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Original Contribution

Neutral sphingomyelinase inhibition decreases ER stress-mediated apoptosis and inducible nitric oxide synthase in retinal pigment epithelial cells

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ABSTRACT

Endoplasmic reticulum (ER) stress and excessive nitric oxide production via the induction of inducible nitric oxide synthase (NOS2) have been implicated in the pathogenesis of ocular diseases characterized by retinal degeneration. Previous studies have revealed the sphingomyelinase/ceramide pathway in the regulation of NOS2 induction. Thus, the objective of this study was to determine the activity of the sphingomyelinase/ceramide pathway, assess nitric oxide production, and examine apoptosis in human retinal pigment epithelial (RPE) cells undergoing ER stress. Sphingomyelinase (SMase) activity; nuclear factor κB (NF-κB) activation; NOS2, nitrite/nitrate, and nitrotyrosine levels; and apoptosis were determined in cultured human RPE cell lines subjected to ER stress via exposure to tunicamycin. Induction of ER stress was confirmed by increased intracellular levels of ER stress markers including phosphorylated PKR-like ER kinase, C/EBP-homologous protein, and 78-kDa glucose-regulated protein. ER stress increased nuclear translocation of NF-κB, NOS2 expression, nitrite/nitrate levels, and nitrotyrosine formation and caused apoptosis in RPE cell lines. Inhibition of neutral SMase (N-SMase) activity via GW 4869 treatment caused a significant reduction in nuclear translocation of NF-κB, NOS2 expression, nitrite/nitrate levels, nitrotyrosine formation, and apoptosis in ER-stressed RPE cells. In conclusion, N-SMase inhibition reduced nitrative stress and apoptosis in RPE cells undergoing ER stress. Obtained data suggest that NOS2 can be regulated by N-SMase in RPE cells experiencing ER stress.

The retinal pigment epithelium (RPE) is a monolayer of cells located outside the neural retina and is important in maintaining retinal function. The proximity of choroidal capillaries enables RPE cells to provide nutrients to maintain visual function and to play a key role in forming the outer blood–retinal barrier that prevents nonspecific diffusion and transport of material from the choroid [1]. Ischemic and hypoxic injuries as well as many retinal diseases affect RPE cells [2]. Apoptosis is a frequent type of cell death observed in RPE cells [3] and is an important feature of age-related macular degeneration, the most common cause of irreversible vision loss especially in the elderly population over 65 years of age [4]. Age-related alterations in the RPE include a reduction in cell density that can be caused by apoptosis resulting from accumulation of toxic substances [5]. Oxidative stress [6], hyperglycemia [7], mitochondrial dysfunction [8], and endoplasmic reticulum (ER) stress [9] are among the many studied proapoptotic factors in RPE cell lines.

Protein folding, maturation, and trafficking; lipid synthesis; and intracellular calcium homeostasis are some of the major functions of the ER [10]. Endoplasmic reticulum stress results in the accumulation of unfolded proteins in the ER lumen leading to disturbed protein homeostasis. Cells activate an adaptive mechanism known as the unfolded protein response (UPR) to eliminate toxic protein components, which relieves ER stress and restores protein homeostasis [10]. However, extended periods of ER stress result in failed UPR and activate the apoptotic cascade [10]. Fundamental mechanisms resulting in the switch of the UPR from a prosurvival to a proapoptotic stimulus are still not clearly understood.

Changes in sphingolipid metabolism and accumulation of ceramide during ER stress have been demonstrated to induce apoptosis [11]. Ceramide can be generated through de novo synthesis,

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hydrolysis of sphingomyelin by sphingomyelinase (SMase), and breakdown of glycosphingolipids [12]. Among the different types of SMase enzymes that are present, acid and neutral SMase (N-SMase) have been investigated extensively in response to cellular stress. The endoplasmic reticulum stress response in insulinoma cells was shown to be associated with both increased message and protein levels of N-SMase [11], whereas, in contrast, ER stress was demonstrated to inhibit N-SMase activity in bovine aortic endothelial cells [13].

Localization of N-SMase in the sphingolipid-rich membrane fraction [12] constitutes the structural basis for the functional interaction between ceramide-generating enzymes and nitric oxide synthases (NOSs) [14]. Both inducible NOS (NOS2) and endothelial NOS (NOS3) proteins may interact with caveolin-3 or caveolin-1 proteins responsible for the localization of these enzymes at plasma membranes [15,16]. Indeed, change in N-SMase activity associated with ER stress has been reported to affect NOS3 and nitric oxide bioavailability [13]. Similarly, it was shown that ceramide production by N-SMase is a key mediator in the induction of NOS2 [17]. Here, we used a retinal epithelial cell model to test the hypothesis that N-SMase plays a role in modulating apoptosis, NOS2, and nitric oxide levels during ER stress.

Materials and methods

Cell culture and treatment conditions

The ARPE-19 cell line is a human RPE cell line and was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium:Ham's nutrient mixture, 1:1 (ATCC), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma–Aldrich, St. Louis, MO, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Wisent, St-Bruno, QC, Canada). Cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The ARPE-19 cells were passaged by trypsinization (0.05% trypsin/0.53 mM EDTA; Wisent) every 3 or 4 days. Cells from passages 10 to 15 were used in the studies.

The compound GW 4869 hydrate (Sigma–Aldrich) is a noncompetitive inhibitor of N-SMase and was prepared in dimethyl sulfoxide (DMSO; Calbiochem, EMD Bioscience, La Jolla, CA, USA) at a stock concentration of 346 μM. In most of the experiments cells were treated with 10 μg/ml tunicamycin (TM; Sigma–Aldrich) for 18 h to induce ER stress. The dose and duration of TM used to treat ARPE-19 cells were based on the results of earlier studies [18,13].

Measurement of sphingomyelinase activity

Acid and N-SMase activity was measured in equal number of cells via a sphingomyelinase assay kit (Molecular Probes, Cat. No. A12220, Eugene, OR, USA; Cayman Chemical, Cat. No. 10006964, Ann Arbor, MI, USA). This assay utilizes sphingomyelin as substrate to specifically monitor SMase activity. First, SMase hydrolyzes sphingomyelin to yield ceramide and phosphorylcholine. Alkaline phosphatase hydrolyzes phosphorylcholine to form choline. Choline oxidase oxidizes free choline to yield betaine and hydrogen peroxide (H_2O_2). Hydrogen peroxide, in the presence of horseradish peroxidase, reacts with 10-acetyl-3,7-dihydroxyphenoxazine in a 1:1 stoichiometry to generate the fluorescent molecule resorufin. The fluorescence intensity of resorufin (ex 530 nm; em 590 nm) was quantified using a fluorescence microplate reader (Synergy Mx, BioTek Instruments, Winooski, VT, USA). A standard curve of fluorescence values of known amounts of phosphorylcholine (Cayman Chemical) standards was generated. One unit of sphingomyelinase activity was defined as the amount of enzyme that caused the formation of 1 nmol of phosphorylcholine per minute at 37 °C. Sphingomyelinase activity in the samples (nmol/min/ml) were calculated from their corresponding fluorescence values via the standard curve.

Immunofluorescent staining

For immunofluorescence cell-imaging studies, cells were plated at a density of 100,000 cells/chamber in an eight-chamber slide (Lab-Tek II, Thermo Fisher Scientific, Roskilde, Denmark) 1 day before staining to achieve 70% confluency. Subsequently, cells were incubated with DMSO, 10 μg/ml TM, 6.92 μM GW 4869, or 10 μg/ml TM + 6.92 μM GW 4869 for 18 h. After treatment the cells were washed with phosphate-buffered saline (PBS), fixed in 4% freshly prepared formaldehyde for 5 min, and permeabilized in PBS with 0.1% Triton X-100 for 5 min at room temperature. The cells were then incubated for 1 h in blocking solution (3% bovine serum albumin/0.08% glycine in PBS) and treated with a rabbit polyclonal antibody against glucose-regulated protein 78 (GRP78; 1:100, ab53068, Abcam, Cambridge, MA, USA), the phosphorylated form of PKR-like ER kinase (pPERK; 1:50, Bioss, Woburn, MA, USA), C/EBP-homologous protein (CHOP/GADD 153; 1:50, sc-575, Santa Cruz Biotechnology, Dallas, TX, USA), nitrotyrosine (1:50, Cayman Chemical), NOS2 (1:50, sc-649, Santa Cruz Biotechnology), or nuclear factor κB (NF-κB) p65 (1:200, ab31481, Abcam) for 2 h at room temperature. The secondary antibody, Alexa Fluor-488-conjugated goat anti-rabbit (1:300, A11008, Life Technologies, Gaithersburg, MD, USA), was applied for 1 h at room temperature and nuclei were counterstained with DAPI (Vector Laboratories, Burlingame, CA, USA) in all experiments. Slides were viewed under a fluorescence microscope (Olympus BX61 fully automated, Tokyo, Japan) and fluorescence intensity was quantified using Metamorph software, version 7.1.0.0 (Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis

Proteins from ARPE-19 cells were denatured at 100 °C in sample buffer (161-0737, Bio-Rad Laboratories, Hercules, CA, USA) and separated on 12% Mini-Protean TGX precast electrophoresis gels (Bio-Rad). Resolved proteins were transferred to nitrocellulose membranes and incubated with rabbit polyclonal primary antibodies to the proteins of interest. Primary antibody incubations were for 1 h at room temperature with GRP78 (1:300, ab53068, Abcam), pPERK (1:200, Bioss), CHOP/GADD 153 (1:200, sc-575, Santa Cruz Biotechnology), NOS2 (1:200, sc-649, Santa Cruz Biotechnology), or actin (1:1000, AANO1, Cytoskeleton, Denver, CO, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution; Zymed Laboratories, San Francisco, CA, USA) was used as a secondary antibody, and immunoreactive proteins were visualized by chemiluminescence via ECL reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK). All Western blots were quantified by densitometric analysis using NIH ImageJ 1.44p software.

Measurement of nitrotyrosine levels

Nitrotyrosine content in ARPE-19 cells was measured via enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Cat. No. STA-305, Cell Biolabs, San Diego, CA, USA). Antigen captured by a solid-phase monoclonal antibody (nitrated keyhole limpet hemocyanin raised in mouse) was detected with a biotin-labeled goat polyclonal anti-nitrotyrosine. A streptavidin–peroxidase conjugate was then added to bind the biotinylated antibody. A TMB substrate was added and the yellow product was measured