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Effects of estrogen on endothelial prostanoid production and cyclooxygenase-2 and heme oxygenase-1 expression

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ABSTRACT

We studied the effects of 17β-estradiol (E_2) (10, 40 nM) on 2 vasoprotective pathways, i.e. cyclooxygenase-2 (COX-2)-dependent prostanoids and the antioxidant heme oxygenase-1 (HO-1), in human umbilical vein endothelial cells (HUVEC) exposed for 6 h to steady laminar shear stress (LSS, 10 dyn/cm^2), characteristic of atherosclerotic lesion-protected areas. COX-2 was induced by LSS versus static condition (SC). E_2 did not significantly affect COX-2 expression in HUVEC cultured in SC or exposed to LSS. Prostacyclin (PGI_2) and prostaglandin (PG)E_2 were induced while $PGF_{2\alpha}$ was reduced by LSS. E_2 caused no effect or a small reduction of prostanoid biosynthesis. In HUVEC cultured in SC or exposed to LSS, E_2 10 nM caused a comparable HO-1 induction (35–45%) while E_2 40 nM was 5-fold more potent in LSS-exposed HUVEC than in SC (290% and 58%, respectively). PGI_2 receptor antagonist RO3244794 did not affect HO-1 induction by E_2. In conclusion, E_2 may restrain oxidant stress in the endothelium through HO-1 induction by a mechanism independent on PGI_2 signaling.

1. Introduction

Premenopausal women are less susceptible to myocardial infarction and stroke than males of the same age group, a difference that does not exist between postmenopausal women and men [1]. This finding is presumably explained by the protective effects of estrogen for the cardiovascular system. In fact, it has been reported that estrogen: (i) lowers plasma lipoproteins [2]; (ii) influences the renin–angiotensin system [3,4]; (iii) has antioxidant properties [5]; (iv) acts as calcium-blocking agents [6]; (v) increases vascular nitric oxide (NO) production and modulation of endothelial NO synthase (eNOS [NOS III]) expression [7–9]. Interestingly, Egan et al. [10] found that, in mice, estrogen acts via its estrogen receptor (ER)α to enhance the biosynthesis of vascular prostacyclin (PGI_2) by the cyclooxygenase(COX)-2 pathway. Activation of plasma membrane PGI_2 receptor (I prostanoid receptor, IP), then, translates into an atheroprotective effect through the inhibition of platelet function and oxidant stress and the induction of heme oxygenase (HO)-1 [10].

HO-1 is a 32-kDa stress-inducible enzyme which protects against oxidative damage and its induction is part of a more generalized protective cellular response that involves phase II enzymes [11]. It catalyzes the rate-limiting step in the metabolic conversion of heme to the bile pigments (i.e. biliverdin and bilirubin) and constitutes a major intracellular source of carbon monoxide [12]. These products mediate the antioxidant and anti-inflammatory actions of HO-1 [12].

Several studies in vitro have shown the capacity of estrogen to modulate the endothelial generation of PGI_2 via "non-genomic" [i.e. rapid in onset and short (a few minutes) duration] and "genomic" mechanisms [i.e. delayed in onset (from hours to days to occur) and prolonged in duration] [reviewed in Ref. #13]. Moreover, Turner and Kinsella found that in the human umbilical vein cell line EA.hy926, in the platelet progenitor megakaryocytic human

Abbreviations: ARE, AU-rich elements; COX-2, cyclooxygenase-2; eNOS, endothelial NO synthase; E_2, 17β-estradiol; ER, estrogen receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HO-1, heme oxygenase-1; HuR, Hu-antigen R; hPGT, human prostaglandin transporter; HUVEC, human umbilical vein endothelial cells; IP, I prostanoid receptor; IL-1β, interleukin-1β; LSS, laminar shear stress; NO, nitric oxide; OD, optical density; (PPAR)-γ, peroxisome proliferator-activated receptor; PGI_2, prostacyclin; RO, RO3244794 (*R*-3-(4-fluoro-phenyl)-2-[5-(4-fluoro-phenyl)-benzofuran-2-ylmethoxycarbonylamino]-propionic acid); SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SC, static conditions; TBS–Tween-20, tris-buffered saline–0.1% Tween-20; 3′UTR, 3′-untranslated region.

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erythroleukemia cell line HEL 92.1.7 and in primary human aortic smooth muscle cells, prolonged incubation with 17β-estradiol (E_2; at ≥5 nM) induced the expression of the human IP receptor through an ERα-dependent mechanism [14].

The effects of E_2 on endothelial prostanoid generation have been studied in cells cultured in static conditions (SC) for different incubation times (from 15 min to 24 h) [reviewed in Ref. #13]. However, it is now generally recognized that the vascular endothelium is a dynamically mutable interface which models itself to various biomechanical forces, generated by the flow of blood which may induce specific phenotypes through the modulation of the expression of both protective and pathological relevant genes [15]. Among vasoprotective genes up-regulated in endothelial cells by steady laminar shear stress (LSS) (characteristically associated with lesion-protected areas of the vasculature), COX-2 is included [16–18]. We have recently shown that in human umbilical vein endothelial cells (HUVEC), the induction of COX-2-dependent PGI_2, in response to steady LSS, restrains the biosynthesis and release of the pro-atherogenic cytokine tumor necrosis factor-α. This effect is mediated by PGI_2-dependent up-regulation of HO-1 in HUVEC [18].

In the study reported here, we aimed to investigate the effects of physiological and high concentrations of E_2 (10 and 40 nM, respectively) on the expression of COX-2 and the generation of PGI_2 and other prostanoids in HUVEC exposed for 6 h to vasoprotective steady LSS (10 dyn/cm^2). We chose this time of exposure since it was previously shown to be appropriate for the induction of COX-2 and the increase of prostanoid biosynthesis in HUVEC [16–18]. The second objective of this study was to verify whether E_2 modulated the induction of HO-1 in HUVEC exposed to LSS. Finally, we addressed whether PGI_2 signaling played a role in E_2-dependent induction of HO-1 by using the IP antagonist RO3244794 [19]. The effects of E_2 in HUVEC cultured in SC were also studied.

2. Materials and methods

2.1. Endothelial cell cultures

HUVEC were isolated from normal-term umbilical cords [20], used at passage level 2 or 3 and grown as previously described [18].

2.2. Exposure of HUVEC to steady LSS

HUVEC were seeded on gelatin covered glass slide (24 mm × 50 mm) at a density of 1.5×10^5/slide and grown for 4–5 days to form confluent ($0.8–1 \times 10^6$ cells per glass slide) monolayer. HUVEC were shear stressed, using a parallel plate flow chamber connected to a constant pressure drop flow loop [21], maintained at 37 °C and gassed continuously with a humidified mixture of 5% CO_2 in air. Endothelial monolayers were continuously perfused in a closed circuit at an estimated shear stress of 10 dyn/cm^2 (flow rate of 2.53 mL/min; shear rate of 1400 s^{-1}) with 7 mL of perfusion DMEM-medium199 (50%, vol/vol), supplemented with 5% fetal calf serum, 1% glutamine, and antibiotics for 6 h. Matched control cells were cultured under SC in parallel.

2.3. Pharmacological treatments

E_2 (Sigma–Aldrich, St. Louis, MO) and IP receptor antagonist RO3244794 (*R*-3-(4-fluoro-phenyl)-2-[5-(4-fluoro-phenyl)-benzofuran-2-ylmethoxycarbonylamino]-propionic acid) [19] (kindly provided by Roche, Palo Alto, CA) were dissolved in DMSO and 7 μL of vehicle (DMSO) or stock solution of the compounds were added to 7 mL of perfusion medium to give final concentrations of 10 nM or 40 nM and 10 μM, respectively. We tested the effects of these compounds in HUVEC cultured under LSS or in SC. Finally, HUVEC were treated with interleukin (IL)-1β (5 ng/mL) (Sigma–Aldrich) and cultured in SC for 6 h, as positive control for COX-2 expression [22].

2.4. Biochemical analyses

6-Keto-$PGF_{1\alpha}$ (the hydrolysis product of PGI_2), PGE_2 and $PGF_{2\alpha}$ levels were measured in cell culture media by previously described and validated radioimmunoassay techniques [23].

2.5. Real-time PCR

Total RNA was extracted from HUVEC using TRIzol reagent (Invitrogen Life Technologies, CA, USA), according to the manufacturer's protocols. One microgram of total RNA was treated with DNAse kit (Fermentas, St. Leon-Rot, Germany) and subsequently reverse transcribed into cDNA using Iscript cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA), according to the manufacturer's protocols.

One hundred nanogram of cDNA was used for the reaction mixture and the amplification of COX-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed using iTaq™ Fast SYBR® Green Supermix With ROX (Bio-Rad) and these couples of primers: COX-2-fwd: 5′GCTCAGCCATACAGCAAATCC; rev: 5′CCAAAATCCCCTTGAAGTGGG; (GAPDH)-fwd: 5′TCACCAGGGCTGCTTTTAAC; rev: 5′ GACAAGCTTCCCGTTCTCAG, using 7900HT Fast Real-Time PCR System (Applied Biosystems, CA, USA). COX-2 mRNA expression levels were normalized with GAPDH levels. Gene expression assays were performed by relative quantification with comparative cycle threshold using ABI Prism, SDS 2.1 software (Applied Biosystems).

2.6. Western blot analysis

HUVEC were lysed and 20 μg total proteins were loaded for 4–12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membrane (Bio-Rad). As previously described, membranes were saturated with a solution of 5% nonfat milk in tris-buffered saline–0.1% Tween-20 (TBS–Tween-20), and then incubated with anti-COX-2 (kindly provided by Dr. Stacia Kargman from Merck Frosst, Kirkland, Quebec, Canada), anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) polyclonal antibodies or, anti-HO-1 monoclonal antibody (Hsp32, Assay Designs, USA) for 1 h at room temperature. Then, the membranes were washed in TBS–Tween-20 and incubated with the secondary antibodies: anti-goat peroxidase-conjugated IgG (Santa Cruz Biotechnology) for β-actin, anti-rabbit peroxidase-conjugated IgG (Sigma–Aldrich) for COX-2, or anti-mouse peroxidase-conjugated IgG (Sigma–Aldrich) for HO-1. Finally, the membranes were washed in TBS–Tween-20, and then all of the blots were developed by using ECL plus detection according to the manufacturer's instructions (GE Healthcare Life Sciences, Bucks, UK). Optical density (OD) quantification of different specific bands was calculated using laser densitometry (Bio-Rad) and normalized to the OD of β-actin [18].

2.7. Statistical analysis

All values were reported as means ± SEM. Statistical analysis was performed with Student's *t* test or 1-way ANOVA and Newman–Keuls multiple comparisons test. Values of $P < 0.05$ were considered statistically significant. Analysis and graphing were performed in GraphPad Prism (version 5.00 for Windows, GraphPad).