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Periodontal disease level-butyric acid amounts locally administered in the rat gingival mucosa induce ER stress in the systemic blood

Marni E. Cueno**, Yuko Saito, Kuniyasu Ochiai*

Department of Microbiology, Nihon University School of Dentistry, Tokyo 101-8310, Japan

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ABSTRACT

Periodontal diseases have long been postulated to contribute to systemic diseases and, likewise, it has been proposed that periodontal disease treatment may ameliorate certain systemic diseases. Short-chain fatty acids (SCFA) are major secondary metabolites produced by oral anaerobic bacteria and, among the SCFAs, butyric acid (BA) in high amounts contribute to periodontal disease development. Periodontal disease level-butyric acid (PDL-BA) is found among patients suffering from periodontal disease and has previously shown to induce oxidative stress, whereas, oxidative stress is correlated to endoplasmic reticulum (ER) stress. This would imply that PDL-BA may likewise stimulate ER stress, however, this was never elucidated. A better understanding of the correlation between PDL-BA and systemic ER stress stimulation could shed light on the possible systemic effects of PDL-BA-related periodontal diseases. Here, PDL-BA was injected into the gingival mucosa and the systemic blood obtained from the rat jugular was collected at 0, 15, 60, and 180 min post-injection. Collected blood samples were purified and only the blood cytosol was used throughout this study. Subsequently, we measured blood cytosolic GADD153, Ca^{2+}, representative apoptotic and inflammatory caspases, and NF-κB amounts. We found that PDL-BA presence increased blood cytosolic GADD153 and Ca^{2+} amounts. Moreover, we observed that blood cytosolic caspases and NF-κB were activated only at 60 and 180 min post-injection in the rat gingival mucosa. This suggests that PDL-BA administered through the gingival mucosa may influence the systemic blood via ER stress stimulation and, moreover, prolonged PDL-BA retention in the gingival mucosa may play a significant role in ER stress-related caspase and NF-κB activation. In a periodontal disease scenario, we propose that PDL-BA-related ER stress stimulation leading to the simultaneous activation of apoptosis and inflammation may contribute to periodontal disease pathogenesis.

1. Introduction

Endoplasmic reticulum (ER) is an important organelle found in eukaryotic cells and is mainly subdivided into: (1) rough ER which functions in protein synthesis and secretion; and (2) smooth ER which is central to fatty acid and phospholipid synthesis, lipid bilayer assembly, carbohydrate metabolism, and regulation of homeostasis [1]. However, when ER function is impaired by either physiological or pathological conditions, this then results to ER stress [1,2] which in turn has been correlated to several systemic diseases [3,4].

Periodontal diseases, such as gingivitis and periodontitis, have been suggested to affect overall health and well-being [3,4]. Short-chain fatty acids (SCFA), are major secondary metabolites produced by oral anaerobic bacteria, like *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, and are detected in high concentrations in the saliva of patients with severe periodontal disease [5]. Among the SCFAs, butyric acid (BA) in high amounts contribute to periodontal disease development [6]. Our previous works showed that periodontal disease level-BA (PDL-BA) amounts cause oxidative stress and, consequentially, neurite retraction in NGF-treated rat pheochromocytoma PC12 cells [7] while prolonged retention of PDL-BA amounts within the gingival tissue can induce both blood mitochondrial and cytosolic oxidative stresses [8,9]. This would imply that PDL-BA presence is associated with oxidative stress induction.

Oxidative stress represents an imbalance between excessive

* Corresponding author. Department of Microbiology, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan.
** Corresponding author. Department of Microbiology, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-8310, Japan.
E-mail addresses: marni.cueno@nihon-u.ac.jp (M.E. Cueno), ochiai.kuniyasu@nihon-u.ac.jp (K. Ochiai).

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reactive oxygen species and low antioxidant activity [10] and, likewise, has been correlated to ER stress [2]. This suggests that PDL-BA-related oxidative stress may likewise contribute to ER stress stimulation, however, this was never fully elucidated. A better understanding of the correlation between PDL-BA and systemic ER stress stimulation may shed light on the possible systemic effects of PDL-BA-related periodontal diseases.

Here, we determined whether PDL-BA presence in the gingival mucosa can induce ER stress in the systemic blood. We found that PDL-BA stimulates ER stress and Ca^{2+} levels in the rat blood cytosol. Moreover, we established that blood cytosolic caspases and NF-κB are simultaneously activated. We attributed these results to PDL-BA-induced ER stress in the rat blood cytosol.

2. Materials and methods

2.1. Animal handling and PDL-BA treatment

Handling and treatment of rats were made according to previously published works [8,9]. Briefly, male Wistar rats (10 weeks old, Japan SLC, Shizuoka, Japan) were individually housed in stainless steel cages with wire-mesh bottoms and, subsequently, placed in a room under controlled temperature (23–25 °C), relative humidity (40–60%), and lighting (12 h). Rats had free access to water and a semi-purified diet based on the AIN93G formulation for an acclimation period of 7 days. All rats were handled in accordance with the guidelines for animal studies of the Kyoto Institute of Nutrition and Pathology. Six acclimated rats were implanted with jugular canulae under sodium pentobarbital anesthesia (40 mg kg^{-1} body weight) and 10 μL of ^{13}C *n*-butyrate solution (1 M) was injected in several batches into the gingival mucosa until obtaining the same PDL-BA levels found among patients suffering periodontitis (5 mM) [11]. Blood BA concentration was verified through LC-MS as previously described [8,9]. Jugular blood was collected prior to BA-injection (0 min) to serve as a negative control. Subsequently, jugular blood was collected 15, 60, and 180 min after BA-injection for use in further downstream analyses. These collection times were selected since these times coincide with our earlier works on rat blood mitochondrial and cytosolic oxidative stresses [8,9]. Similarly, to serve as a control for gingival injection, 1 M PBS (phosphate buffer saline) was injected in 10 μL-batches into the gingival mucosa until reaching the same PDL-BA concentration (5 mM) as earlier described and blood was collected 180 min after PBS-injection.

2.2. Sample preparation and purity

Blood cytosol isolation and purity were determined as previously described [9]. Briefly, Cytosol/Particulate Rapid Separation Kit (BioVision Inc., California, USA) was used to isolate blood cytosol while Pierce® Detergent Removal Spin Columns (Thermo Scientific, California, USA) was used to purify samples from detergents. Pierce® Microplate BCA Protein Assay Kit-Reducing Agent Compatible Kit (Thermo Scientific) was used to standardize the protein concentration. All kits were used according to manufacturer's recommendation.

Western blotting was performed to establish blood cytosol purity. Blood cytosolic proteins were separated by SDS-PAGE and transferred to Hybond-C nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). Membranes were subsequently blocked with Difco™ Skim Milk (BD Company, New Jersey, USA), probed with antibodies, and immunoreactive proteins were visualized using SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Illinois, USA). Anti-GAPDH (GeneTex Inc, Texas, USA) was used to detect the glyceraldehydes-3-phosphate in the blood cytosol to serve as control. Anti-HSP60 (StressMarq Biosciences Inc., British Columbia, Canada) was used to determine cytosolic heat-shock protein 60 in the blood cytosol extract to verify the purity of the cytosolic samples. Anti-MTC02 (Novus Biologicals, Colorado, USA) is a mitochondria-specific antibody used to confirm the absence of mitochondrial components. Anti-PCNA (Thermo Scientific) is used to detect the proliferating cell nuclear antigen and we used this antibody to verify the absence of nuclear proteins. Anti-PMCA (Thermo Scientific) is an antibody specific for the plasma membrane calcium ATPase which we used to validate the absence of plasma membrane proteins. Anti-GRP78 (GeneTex Inc.) is an ER-specific antibody that detects the glucose-regulated protein 78 and functions in ER stress response. We utilized this antibody for confirming ER absence in the processed sample.

2.3. Detection of ER stress and calcium levels

In order to determine ER stress induction, GADD153 levels in the blood cytosol were quantified through ELISA. Briefly, antigen coating (at 1 μg mL^{-1} concentration) on polystyrene plates was done using sodium bicarbonate-sodium carbonate buffer (Polysciences, Inc., Taipei, Taiwan). Blocking (24 h) was performed using PBS with 1% BSA blocking buffer (GeneTex Inc.). GADD153 protein (GeneTex Inc.) was used to establish a protein standard while HRP-conjugated GADD153/CHOP antibody (Novus Biologicals) was utilized to detect GADD153 in the blood cytosol. SIGMA*FAST*™ OPD tablets (Sigma–Aldrich Co., Missouri, USA) were used as substrate for peroxidase detection. Washing in-between steps was done using the PBS/Tween® Solution (AppliChem GmbH, Darmstadt, Germany). Hydrochloric acid (1.0 M) was used as stop solution. ELISA measurements were done in Abs 450 nm. Subsequently, Calcium Colorimetric Assay Kit (BioVision Inc.) was used to quantify blood cytosolic calcium amounts. All kits were used according to manufacturer's recommendation.

2.4. Quantification of caspase activities and NF-κB amounts

Activation of representative apoptotic caspases [caspase-9 (CASP9), caspase-8 (CASP8), and caspase-3 (CASP3)] were established using the Caspase-9 Colorimetric Assay Kit, FLICE/Caspase-8 Colorimetric Assay Kit, and Caspase-3/CPP32 Colorimetric Assay Kit, respectively. Activation of representative inflammatory caspases [caspase-12 (CASP12), caspase-4/11 (CASP4/11), and caspase-1 (CASP1)] were determined using the Caspase-12 Fluorometric Assay Kit, Caspase-4 Colorimetric Assay Kit, and Caspase-1/ICE Colorimetric Assay Kit, respectively. All kits were purchased from BioVision Inc. and performed according to manufacturer's recommendation. Measurements of blood cytosolic NF-κB amounts were performed using NF-κB/p65 ActiveELISA™ Kit (Novus Biologicals) according to manufacturer's recommendation.

2.5. Statistical analyses

Statistical analysis was performed in all assays made throughout the study. The statistical significance of differences was determined by Student's *t* test (two-tail). A significance level of 95% ($P < 0.05$) was considered statistically significant.

3. Results

3.1. PDL-BA in the gingival mucosa increases GADD153 and Ca^{2+} amounts in the systemic blood

To establish whether PDL-BA induce ER stress in the rat blood cytosol, both blood cytosolic GADD153 and Ca^{2+} levels were