Journal of Steroid Biochemistry and Molecular Biology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Role of GPER in estrogen-dependent nitric oxide formation and vasodilation

Natalie C. Fredette[1,3], Matthias R. Meyer[1,2], Eric R. Prossnitz[1,*]

[1] Department of Internal Medicine, University of New Mexico Health Sciences Center, Albuquerque, NM 87131, USA
[2] Institute of Primary Care, University of Zurich, Zurich, Switzerland
[3] Current address: Department of Pathology, University of Florida, Gainesville, FL, USA

ARTICLE INFO

Keywords:
eNOS
Estrogen
Endothelium
GPER
GPR30
NO
SERD
SERM
Vascular
Vasodilation

ABSTRACT

Estrogens are potent regulators of vasomotor tone, yet underlying receptor- and ligand-specific signaling pathways remain poorly characterized. The primary physiological estrogen 17β-estradiol (E2), a non-selective agonist of classical nuclear estrogen receptors (ERα and ERβ) as well as the G protein-coupled estrogen receptor (GPER), stimulates formation of the vasodilator nitric oxide (NO) in endothelial cells. Here, we studied the contribution of GPER signaling in E2-dependent activation of endothelial NO formation and subsequent vasodilation. Employing E2 and the GPER-selective agonist G-1, we investigated eNOS phosphorylation and NO formation in human endothelial cells, and endothelium-dependent vasodilation in the aortae of wild-type and *Gper*-deficient mice. Both E2 and G-1 induced phosphorylation of eNOS at the activation site Ser1177 to similar extents. Endothelial NO production to E2 was comparable to that of G-1, and was substantially reduced after pharmacological inhibition of GPER. Similarly, the clinically used ER-targeting drugs 4OH-tamoxifen, raloxifene, and ICI182,780 (faslodex, fulvestrant™) induced NO formation in part via GPER. We identified c-Src, EGFR, PI3K and ERK signaling pathways to be involved in GPER-dependent NO formation. In line with activation of NO formation in cells, E2 and G-1 induced equally potent vasodilation in the aorta of wild-type mice. *Gper* deletion completely abrogated the vasodilator response to G-1, while reducing the response to E2 by ~50%. These findings indicate that a substantial portion of E2-induced endothelium-dependent vasodilation and NO formation is mediated by GPER. Thus, selective targeting of vascular GPER may be a suitable approach to activate the endothelial NO pathway, possibly leading to reduced vasomotor tone and inhibition of atherosclerotic vascular disease.

1. Introduction

Epidemiological studies show a lower incidence of coronary artery disease, hypertension and stroke in premenopausal women compared to age-matched men; however, these sex differences lessen with the onset of menopause [1,2]. Such observations suggest that the loss of ovarian sex hormones coincides with a loss of protection against vascular diseases, implicating an important functional role for natural estrogen (predominantly 17β-estradiol, E2) in arterial health [3–5]. In particular, endothelial cells, which form the luminal cell monolayer of the vascular wall, have emerged as critical mediators of estrogen's salutary effects in the cardiovascular system [3,6,7].

Endothelial cells release multiple vasoactive substances including the endothelium-derived relaxing factor (EDRF) [8], which was identified as nitric oxide (NO) [9,10]. The enzyme that catalyzes NO formation (utilizing L-arginine and molecular oxygen as substrates) is NO synthase (NOS) [11,12]. Three NOS isoforms exist, with one isoform (NOS III) being predominantly expressed in endothelial cells and hence referred to as endothelial NOS (eNOS) [11,12]. eNOS-derived NO is a potent vasodilator, but also conveys vasoprotection through multiple mechanisms such as inhibition of leukocyte adhesion and migration, platelet aggregation and thrombosis, as well as mitigating proliferation and migration of the underlying vascular smooth muscle cells [12]. E2 has been shown to stimulate NO formation in cultured human endothelial cells [13]. In particular, E2 binding to estrogen receptors (ER) triggers signaling cascades that include activation of the kinases c-Src [14], ERK [15,16], PI3K [17], and Akt [18], the latter eliciting phosphorylation of the eNOS activation residue Ser1177 [19–22]. Studies in murine aortae have confirmed the involvement of these pathways in endothelium-dependent, NO-mediated vasodilation induced by E2 [19,23,24]. Different ER subtypes (the holoreceptor ERα, its truncated isoform ER46, and possibly ERβ) localized to the plasma membrane have been associated with these actions [25–27].

In 1997, an orphan G protein-coupled receptor termed GPR30 was cloned from human endothelial cells exposed to fluid shear stress [28], a prototypic physiologic stimulus of eNOS activation [29]. Following

* Corresponding author at: Dept. of Internal Medicine, University of New Mexico Health Sciences Center, 2325 Camino de Salud NE, Albuquerque, NM 87131, USA.
E-mail address: EProssnitz@salud.unm.edu (E.R. Prossnitz).

http://dx.doi.org/10.1016/j.jsbmb.2017.05.006
Received 21 February 2017; Received in revised form 8 May 2017; Accepted 16 May 2017

Table 1
Sets of primers used for amplification of gene-specific cDNA fragments by qPCR.

Gene (Accession number)	Forward Primer	Reverse Primer
Human GPER (NM_001098201.1)	5′-GTA CCC AGA AGT GAG CAG CT-3′	5′-GTG CAT CCG TGG AGG CGA GG-3′
Human eNOS (NM_008713)	5′-AGA GCC TGC AAT TAC TAC CA-3′	5′-GTG GAT TTG CTG CTC TGT AG-3′
Human ERα (NM_000125.3)	5′TGA TTG GTC TCG TCT GGC G-3′	5′-CAT GCC CTC TAC ACA TTT TCC C-3′
Human GAPDH (NM_00804)	5′-TTC ACC ACC ATG GAG AAG GC-3′	5′-GGC ATG GAC TGT GGT CAT GA-3′

the demonstration of binding and signaling in response to E2 [17,30], GPR30 was renamed G protein-coupled estrogen receptor (GPER) [31]. Experiments utilizing GPER-deficient (*Gper*$^{-/-}$) mice [32] and GPER-selective ligands, the agonist G-1 [33,34] and antagonists G15 and G36 [35,36], have established actions of this third estrogen receptor in vascular biology [37–45] as well as many other aspects of physiology [46–51]. We and others have previously shown that G-1 induces eNOS activation in human endothelial cells [52–54], as well as endothelium-dependent, NO-mediated vasodilation of multiple human and rodent arteries [3,53,55,56]. These findings established GPER as an ER mediating eNOS activation; however, the extent to which GPER contributes to the overall E2-dependent response and the activation of eNOS by multiple ER-targeting drugs remain unresolved. Hence, we set out to determine the role of GPER in eNOS phosphorylation and the mechanisms of NO production in human endothelial cells, as well as vasodilation in the aorta of wild-type and *Gper*$^{-/-}$ mice. To this end, we utilized the GPER-selective agonist G-1 [33] and the non-selective ER agonist E2, as well as the selective ER modulators (SERMs) 4OH-tamoxifen and raloxifene and the selective ER downregulator (SERD) ICI182,780 (faslodex, fulvestrant™), which have been shown to act as GPER agonists in other systems [57].

2. Methods

2.1. Human endothelial cells

Telomerase-immortalized human umbilical vein endothelial (TIVE) cells were a generous gift from Rolf Renne, PhD (University of Florida, Gainesville, FL), and their derivation has been described previously [58]. Sex of the cells was determined by fluorescence in situ hybridization (FISH) analysis (TriCore Reference Laboratories, Albuquerque, NM). Primary single donor human umbilical vein endothelial cells (HUVEC) were obtained from Lonza (cat # C2517AS). TIVE and HUVEC cells were cultured in M199 basal media supplemented with 20% FBS, 100 μg/mL bovine neural-derived endothelial growth factor and antibiotics (50 U/mL penicillin, 50 μg/mL streptomycin). All tissue culture vessels were coated for 30–60 min at room temperature with 0.1% sterile gelatin in sterile milliQ-filtered water prior to seeding cells. The expression pattern of endothelial cell-specific markers remains unchanged from passages 2–9 in this cell line [58], and thus TIVE cells were used below passage 10 for experiments. HUVEC were used below passage 4.

2.2. Quantitative real-time polymerase chain reaction (qPCR)

Gene expression of GPER and eNOS in TIVE cells was analyzed at passages 3, 6, 9 and 12 and in HUVEC at passage 3. Total cellular RNA was extracted using Trizol Reagent and eluted using Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) according the manufacturer's instructions. RNA (400 ng) was reverse transcribed using the First Strand cDNA synthesis kit (Applied Biosystems, Carlsbad, CA). SYBR green-based detection of amplified gene-specific cDNA fragments was performed on a 7500 FAST real-time PCR system (Applied Biosystems). Primer sequences are provided in Table 1. GAPDH served as a housekeeping control.

2.3. Fluorescence microscopy

Cells were seeded onto gelatin-coated coverslips (approximately 30,000 cells per coverslip in a 24 well cell culture plate) for 48 h and fixed in PBS containing 4% paraformaldehyde for 15 min at room temperature. For examining GPER localization, cells were treated with either permeabilizing (PBS containing 3% BSA and 0.1% Triton X-100) or non-permeabilizing (PBS containing 3% BSA) blocking buffer for 1 h at room temperature, and incubated with a rabbit anti-mouse GPER antibody targeting a sequence within the second extracellular loop (acetyl-FADVREVQWLEVTLGFIC, 1:10,000) [18] or negative control pre-immune rabbit serum overnight at 4 °C. Slides were then washed 3 times with permeabilizing (0.1% Triton X-100) or non-permeabilizing PBS, incubated with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:500) for 1 h at room temperature, washed three times with PBS, and mounted in Vectashield supplemented with DAPI (200 ng/mL). To evaluate ERα and GPER staining in TIVE and HUVEC, cells were permeabilized and incubated with 1:50 mouse IgG antibody targeting ERα (Santa Cruz sc8002 clone F10) overnight at 4 °C, washed three times with PBS-T and incubated with 1:500 rabbit anti-mouse Alexa Fluor 488. Cells were co-stained using a GPER antibody targeting the N-terminus [17] at 1:10,000 and 1:500 anti-rabbit Alexa Fluor 568. DAPI was used as a nuclear counterstain described above. Fluorescence signals were visualized using a Zeiss LSM510 Meta or Zeiss 720 confocal fluorescent microscope.

2.4. Western blotting

TIVE cells (200,000 cells per well) were seeded and grown to 70–80% confluence in 6-well plates and serum starved overnight. The next morning, cells were treated with the non-selective estrogen receptor agonist E2 (100 nM), the GPER-selective agonist G-1 (1–100 nM) or vehicle (DMSO 0.01%) for up to 15 min [52], briefly washed with ice cold PBS and lysed on ice with 50 μL of ice cold NP-40 lysis buffer supplemented with 1% SDS, 5 μM $NaVO_4$ and 5 μM NaF to preserve phosphorylated proteins. Cell debris was pelleted at 12,000 × *g* at 4 °C and the supernatant of soluble proteins was collected, aliquoted and stored at −80 °C. Protein concentrations were determined by Coomassie (Bradford) assay (Pierce, Rockfield, IL). For each sample, 20 μg of total protein was resolved by 10% SDS PAGE and blotted to PVDF membrane. Blots were blocked for 1 h at room temperature in TBS-T 0.01% supplemented with 3% newborn calf serum and incubated overnight at 4 °C with mouse anti-human pSer1177-eNOS (1:500, antibody 612392, BD Biosciences, Sparks, MD) or mouse anti-human β-actin (1:10,000, antibody MAB1501, Millipore, Darmstadt, Germany) antibodies. Blots were then washed, incubated with secondary HRP-conjugated antibodies (1:5000) for 1 h at room temperature, and developed with PicoWest Chemiluminescence detection (ThermoScientific) for one minute at room temperature. Blots were imaged on Kodak X ray film and quantified using ImageJ densitometry analysis software (National Institutes of Health).

2.5. Detection of NO

NO was determined by detection of the stable NO metabolites NO_2/