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Vasodilatory effects of the selective GPER agonist G-1 is maximal in arteries of postmenopausal women

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ABSTRACT

G-protein-coupled estrogen receptors (GPERs) have been proposed to mediate estrogen-mediated vasodilation. The presence of GPER-dependent vasodilation in human resistance-sized arteries (HRAs) or its signal transduction pathways have not been investigated. HRAs in subcutaneous fat tissues (biopsies from postmenopausal women (PMW), age-matched men (M) and pregnant women (PGW)) were mounted for in vitro isometric force recording. Vasodilation induced by G-1 (selective GPER-agonist, 3 μM) from HRAs pre-contracted with norepinephrine amounted to $40 \pm 5\%$ in PMW, significantly larger than those obtained from M ($20 \pm 5\%$) or PGW ($20 \pm 5\%$). L-NAME (nitric oxide (NO) synthase inhibitor) abolished these relaxations in PGW, attenuated them in PMW and had no effect in M. Immunohistochemical analysis confirmed the presence of GPER in both smooth muscle and endothelial cells of HRA with maximum expression in PGW. In cultured human umbilical vein endothelial cells (HUVECs), G-1 increased NO-synthesis concentration-dependently through higher expressions of endothelial NO-synthase (eNOS) and through enhanced phosphorylation of eNOS on Ser^{1177}. In conclusion, GPER vasodilates human resistance arteries through various activating mechanisms of the eNOS-signaling pathway.
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1. Introduction

G-protein-coupled receptors are integral membrane proteins responding to a wide variety of extra-cellular signals such as hormones, neurotransmitters, chemokines and autocoids [1]. The estrogen-mediated signaling mechanisms have traditionally been thought to involve binding of estrogens with the nuclear estrogen receptors ERα and ERβ [2,3]. More recently, a third membrane-bound estrogen receptor (ER) has emerged: the G protein-coupled receptor 30 or G protein-coupled estrogen receptor (GPER). Several groups have reported that activation of GPER reduces cell proliferation in cell culture and reduces blood pressure in animal models [4]. This receptor appears to mediate estrogen-dependent responses in the cardiovascular system without delays or in a very short time frame. Importantly, evidence from gene ablation in murine models suggests its involvement in hyperglycemia, impaired glucose tolerance, reduced body growth and increased blood pressure [5]. It has been reported that GPER immunoreactivity is observed in endothelial and vascular smooth muscle cells of both male and female rat carotid arteries [6], rat aorta [7] and in human internal mammary arteries and saphenous veins [8] implying that GPER is expressed throughout the arterial wall and GPER-agonists could elicit endothelial-dependent and independent relaxation. Additionally, deletion of this GPER increased endothelial-dependent vasoconstriction [9]. The selective GPER agonist G-1 was identified in 2006 as a vasodilator, with a variable dependence on nitric

Abbreviations: ACh, acetylcholine; BK, bradykinin; CVD, cardiovascular diseases; DAB, 3,3′-diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; eNOS, endothelial nitric oxide synthase; ERs, estrogen receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; EDHF, endothelium-derived hyperpolarizing factor; FBS, fetal bovine serum; G-1, GPER-agonist; GPER, G-protein-coupled estrogen receptor; HRA, human resistance-sized arteries from subcutaneous fat biopsies; HUVEC, human umbilical vein endothelial cells; KPSS, high potassium physiological salt solution; L-NAME, N-nitro-L-arginine-methyl ester; M, age matched men; NO, nitric oxide; NOS, nitric oxide synthase; NE, norepinephrine; PMW, postmenopausal women; PGW, pregnant women; PSS, physiological salt solution.

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oxide (NO) [6,10–12]. In female mRen2 Lewis rats, it was shown that G-1 mimicked the vasodilation induced by estradiol [11]. The vasodilatory, endothelium-dependent effects of G-1 have hitherto not been investigated in human resistance arteries.

The characteristic menopausal loss of estrogen has been assumed to mediate the increased risk of cardiovascular diseases (CVD) [13,14]. The cardiovascular protective effects of 17β-estradiol, the main contributing estrogen, are thought to be controlled through the stimulation of NO formation, through the attenuation of vascular inflammation and through the prevention of vascular smooth muscle cells proliferation [15,16]. Small arteries with a diameter between 100 and 300 μm participate actively in the regulation of peripheral blood pressure, of vascular resistance and of flow to target organs [17]. Flow is regulated by active changes to the lumen diameter of these arteries. Neurotransmitters, paracrine and autocrine substances, hormones (including sex hormones), neurohumoral substances (bradykinin, acetylcholine, histamine, vasopressin, angiotensin-II) and physical stimuli (pressure, shear stress) all can influence the diameter of these arteries through interaction with the endothelial cells, smooth muscle cells or both. Interaction between the endothelium and the above-mentioned substances usually results the release of endothelial derived vasoactive substances such as NO, thromboxane, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF). Endothelial functions gradually decline during and after the menopause. This phenomenon is accompanied by alterations of certain biochemical markers [18], by impaired flow-mediated dilatation [19], and most importantly, by a decline of endothelial nitric oxide synthase (eNOS) activity and NO bio-availability [20]. Whether the beneficial effects of estrogen are mediated through the nuclear receptors or alternatively, through the GPER-pathway, are currently unclear.

We aimed to assess whether selective activation of GPER could induce vasodilation in small resistance arteries and which signal transduction pathways could be involved in such a phenomenon. We specifically hypothesized that GPER expression and function could be different in isolated small arteries from men or women and within the female group, between the status of postmenopause or pregnancy. This hypothesis was tested in the following ways: (i) the NO-dependence of vasodilatory effects of G-1 in vitro on isolated small arteries using isometric force recording with wire myography; (ii) immunohistochemical analysis of GPER-presence in DAB-stained cross-sections of cryosectioned small arteries. Further elucidation of the signal transduction pathways involved in the effects of G-1 and confirmation of its non-nuclear effects was obtained from human umbilical vein endothelial cells in culture (HUVECs) through the quantification of G-1 dependent NO-release and immunoblot analysis of G-1 dependent phosphorylation of eNOS.

2. Methods

2.1. Experiments on human resistance-sized arteries

2.1.1. Material

Three groups of subjects were recruited from Karolinska University Hospital, Huddinge: postmenopausal women (PMW, $n = 20$), pregnant women (PGW, $n = 19$), and age-matched men (M, $n = 13$). The investigation was undertaken with the approval by the Regional Ethical Review Board in Stockholm. Subcutaneous lower abdominal fat biopsies (weight between 2 and 10 g) were obtained from PMW and M during elective surgery (hernia repair or laparoscopic cholecystectomy). Similar biopsies were obtained from healthy PGW undergoing planned cesarean section. All subcutaneous fat biopsies were immediately placed in cold physiological salt solution (PSS, composition in mM: NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.026, and glucose 5.5) and were kept on ice.

2.1.2. Vascular reactivity

Arterial segments (diameter ∼200 μm, length ∼2000 μm) were identified with a stereo-zoom microscope and dissected out of the surrounding non-vascular tissues with fine ophthalmological instruments. Two stainless steel or tungsten wires (diameter respectively 40 or 25 μm) were carefully inserted through the lumen. These wires were attached to two specimen holders of a wire myograph organ bath for subsequent registration of isometric force (Danish Myotechnology, Model 610, www.dmt.dk). Each organ bath contained warmed 37 °C PSS, bubbled with 5% CO_2/95% O_2. For data registration Myodac Software was used (version 2.1, Danish Myo Technology, www.dmt.dk). The standardization, conditioning and viability-assessment protocol consisted of two stages, an initial stretching protocol followed by 5 discrete smooth muscle activation challenges [21]. In order to optimize the resting tension for subsequent isometric active force development, vessels were gradually stretched until the internal diameter corresponded to an effective transmural pressure of 100 mm Hg. The activation challenges lasted 3 min and were each followed by a thorough wash with standard PSS, allowing isometric force to return to pre-challenge levels. In the first and second challenge, the vessels were stimulated with a mixture of high potassium physiological salt solution (KPSS, equimolar substitution of 125 mM Na^+ with K^+) and 10 μM norepinephrine (NE). The third stimulation consisted of 10 μM NE, during which the presence of a functional endothelium was tested by the addition of 1 μM acetylcholine (ACh) or 0.1–0.3 μM bradykinin (BK). During the final 2 challenges, vessels were stimulated with KPSS only. Arteries that failed to produce active tension equivalent to 100 mm Hg or failed to reach a relaxation of >50% were rejected at that point and not carried forward to the protocol below [21]. To determine G-1 dependent relaxations, arteries were activated with 3 μM NE. After a stable plateau was reached (Fig. 1), the responses to cumulative additions of G-1 (10 nM to 3 μM, half log unit steps) were determined. Each higher concentration of G-1 was added as soon as the effects of the previous concentration reached their nadir. After a thorough and repeated wash-out with warmed PSS, the preparation was then incubated for 20 min with the nitric oxide synthase (NOS) inhibitor, L-NAME (300 μM) and the above-described protocol was repeated (Fig. 1) [22–24]. At the end using Myodac Software the percentage of relaxation was determined.

2.1.3. Immunohistochemistry

Arteries were isolated as described, flash-frozen on dry ice and stored at −80 °C until transversal sectioning (cryostat, 8 μm thickness). Sections were mounted on glass slides and stored at −80 °C until staining. Upon staining, all the tissue sections were first blocked with 0.75% H_2O_2 for 15 min after which GPER-antibody (1:400) (www.abcam.com) with 4% goat serum (www.sigmaaldrich.com) was added for an overnight incubation at 4 °C. Antibody binding was subsequently detected by using a DAB staining kit (www.thermoscientific.com). For negative controls, the primary antibody was replaced with 4% normal goat serum. Finally, the slides were counterstained with hematoxylin.

2.2. Cell cultures and treatments

2.2.1. Materials

Human umbilical cords were obtained from healthy women who underwent uncomplicated term pregnancies. Written informed consent was obtained from each subject in accordance

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