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Original article

Resolvin D1 down-regulates CYP1A1 and PTGS2 gene in the HUVEC cells treated with benzo(a)pyrene

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ABSTRACT

Background: Polycyclic aromatic hydrocarbons (PAHs) can interact with lipids and their derivatives and have been known to induce atherosclerosis. The aim of this study was to evaluate the impact of Resolvin D1 (RvD1) on inflammatory-state realted proteins and genes in the human primary umbilical vein endothelial HUVEC cells exposed to benzo(a)pyrene (BaP).
Methods: We analyzed the influence of RvD1 and/or BaP on cyclooxygenase-2 (COX-2), cytosolic prostaglandine E2 synthase (cPGES), glutathione S transferase (GSTM1) and aryl hydrocarbon receptor (AhR) protein expression by Western blot. Additionaly, phospholipase A2 (cPLA2) and cytochrome P450 (CYP1A1) activity, as well as AhR, CYP1A1, phospholipase A2 (PLA2G4A) and prostaglandin synthase 2 (PTGS2) gene expression by qRT-PCR was studied.
Results: RvD1 down-regulates cytochrome P450 (CYP1A1) and prostaglandin synthase 2 (PTGS2) gene expression in HUVEC cells exposed to BaP. Repressesion of COX-2, cPGES and overexpressesion of GSTM1 protein was noted after co-treatment with RvD1 and BaP. After incubation with RvD1 an increase of cPLA2 and a decrease of CYP1A1 activity was observed when compared to BaP treated alone endothelial cells.
Conclusions: Our data suggests that RvD1 can significantly contributes on vascular function and alleviates the harmful effects caused by BaP, which might potentially aid in the repair of the injured endothelium.
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Introduction

Environmental contaminants exposure has been implicated in the etiology of atherosclerosis [1,2]. Polycyclic aromatic hydrocarbons (PAHs) are lipophilic pollutants widespread in the air, soil and grilled food. Benzo(a)pyrene (BaP) is a principle indicator of carcinogenic PAHs. The carcinogenic effects of PAHs has been well studied, while the mechanisms involving inflammatory and atherogenic PAHs capacities is still lacking [3,4].

The endothelium is a first line of defence between the vascular wall and circulating harmful substances and their metabolities. Endothelial cells are highly sensitive toward environmental stress injury induced by dioxins, PCBs and PAHs as aryl hydrocarbon

Abbreviations: AhR, aryl hydrocarbon receptor; BaP, benzo(a)pyrene; Bbf, benzo(b)fluoranthene; Bkf, benzo(k)fluoranthene; COX-2, cyclooxygenase-2; cPGES, cytosolic prostaglandin E2 synthase; CYP1A1, cytochrome P450 1 A1; DHA, docosahexaenoic acid; FA, fatty acids; GSTM1, gluthathione S transferase Mu1; NF-κB, nuclear factor κB; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PAHs, polycyclic aromatic hydrocarbons; PLA2G4A, phospholipase A2 IV; PPARs, peroxisome proliferator activated receptors; PTGS2, prostaglandin synthase 2; Pyr, pyrene; RvD1, Resolvin D1.

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receptor (AhR) agonists [3,5]. This effect is connected with oxidative stress and associated with activation of AhR-mediated cytochrome P450 CYP1A1 enzyme as well as nuclear factor NF-κB signaling [1–5].

Environmental stresses, genetic factors, diet and lifestyle can accentuate atheroma formation [6]. Endothelial cells dysfunction, early and chronic inflammation within the subendothelial compartment is a crucial factor in the initiation and progression of cardiovascular diseases such as atherosclerosis [6,7]. Cellular activation leads to signaling through transcription factors, which is involved with an increase of cytokines and adhesion molecules expression and promotion of the pro-inflammatory state [7].

Atherosclerosis is a chronic inflammatory disease with failed resolution of inflammation [6,7]. The resolution of inflammation is an active process in that participate lipid mediators [8]. Docosahexaenoic acid (DHA), a polyunsaturated $n-3$ fatty acid, has numerous beneficial actions in the cardiovascular system. DHA reduces adhesion molecules (VCAM-1 and ICAM-1) expression which are induced by pro-inflammatory factors. DHA also regulates leukocyte – endothelial interactions [8,9]. Hypoxic endothelial cells treated with aspirin convert DHA to 17R-hydroxydocosahexaenoic acid which is transformed into D-series resolvins by leukocytes [9]. At the physiological state, 7(S),8(R),17(S)-trihydroxy-DHA (Resolvin D1, RvD1) is a product of transcellular biosynthesis with leukocytes and endothelial cells, from the enzymatic, sequential oxygenation of DHA by 15- and 5-lipoxygenase [10–12]. Resolvins possess potent anti-inflammatory and immunoregulatory actions, the mechanisms of actions involve repression of NF-κB and AP-1 transcription factors as well as activation of PPARs signaling pathways [1,13–16].

In our previous study we observed in HUVEC cells incubated with DHA and activated with PAHs down-regulation of AhR, CYP1A1 and up-regulation of PTGS gene, which may be due to the antioxidant and pro-resolving properties of DHA [6].

The aim of this study was to evaluate the impact of RvD1, as a DHA derivative, on the inflammatory state-related proteins and genes in HUVEC cells exposed to benzo(a)pyrene (BaP).

Materials and methods

Cultured cells

HUVEC cells (PCS-100-010, ATCC, Mannasas, VA, USA) at the 4th passages were cultured in Vascular Cell Basal Medium (ATCC) with an Endothelial Cell Growth Kit – VEGF (ATCC) and Gentamicin-Amphotericin B Solution (ATCC). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in the air and were seeded into a 6-well plate (Sarstedt AG&Co., Nümbrecht, Germany) at a density of 5×10^5 cells/well in 1 mL of medium. During every step of the procedure, cell morphology was analyzed using an inverted light microscope (Olympus, Tokyo, Japan). Cell viability during culturing was assessed with a Trypan Blue Exclusion Test. HUVEC cells were treated with 100 nmol of BaP (Sigma–Aldrich, Saint Louis, MO, USA) for 2 h. After treatment, 1 nmol of Resolvin D1 (RvD1) (Cayman Chemical, Ann Arbor, MI, USA) was added and cells were incubated for an additional 4 h. RvD1 was dissolved in ethanol while BaP were dissolved in dimethylosulfoxide (DMSO). Control cultures (vehicle) received the same concentration of ethanol and DMSO as experimental cells.

Western blot for quantity of COX-2, cPGES, GSTM1 and AhR expression

Cell lysate was prepared using mammalian protein extraction reagent M-PER (Thermo Fisher Scientific, Waltham, MA, USA) with protease inhibitor cocktail set III (Merck, Darmstadt, Germany) and phosphatase inhibitors (Cayman Chemical). Protein concentrations were determined using the Bradford reaction. Samples were subjected to 10% SDS-polyacrylamide gel electrophoresis as described below [17]. We used the following primary antibodies: cyclooxygenase-2 (COX-2), prostaglandin E2 synthase, glutathione S transferase Mu1 (GSTM1), aryl hydrocarbon receptor (AhR) and β-actin diluted 1:1000 and secondary antibody Easy Blot anti rabbit IgG (HRP) diluted 1:2000 in Signal+ for Western Blot (GeneTex Inc., Irvine, CA, USA). Proteins were detected using the Western blot detection kit produced by Clarity Western ECL Luminol Substrate (Bio-Rad, Hercules, CA, USA). The integrated optical density of the bands was quantified using a Chemi Doc Camera with Image Lab software (Bio-Rad).

Quantitative real-time PCR

RNA was extracted from cells using a Maxwell 16 Cell LEV total RNA purification kit (Promega) on a Maxwell instrument (Promega). After quantity and quality evaluation, RNA concentration was normalized to 15 ng/μL [6]. Reverse transcription was done with High-Capacity Reverse Transcription Kit (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). qPCR 384-well reaction plates were prepared on Bravo Biorobot (Agilent, Santa Clara, CA, USA), and subsequently primers and probes for phospholipase A2, (PLA2G4A), prostaglandin synthase 2 (PTGS2), aryl hydrocarbon receptor (AhR), glutathione S transferase (GSTM1) and cytochrome P450 (CYP1A1) were performed with TaqMan (Life Technologies) according to the manufacturer's protocol on CFX384 Touch Real Time PCR Detection System (Bio-Rad). Endogenous control genes (GAPDH and TBP) were selected on the basis of the pilot experiment. Relative expression was calculated using ΔΔCq method.

CYP1A1 activity

Cells were seeded in a white 96 well plate (4×10^4 cells/well) on day 1. On day 2 BaP and/or RvD1 was added to the cells at the indicated concentrations. CYP1A1 activity was performed with 12 mmol of the pro-luciferin substrate, after 3 h of incubation using a lytic P450-Glo™ Assay with an aliquot of intact-cell supernatant as per manufacturer instructions (Promega). Intracellular CYP enzymes convert the substrate to luciferin product, which passes out of cells and then can be detected with the Luciferin Detection Reagent. The light output of the luciferase reaction is proportional to CYP activity. Luminescence was measured using the POLARstar Omega plate reader (BMG LABTECH). Data were expressed as fold changes compared to control.

PLA2 activity

HUVEC cells (1×10^6 cells/mL) were treated with BaP and after 2 h, RvD1 was added and cells were incubated for 4 h. HUVEC were collected by centrifugation at $1000 \times g$ for 10 min at 4 °C. Cell pellets were homogenized using 1 mL of 50 mmol Hepes buffer, pH 7.4, containing 1 mmol EDTA. Samples were centrifuged at $10{,}000 \times g$ for 15 min at 4 °C. The supernatants were removed and stored on ice. 5 μL of 1 mmol bromoenol lactone was added to the samples prior to assaying and samples to inhibit any iPLA2. Arachidonoyl thio-PC was used to detect phospholipase activity. Hydrolisys of the arachidonoyl thioester bond at the *sn*-2 position by PLA2 releases a free thiol which was detected by Ellman's reagent (DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid) (Cayman Chemical)). Absorbance after 60 min of incubation with Arachidonoyl thio-PC was measured at 414 nm using the POLARstar-Omega plate reader (BMG LABTECH). Absorbance changes during the time were calculated using cPLA2Triple software (Cayman Chemical). Data were expressed as μmol/min/mL.

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