Endoplasmic Reticulum Stress Pretreatment Alleviated Advanced Glycation End Products-induced Cytotoxicity

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Abstract. Purposes: Increased advanced glycation end products (AGEs) plays critical role in the exacerbation of periodontitis in diabetic patients. Though our previous findings confirmed the importance of endoplasmic reticulum stress (ERS) in diabetic-relative periodontitis, the regulating action of ERS on AGEs-induced apoptosis remains uncertain. Methods: human periodontal ligament cells (hPDLCs) were pretreated with ERS inducer tunicamycin or thapsigargin, followed by AGEs. Cell viability and expression of GRP78 were analyzed. Results: AGEs decreased cell viability. Tunicamycin or thapsigargin pretreatment inhibited AGEs-induced cytotoxicity and increased the expression of GRP78. Knockdown of GRP78 significantly blunts the protective effect of tunicamycin or thapsigargin. Conclusion: These data suggest that tunicamycin or thapsigargin suppresses AGEs-induced cytotoxicity by upregulation of GRP78.

Introduction

Periodontal disease has been recognized as an important dental public health problem which is largely responsible for tooth loss with reduced chewing function and impaired aesthetics (1). Periodontal ligament cells produce pro-inflammatory cytokines in response to a series of inflammatory stimuli such as lipopolysaccharide, microorganism, or nicotine, resulting in destruction of tooth supporting tissue and absorption of alveolar bone. Diabetes mellitus is also an important pathogenic factor of periodontitis according to the majority of studies and several systematic reviews (2). The risk of periodontitis is approximately 3-4 times higher in people with diabetes than in non-diabetic subjects (3). Under hyperglycaemic condition, advanced glycosylation end products (AGEs) excessively form in the periodontal tissues of diabetic patients and AGEs accumulation, rather than hyperglycemia, ultimately triggers the onset of diabetic periodontitis (4). AGEs can induce cells apoptosis through the upregulation of reactive oxygen species and activation of NF-κB in various cell types, including vascular smooth muscle cells (5), endothelial cells (6), and epithelial cells (7). Periodontal ligament cells treated with AGEs express higher levels of caspase-3, reduced cell viability and significantly increased apoptosis (8).

Endoplasmic reticulum is a multi-functional organelle which maintains lipid, protein and calcium homeostasis and it is also a well-known translational attenuator under stress. Endoplasmic reticulum-located molecular chaperone GRP78 is completely inactive when tightly bound to three endoplasmic reticulum intermembrane transducers under normal condition. But it will dissociate from transducers in response to stress and the dissociated transducers have the ability to initiate unfolded protein response and activate survival signal pathways. A great number of studies show that endoplasmic reticulum stress preconditioning provides protection against neurotoxins (9) or nephrotoxins (10) induced cytotoxicity. In the present study, we investigated the effect and mechanism of endoplasmic reticulum stress inducer pretreatment on AGEs induced cytotoxicity in human periodontal ligament cells. We hypothesized that the cytoprotection provided by endoplasmic reticulum stress preconditioning was a common property and would be effective in periodontal ligament cells.
Material and Methods

AGEs preparation: GEs was prepared according to methods described previously (11). Briefly, 50 mg/ml bovine serum albumin (Sigma Aldrich, St. Louis, MO) was incubated with 0.5 mol/L D-glucose and 0.1 mol/L phosphate-buffered saline (pH 7.4) at 37 °C for 10 weeks. After incubation, unbound sugar was removed by dialyzing against phosphate-buffered saline for 48 hours. To identify and estimate the concentration of prepared AGEs, the fluorescence intensity of AGEs and untreated phosphate-buffered saline was measured and compared using a fluorescence spectrophotometer at an excitation wavelength of 370 nm and emission wavelength of 440 nm. AGE showed 37.6 fluorescence U/mg proteins, about 26.8-fold fluorescence intensity of that of phosphate-buffered saline. This strongly indicated that AGE was prepared.

Cell culture: Human periodontal ligament cells were prepared as previously (11) and grown in Dulbecco’s modified Eagle’s medium (Gibco, Thermo Fisher Scientific) supplemented with 15% heat-inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific) and 100 μg/mL penicillin (Gibco, Thermo Fisher Scientific), as well as streptomycin (Gibco, Thermo Fisher Scientific).

Cell viability assay: Cells were seeded in 96-well plate at the density of 1x10⁴ cells/well. The next day, cells were exposed to different concentrations (25, 50, 100, 200 μg/ml) of AGEs. Cell viability was determined using MTT assay. To investigate the protective effect of endoplasmic reticulum stress inducers, cells were incubated with tunicamycin or thapsigargin at different concentrations for 2 h. Then, the media were changed and replaced with fresh media containing 200 μg/ml AGEs for 24 h.

Western blot analysis: The cells were rinsed with triethanolamine buffered saline solution for 2 or 3 times. The cell lysis supplemented with protease inhibitor phenylmethanesulfonyl fluoride was added to six-well plates to lyse cells. Then, the cells and lysis were harvested and transferred to the polyethylene tube using a cell scraper. After incubation on ice for 30 min and centrifugation, the supernatant was the extracted protein. The concentration of the protein was determined by BCA Protein Assay kit, Beyotime Technologies, China. The protein solution was electrophoresed and transferred to nitrocellulose membranes at 4 °C. The membranes were incubated with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA.) at 4 °C followed by horseradish peroxidase-conjugated secondary antibodies (KPL, Seracare, Gaithersburg, MD) at room temperature. The protein bands were detected by electrochemiluminescence colouration system and the intensities were quantified by appropriate software (AlphaEaseFC, Alpha Innotech, CA).

Small interfering RNA transfection: Human periodontal ligament cells were seeded at 2x10⁵ cells/ml in 6-well tissue culture plates and incubated for 24 h until the cells were 90% confluent. Diluted Lipofectamine 2000® Transfection Reagent (Invitrogen, Thermo Fisher Scientific) and GRP 78 small interfering RNA (Santa Cruz, USA) by Opti-MEM® Reduced Serum Medium (Invitrogen, Thermo Fisher Scientific) were prepared, mixed in equal proportions, and stood at room temperature for 20 min. Then, this complex was added to cells and incubated for 6 h. Finally, the culture medium was changed to fresh complete medium.

Statistical Analyses: Data was presented as means ± standard error of the mean. Differences were analysed using one way analysis of variance and Tukey tests were used following significant results with ANOVA. P<0.05 was considered statistically significant.

Results

200μg/mL AGEs induced cytotoxicity in hPDLCs: hPDLCs were incubated with 25, 50, 100, 200 μg/mL AGEs for 24 h, cell viability was tested by MTT assay. Results showed that as the concentration of AGEs increased, the cytotoxicity also increased. When concentration was 200 μg/mL, the cell viability was 54.38±3.16%. Thus, we chose 200 μg/mL as the concentrations of AGEs in a follow-up experiment.

Tunicamycin and thapsigargin pretreatment protects against AGEs-induced cytotoxicity: hPDLCs were incubated with 0.1, 0.25, 0.5, 1 μg/mL tunicamycin or 0.1, 0.25, 0.5, 1 μM thapsigargin for 2 h.
The results of MTT assay demonstrated minimal cell toxicity. We next analyzed the ability of tunicamycin and thapsigargin pretreatment to provide protection against AGEs. All two endoplasmic reticulum inducers caused significant cytoprotection against AGEs toxicity in hPDLCs. Tunicamycin showed much greater protection than thapsigargin.

**Endoplasmic reticulum stress inducers pretreatment** increased AGEs-induced **GRP78 expression**: The present study also determined whether GRP78 was involved in the protective effect of endoplasmic reticulum stress inducers on AGEs-induced cell damage. Western blot analysis demonstrated that exposure to AGEs upregulated GRP78 expression, suggesting that AGEs triggered endoplasmic reticulum stress. Tunicamycin and thapsigargin pretreatment strongly increased AGEs-enhanced expression of GRP78.

**Knockdown of GRP78 blunted the protective effect of tunicamycin and thapsigargin on AGEs-induced cell damage**: To explore whether GRP78 was the key factor of tunicamycin and thapsigargin on the protection of cell damage, specific GRP78 siRNA was applied to knockdown the GRP78 expression in hPDLCs cells. Confirmed by western blot assay, GRP78 was blocked under tunicamycin and thapsigargin stimulation (Fig 5A). GRP78 silence significantly enhanced cell damage triggered by AGEs (Fig 5B). The protective effect of tunicamycin and thapsigargin on AGEs-induced cell damage was also completely impaired by GRP78 knockdown (Fig 5C). These results clearly indicated that GRP78 was a negative regulator for AGEs-induced cell damage in hPDLCs cells and endoplasmic reticulum stress inducers pretreatment alleviated cytotoxicity triggered by AGEs via upregulation of GRP78.

**Discussion**

AGEs are non-enzymatically modified proteins that degrade difficultly and tend to accumulate in the tissue increasing the risk for subclinical inflammation. A narrative review discussing AGEs linkage to dental or periodontal diseases suggests that AGEs are responsible for initiating and/or aggravating oral pathology (12). Exposure to AGEs can lead to deformation and contraction of periodontal ligament fibroblasts, alteration in cellular permeability and function (8), generation of proinflammatory cytokines (13). AGEs have been shown to induce cell injury, cell death, or cell apoptosis, impair cell function through activation of endoplasmic reticulum stress (14). Intracellular AGEs accumulation increases the expression of GRP78 and induces co-localization with GRP78, contributing to modification of unfolded protein response-related protein and formation of high-molecular-weight complex which is involved in the occurrence and progression of pathology (15). Thus, alleviation of AGEs-induced endoplasmic reticulum stress may prove of therapeutic potential in the AGEs-triggered pathogenesis of general diseases. However, the exposure of cells to sublethal endoplasmic reticulum stress without impairing endoplasmic reticulum function and causing apoptotic events provides adaptation to subsequent more persistent and severe stress induced by lipopolysaccharide, inflammatory factors or toxic reagents. In cytotoxicity, endoplasmic reticulum stress preconditioning provides protection against 6-hydroxydopamine-induced neurotoxicity and clinically relevant drugs-induced nephrotoxicity. In inflammation, endoplasmic reticulum stress preconditioning almost completely suppresses TNF-stimulated NF-κB activation and adhesion molecular expression (16). Endoplasmic reticulum stress preconditioning also has been shown to exert inhibitory effects on inducible nitric oxide synthase stimulated by lipopolysaccharide (17, 18). Since the protective effects of endoplasmic reticulum stress preconditioning were investigated in many cell lines, we attempted to detect whether or not the effects observed reflect a general property of endoplasmic reticulum inducers and whether the similar response would be happened in AGEs-damaged hPDLCs. In this study, we chose two known endoplasmic reticulum stress inducers, tunicamycin and thapsigargin, for their different action mechanisms. Tunicamycin prevents protein maturation at post-translational level through N-linked glycosylation inhibition, while thapsigargin disrupts calcium homeostasis through inhibiting calcium ATPase. The first step of the study is to observe the response of PDLCs to the different endoplasmic reticulum stress inducers and AGEs. After 2 h exposure, both tunicamycin and thapsigargin displayed much little toxicity at those tested concentrations, while AGEs at
concentrations of 200 μg/ml exhibited cytotoxicity. The susceptibility of the cells to the cytotoxicity of the inducers was significantly different. At the highest concentration, thapsigargin had less cytotoxicity than tunicamycin. There was a dynamic balance between activation of endoplasmic reticulum stress survival response and initiation of endoplasmic reticulum-associated cell apoptosis. Survival pathway will support cytoprotective effects while apoptosis pathway will promote cell death. To assess the protection of endoplasmic reticulum stress inducers pretreatment against AGEs-triggered cell damage, PDLCs were exposed to inducers for 2 h followed by 200 μg/ml AGEs for 24 h. All two inducers resulted in significant cytoprotection against AGEs toxicity in hPDLCs. Protection was much greater with thapsigargin than with tunicamycin. These data demonstrate that the susceptibility of PDLCs to the inducers is different and the cytoprotective ability of endoplasmic reticulum stress preconditioning in hPDLCs is similar to other cell lines. GRP78, localized in the endoplasmic reticulum, plays a key role in the cellular defense system in response to endoplasmic reticulum stress-induced accumulation of unfolded proteins. The data of previous experiments demonstrated that the induction of GRP78 was closely related to the cytoprotective effects of endoplasmic reticulum stress preconditioning. Thus, we analyzed the difference of GRP78 expression between AGEs group and inducers preconditioning group. As predicted, tunicamycin or thapsigargin preconditioning promoted GRP78 protein accumulation in hPDLCs. This result indicated that 2 h preconditioning with tunicamycin or thapsigargin was sufficient to initiate endoplasmic reticulum stress survival pathway to provide cytoprotective effect through up-regulation of GRP78. To confirm above presumption, GRP78 knockdown was applied and the number of viable cells was measured. Our data displayed that GRP78 knockdown not only decreased the viability of hPDLCs but also dramatically weakened the protective effect of endoplasmic reticulum stress preconditioning on AGES-induced cell damage. Our findings are consistent with previous reports, which show that induction of GRP78 by “preconditioned” cells protects them against cell injury. Although we tested AGES-induced cytotoxicity in this study, it is possible that tunicamycin or thapsigargin might protect cells against other periodontitis inducer such as lipopolysaccharide, Porphyromonas gingivalis, or nicotine et al. Nevertheless, further investigations are needed to clarify it. In summary, tunicamycin or thapsigargin preconditioning exerts protective effect on AGES-induced cell damage. Our research findings provide new insights in understanding the role of endoplasmic reticulum stress on AGES-induced cytotoxicity.

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References


