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Red alga polysaccharides attenuate angiotensin II-induced inflammation in coronary endothelial cells

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ABSTRACT

The pro-inflammatory vasoconstrictor Angiotensin II can cause endothelial dysfunction and is considered to be one of the mediators of atherosclerosis. Our former results demonstrated that polysaccharides derived from the red alga *Porphyridium* sp. attenuate inflammatory processes by interfering with tumor necrosis factor-alpha-induced inflammation, in human coronary artery endothelial cells. However, the anti-inflammatory effect of these polysaccharides on inflammation processes occurring under Angiotensin II stimulation is yet unknown.

Herein, we studied the polysaccharide's anti-inflammatory effect by quantification of inflammatory markers in Angiotensin II- stimulated Human Coronary Artery Endothelial Cells following pre-treatment with polysaccharides. Inflammatory atherosclerotic pathways up-regulated by Angiotensin II, including adhesion molecule expression and nuclear factor kappa-light-chain-enhancer of activated B cells translocation, were significantly attenuated or diminished in cells pre-treated with the polysaccharides. In addition, the polysaccharides increased the antioxidant response elements activity through the nuclear factor-E2-related factor 2- antioxidant protection system.

These polysaccharide's promising abilities may be considered as a basis for future use as a therapeutic agent aimed at improving vascular health by attenuation of the inflammatory atherosclerotic process.

1. Introduction

The endothelium plays a prominent role in the regulation of vascular permeability, tone, thrombogenesis, and inflammatory processes [1]. Disturbance of the healthy endothelial activity may cause vascular endothelial dysfunction, which is known to be one of the hallmarks of hypertension and atherosclerosis pathogenesis. Such a disturbance can be characterized by impaired nitric oxide (NO) bioavailability and increased concentration of endogenous vasoconstrictors such as angiotensin II (Ag-II) and Endothelin-1 (ET-1). Ag-II is a peptide hormone, which is part of the renin-angiotensin system (RAS); it is a prominent pro-inflammatory mediator that increases inflammation and oxidative stress in the endothelium, resulting in endothelial dysfunction [2–4]. One of the inflammatory processes within the endothelial milieu in which Ag-II is involved in, is the nuclear factor kappa B (NF-κB) pathway. Ag-II stimulates the degradation of nuclear factor inhibitor (IκB) and therefore increases NF-κB activation in the nucleus, resulting in the expression of leukocyte adhesion molecules-vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), which contribute to inflammation aggravation [3–6]. Thus, potential inhibitors of the Ag-II signaling pathway, may lead to attenuation of oxidative stress and inflammation, and thereby improve endothelial function [7].

Several studies have reported on the beneficial anti-inflammatory effect of algae polysaccharides in various inflammation models [8–16]. However, there is only limited knowledge regarding the polysacharide's mode of action in endothelial inflammatory processes [15,16]. Recently we demonstrated that the

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polysaccharides derived from the red microalga *Porphyridium* sp. (PSs) act as anti-inflammatory agents in TNF-α- induced coronary endothelial cells, which were chosen as a model for dysfunctional endothelium [16]. The PS's composition, structure and physico-chemical nature have been studied for several years; They are composed mainly of Glucose, Galactose and Xylose, and are characterized by their negative charge due to sulphate and glucuronic acid residues [18]. They contain a disaccharide building block of aldobiouronic acid 3-O-(α-*d*-glucopyranosyluronic acid)-l-galactopyranose [17,18] and are stable (their viscosity remains unchanged) under a wide range of conditions: temperatures (30–160 °C), pH values (2–9), and salinities [17,18]. In addition, the PSs have been shown to have a variety of bioactivities such as antiviral, anticoagulant, antioxidant and anti-inflammatory effects [18]. Nowadays, the PSs have become a valuable ingredient in various cosmetic agents as an anti-aging supplement [17,18]. In accordance with the PSs' anti-inflammatory properties, particularly in the way they confer endothelial function protection under TNF-α induction, we investigated whether PSs can protect human coronary artery cells (HCAECs) from the pro-inflammatory effects of Ag-II, and their involvement in the NF-κB pathway.

2. Materials and methods

2.1. Polysaccharide preparation

Porphyridium sp. polysaccharide (0.9% w/v) was a gift for research purposes from Frutarom Ltd. (Beer-Sheva, Israel). The sample was further dialyzed (MW cutoff 8000) against DDW at 4 ^{O}C, until the conductivity of the water reached 300 μS. The dialyzed polysaccharides were then freeze-dried and re-dissolved in DDW to reach 0.5% w/v, comprising the PS stock. The PS stock did not contain any detectable amounts of lipopolysaccharide (as determined by Limulus assay).

2.2. Cell culture

Human coronary artery endothelial cells (HCAECs, PromoCell GmbH, Sickingen str., Heidelberg, Germany) were maintained until passage 5 in DMEM supplemented with 10% bovine calf serum (BCS; HyClone, South Logan, UT, USA), 2 mmol/L L-glutamine, 100 U/mL penicillin,0.1 mg/mL streptomycin, 12.5 mg/mL nystatin, 2 mmol/L HEPES buffer (Sigma, St. Louis, Missouri, USA). Cells were maintained sub confluent at 37 ^{O}C in humidified air containing 5% CO_2 and harvested by treatment with trypsin.

2.3. Transient transfection and reporter gene assay

HCAECs were seeded in 24-well microtiter-plates ($2 \cdot 10^4$ cells/well) in DMEM culture medium supplemented with 3% w/v BSA and were transfected with 50 mL Jet-PeI™ medium (Polyplus transfection, Illkrich Cedex,France) containing 0.2 mg/well of reporter plasmid NF-κB -luciferase or reporter plasmid of antioxidant reactive response element(ARE)-luciferase with 0.05 mg/well of Renilla-luciferase expression vector (Dual-Luciferase Reporter Assay System, Promega, Madison,WI, USA) overnight. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega), as described previously [19].

2.4. Cell treatments

The various measured parameters were followed under three different experimental settings: 1) *basal*: cells that were cultured without Ag-II and PSs; 2) *vehicle:* cells that were cultured without PSs and were stimulated by Ag-II for 6 h prior to harvesting; and 3) *PSs and Ag-II treatments*: cells that were pre-treated with PSs for 18 h at various concentrations (50–500 μg/mL) and then stimulated with Ag-II for 6 h prior to harvesting; 4) *PS-500*: cells that were treated with PSs at 500 μg/mL for the entire 24 h. HCAECs at 75% confluence were incubated in 24-well plates for the transfection experiements (2×10^4 cells/mL) and in 6-well plates for Western blotting experiements (2×10^6 cells/mL) over 24 h in DMEM supplemented-medium. Where Ag-II was needed, 10 μmol/L Ag-II (R&D Systems, Minneapolis, MN, USA) was applied. The various parameters measured in each treatment (following the 24 h of each treatment period) were compared to the *vehicle* and to the *basal* treatments. Each treatment was designated in accordance with the PS concentration, e.g., PS-50 means PS pre-treatment of 50 μg/mL.

2.5. Cell lysate preparation and western blotting

At the end of each experiment (following the 24 h treatment), different fractions were prepared. Briefly, the cells were washed twice with cold PBS and then the various fractions were prepared using the appropriate buffer/reagents. The total cell lysate, nuclear and cytosolic fractions were prepared using mammalian cell lysis buffer, and NE-PER nuclear and cytoplasmic extraction reagents, respectively, following the manufacturer's suggested protocols (Pierce Rockford, IL, USA). The total protein content in each fraction was quantified by Pierce™ BCA Protein Assay Kit (Pierce Rockford, IL, USA). In accordance with the protein concentration, each well in the SDS page was loaded to reach 30 μg protein extract dissolved in sample buffer and run on 7.5% SDS-PAGE. Following SDS-PAGE, electroblotting and blocking, nitrocellulose membranes were incubated with appropriate primary antibodies. Then, the blots were washed and exposed to horseradish peroxidase (HRP)-conjugated secondary antibody. The protein and immune-reactive bands were visualized with enhanced chemiluminesence (Amersham ECL Western Blotting Detection Kit). Quantitation of protein band optical densities was performed by densitometry analysis using ImageGauge software (Ver. 4.0, Fuji Photo Film, Tokyo, Japan). The Western immunoblotting was normalized to β-actin for the total cell lysate/cytosolic fractions and to lamin for the nuclear fraction. The antibodies for human ICAM-1 (sc-7891), VCAM-1 (sc-13610), IkB (C-21), NF-κB P-65 (sc-372), superoxide dismutase (SOD) (sc-17767), Nrf-2 (sc-365949), and β-actin (sc-8432) and lamin (sc-6215) primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-mouse IgG-HRP, and rabbit anti-goat IgG-HRP were obtained from Jackson Immunoresearch (West Grove, PA, USA).

2.6. Fold effect calculation

The fold effect, designated as fold of *basal*, was calculated as the ratio between the measured parameter level under treatment in relation to that in the *basal*.

2.7. Statistical analysis

All experiments were repeated four times. The data are expressed as the mean ± SEM. The significance of the differences between the means of various subgroups was assessed by unpaired two-tailed Student's *t*-test. The statistical analysis was performed with Graph-Pad Prism 7.02 Software (San Diego, CA). $P < 0.05$ was considered statistically significant.

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