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Docosahexaenoic acid attenuates oxidative stress and protects human gingival fibroblasts against cytotoxicity induced by hydrogen peroxide and butyric acid

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ABSTRACT

Objective: The oxidative burst of the host cells associated with bacterial pathogen infection contributes to the destruction of periodontal tissue. The present study investigates the effect of docosahexaenoic acid (DHA) on human gingival fibroblast (HGF) viability and ROS generation.
Methods: The cell viability by MTT assay, ROS level using H_2DCF-DA probe, and protein thiol content were measured in HGFs after 24 h preincubation with different concentrations of DHA followed by treatment with H_2O_2. The cell death rate was determined by Annexin V/propidium iodide staining, and mitochondrial membrane potential ($\Delta\Psi m$) was examined by MitoTracker Red probe in H_2O_2- and butyric acid-treated HGFs. The fatty acid composition of plasma membranes after incubation with DHA was determined by gas chromatography mass spectrometry.
Results: DHA preincubation in a dose-dependent manner increased the viability of HGFs exposed to H_2O_2 and decreased ROS generation compared to the control cells. In HGFs preincubated with 30 μM DHA, the $\Delta\Psi m$ significantly increased in both H_2O_2- and butyric acid-treated cells. Moreover, incubation with DHA preserved the protein thiol level as effectively as N-acetylcysteine. Application of 50 μM DHA increased the quantity of viable cells, decreased the number of necrotic cells after H_2O_2 treatment, and protected HGFs from apoptosis induced by butyric acid. DHA in the plasma membranes of these HGFs represented about 6% of the total amount of fatty acids.
Conclusions: These results demonstrate that enrichment of HGFs with DHA reduces ROS generation and enhances the mitochondrial membrane potential protecting the fibroblasts against cytotoxic factors.

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Abbreviations: DHA, docosahexaenoic acid; OL, oleic acid; PA, palmitic acid; BA, butyric acid; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; HGF, human gingival fibroblast; $\Delta\Psi m$, mitochondrial membrane potential; MTT, 3[4,5-dimethylothiazol-2-yl]-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species.

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1. Introduction

Destructive periodontal disease is multifactorial in its aetiology. Active disease results from a combination of host susceptibility and dental plaque bacteria. Periodontopathogens injure tissue directly, through harmful toxic products that induce cell death and tissue necrosis, and indirectly, through the activation of inflammatory cells which interact with the phagocytic and gum tissue cells.[1] During the course of inflammation in response to pro-inflammatory cytokines and mediators, the gingival fibroblasts produce IL-1, IL-6, IL-8, TNF-α and TGF-β.[2–4] These cytokines recruit polymorphonuclear leukocytes to the site of infection[5] which produce proteolytic enzymes and reactive oxygen species (ROS) via the oxidative burst, catalyzed by NADPH oxidase.[6] Oxidative DNA damage in fibroblasts and increased hydrogen peroxide production in polymorphonuclear leukocytes have been reported in induced periodontitis in animal studies.[7,8] Clinical studies in patients with periodontitis demonstrated elevated levels of oxidative stress markers in saliva, serum and gingival crevicular fluid.[9,10] Moreover, positive correlations were observed between periodontal parameters and lipid peroxidation in crevicular fluid and saliva.[11,12] Overwhelming evidence indicates that oxidative stress play a central role in the periodontal tissue and alveolar bone destruction.[13–15]

Docosahexaenoic acid (DHA, C22:6), n-3 unsaturated fatty acid (n-3 PUFA), has potent anti-inflammatory effects. It has been demonstrated that exposure of endothelial cells to DHA reduced expression of COX-2 and NADPH oxidase 4, blocked nuclear p65 NF-κB subunit translocation into the nucleus, and diminished IL-1-stimulated ROS generation.[16,17] DHA has also been reported to have a dose-dependent effect on GSH content and antioxidant enzyme activity.[18] The action of DHA in the counter-regulation of inflammation seems to be complex. DHA, being highly unsaturated, can be converted by various lipoxygenases to bioactive di- and trihydroxy derivatives, resolvins and protectins, which promote resolution of inflammation.[19] When added to the cell culture medium and incorporated into membrane phospholipids,[18,20] DHA may compete with arachidonic acid and attenuate chronic inflammatory diseases by reducing pro-inflammatory mediator production.[21]

Although DHA has only been reported to be present in significant amounts in the retina and neurons,[22] dietary supplementation with fish oil caused a rapid incorporation of n-3 PUFA into neutrophils and the epidermis.[23] Furthermore, a cross-sectional study of the U.S. adult population suffering from periodontitis demonstrated that increased dietary DHA was associated with a lower prevalence of periodontitis.[24] Therefore, an assessment of the role of DHA in the protection of gingival fibroblasts against cytotoxic factors was conducted.

Hydrogen peroxide (H_2O_2) and butyric acid were chosen as the cytotoxic compounds. Physiologically, hydrogen peroxide is produced in abundance by NADPH oxidase in stimulated neutrophils, and can locally reach micromolar concentrations.[25] Some toothpastes and mouth rinses contain low concentrations of hydrogen peroxide as a disinfectant to prevent plaque and inflammation of the gums. However, hydrogen peroxide at higher concentrations is a whitening ingredient in many home-use and in-office use tooth whitening products.[26–28] Hydrogen peroxide is harmful to cellular proteins, and in the presence of transition-metal ions[29] is a source of more reactive hydroxyl free radicals ($^{\bullet}OH$), which strengthen the harmful effect of oxidants.[30,31] Butyric acid, the local metabolite of pathogenic periodontal bacteria can induce caspase-dependent apoptosis in gingival fibroblasts.[32] Here, we report that enrichment of HGFs with DHA attenuates oxidative stress and protects the cells from death induced by both cytotoxic compounds.

2. Materials and methods

2.1. *Reagents*

Dulbecco's modified Eagle's medium (DMEM), DMEM without phenol red, fetal bovine serum (FBS), penicillin/streptomycin, phosphate buffered saline (PBS), and trypsin/EDTA were purchased from Biochrom AG (Deutschland); ProLong Gold antifade reagent with DAPI, MitoTracker Red CMXRos, DTNB (5,5′-dithio-bis-(2-nitrobenzoic acid), L-cysteine, and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) were obtained from Invitrogen (Poland); M-PER mammalian protein extraction reagent was from Pierce (Rockford, IL); Annexin V Binding Buffer, Annexin V-FITC were purchased from BD Biosciences (Poland); Hydrogen peroxide (30%) came from Chempur (Poland); 3-[4,5-dimethylothiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), formaldehyde, N-acetyl-L-cysteine (NAC), propidium iodide (PI), dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), palmitic acid (16: 0), oleic acid (18: 1n-9) and docosahexaenoic acid (22: 6n-3) were obtained from Sigma Aldrich (Poland). Sensitive to oxidation DHA was dissolved in ethanol to make 100 mM stock solution, and stored at $-80\,^{\circ}$C in a nitrogen atmosphere. The stock solutions were diluted to reach a final concentration immediately before use.

2.2. *Cell culture*

Primary human fibroblasts were obtained from gingival tissue of healthy patients undergoing extraction of the third molar after their informed consent. The protocol of the study was approved by Ethics Committee of the Medical University of Lodz. The gingival fragments were cut into small pieces (2 mm $\times$ 2 mm) and rinsed several times in PBS with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml and amphotericin 2.5 mg/ml). The tissue was then cultured in DMEM supplemented with 10% FBS, and 1% penicillin–streptomycin solution in Petri dishes. The growth medium was changed every two to three days. When the cells surrounding the tissue explants had formed a confluent monolayer, they were washed with PBS w/o Ca^{2+} and Mg^{2+}, and detached using 0.25% trypsin – EDTA (0.02% in PBS). The cells were collected by centrifugation at 200 $\times$ g for 5 min and the pellet was dispersed in DMEM with 10% FBS. Viable cells were counted using trypan blue dye, transferred into 25-cm^2 or 75-cm^2 cell culture flasks and cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 $^{\circ}$C. Cells from passages 3–7 were used for all of the experiments.

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