentiation, by the mevalonate pathway. Thus, the increased expression of ACAT1 in GD cells after resveratrol treatment indicates that acetyl-CoA, the byproduct of GluCer degradation, is converted into acetoacetyl-CoA, which can produce the molecules used for cell growth, proliferation, and differentiation by the mevalonate pathway.

In this study, we found that resveratrol decreased GluCer accumulation and apoptotic factors, and increased the expression of enzymes associated with glucose and lipid metabolism in type II GD cells. Resveratrol can cross the blood–brain barrier, and it has potential protective activities in neurodegenerative disorders, such as Alzheimer’s disease and Parkinson’s disease. These findings support the further investigation of resveratrol as a novel therapeutic agent, because of its potential use in the treatment of currently intractable neurological symptoms in patients with GD.

Acknowledgments The authors gratefully acknowledge the cooperation of Dr. Seun-Min Kim of Korea Advanced Institute of Science and Technology for valuable technical assistance. This work was supported by a Grant (KRF-2014-081-E0014) from the Korea Research Foundation funded by the Korean Government (MOEHRD, Basic Research Promotion Fund).

Conflict of Interest The authors declare no conflict of interest.

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![Fig. 4. Measurement of Intracellular Glucosylceramide by TLC (A) and Densitometric Analysis (B)](Image)


