G protein-coupled estrogen receptor inhibits vascular prostanoid production and activity

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Abstract

Complications of atherosclerotic vascular disease, such as myocardial infarction and stroke, are the most common causes of death in postmenopausal women. Endogenous estrogens inhibit vascular inflammation-driven atherogenesis, a process that involves cyclooxygenase (COX)-derived vasoconstrictor prostanoids such as thromboxane A2. Here, we studied whether the G protein-coupled estrogen receptor (GPER) mediates estrogen-dependent inhibitory effects on prostanoid production and activity under pro-inflammatory conditions. Effects of estrogen on production of thromboxane A2 were determined in human endothelial cells stimulated by the pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α). Moreover, Gper-deficient (Gper−/−) and WT mice were fed a pro-inflammatory diet and underwent ovariectomy or sham surgery to unmask the role of endogenous estrogens. Thereafter, contractions to acetylcholine-stimulated endothelial vasoconstrictor prostanoids and the thromboxane-prostanoid receptor agonist U46619 were recorded in isolated carotid arteries. In endothelial cells, TNF-α-stimulated thromboxane A2 production was inhibited by estrogen, an effect blocked by the GPER-selective antagonist G36. In ovary-intact mice, deletion of Gper increased prostanoid-dependent contractions by twofold. Ovariectomy also augmented prostanoid-dependent contractions by twofold in WT mice but had no additional effect in Gper−/− mice. These contractions were blocked by the COX inhibitor meclofenamate and unaffected by the nitric oxide synthase inhibitor L-NG-nitroarginine methyl ester. Vasoconstrictor responses to U46619 did not differ between groups, indicating intact signaling downstream of thromboxane-prostanoid receptor activation. In summary, under pro-inflammatory conditions, estrogen inhibits vasoconstrictor prostanoid production in endothelial cells and activity in intact arteries through GPER. Selective activation of GPER may therefore be considered as a novel strategy to treat increased prostanoid-dependent vasomotor tone or vascular disease in postmenopausal women.

Key Words
- acetylcholine
- atherosclerosis
- cyclooxygenase
- estrogen
- GPER
- GPR30
- inflammation
- menopause
- ovariectomy
- vasoconstriction

Introduction
Complications of atherosclerotic vascular disease such as myocardial infarction and stroke are the most common cause of death in women, although they occur 10 years later in life than in men because premenopausal women are largely protected (Schenck-Gustafsson et al. 2011, Barrett-Connor 2013). Such epidemiological findings point toward potent inhibition of atherogenesis by endogenous estrogens such as 17β-estradiol (Schenck-Gustafsson et al. 2011, Barrett-Connor 2013), and experimental evidence further supports that estrogens exert pleiotropic salutary effects on the vascular wall (Murphy 2011). Estrogen signaling pathways are complex because 17β-estradiol nonselectively activates soluble transcription factors including estrogen receptor α (Green et al. 1986, Greene et al. 1986) and estrogen receptor β (Kuiper et al. 1996), as well as the 7-transmembrane, intracellular G protein-coupled estrogen receptor (GPER) (Revankar et al. 2005, Thomas et al. 2005). GPER is highly expressed in the cardiovascular system (Iseensee et al. 2009) and has been implicated in the regulation of vascular tone and inflammation (Haas et al. 2009, Lindsey et al. 2009, Meyer et al. 2010, Chakrabarti & Davidge 2012, Meyer et al. 2012a,b, 2014a), although the mechanisms involved are only partially understood.

The endothelium is a key regulator of vascular tone through the release of multiple vasoactive substances, including both relaxing factors, such as nitric oxide (NO), and contracting factors, such as cyclooxygenase (COX)-derived vasoconstrictor prostanooids and endothelin-1 (Feletou & Vanhoutte 2006). Studies on endothelial function widely rely on acetylcholine as a muscarinic agonist that initiates two distinct endothelium-dependent responses: relaxation mediated predominantly by NO at low concentrations (1–100 nmol/l) and contraction mediated by vasoconstrictor prostanooids at high concentrations (≥100 nmol/l) (Kauer & Rubanyi 1995, Traupe et al. 2002a, Zhang & Kosaka 2002, Zhou et al. 2005, Feletou & Vanhoutte 2006). Prostanoids, such as thromboxane A2, released by the endothelium in response to acetylcholine elicit contraction of the underlying vascular smooth muscle by activating thromboxane-prostanoid (TP) receptors (Feletou & Vanhoutte 2006). In fact, intracoronary infusion of acetylcholine induces vasoconstriction in patients with mild and advanced atherosclerosis independent of sex (Horio et al. 1986, Ludmer et al. 1986), indicating that the release of prostanoids in humans modulates vasoconstriction. Because COX-derived prostanoids are also important modulators of vascular inflammation involved in atherogenesis (Ricciotti & FitzGerald 2011), biosynthesis of thromboxane A2 is increased in atherosclerotic lesions (Mehta et al. 1988).

Although endogenous estrogens contribute to the inhibition of vasoconstriction, vascular inflammatory processes, and atherosclerosis (Murphy 2011) in part through a reduction of vasoconstrictor prostanoid production and activity (Kauer & Rubanyi 1995, Davidge & Zhang 1998, Dantas et al. 1999, Zhang & Kosaka 2002), the specific estrogen receptor that modulates these responses is unclear. Given that GPER activation inhibits vascular inflammation in mice (Meyer et al. 2014a), we hypothesized that endogenous estrogens might reduce the production and activity of vasoconstrictor prostanoids through GPER. We therefore set out to determine the effects of 17β-estradiol on vasoconstrictor prostanoid production in human endothelial cells under quiescent and pro-inflammatory conditions. In addition, functional responses to acetylcholine-stimulated vasoconstrictor prostanoids were compared between ovari-intact and ovariectomized WT and GPER-deficient (Gper−/−) mice fed a high-fat, cholate-containing diet known to induce vascular inflammation (Paigen et al. 1987, Lin et al. 2007, Chen et al. 2010, Denes et al. 2012, Meyer et al. 2014a).

Materials and methods
Materials
L-N6G-nitroarginine methyl ester (l-NNAME), 2-((2,6-dichloro-3-methylphenyl)amino)-benzoic acid (meclofenamate), and 9,11-dideoxy-9α,11α-methaneoepoxy prostaglandin F2α (U46619) were from Cayman Chemical (Ann Arbor, MI, USA). Endothelin-1 was from American Peptide (Sunnyvale, CA, USA), and tumour necrosis factor alpha (TNF-α) was from R&D Systems (Minneapolis, MN, USA). G36 was synthesized as described (Burai et al. 2010, Dennis et al. 2011) and provided by Jeffrey Arterburn (New Mexico State University, Las Cruces, NM, USA). All other drugs were from Sigma–Aldrich (St Louis, MO, USA). For vascular reactivity studies, stock solutions were prepared according to the manufacturer’s instructions and diluted in physiological saline solution (PSS, composition in mmol/l: 129.8 NaCl, 5.4 KCl, 0.83 MgSO4, 0.43 NaH2PO4, 19 NaHCO3, 1.8 CaCl2, and 5.5 glucose; pH 7.4) to the required concentrations before use. Concentrations are expressed as final molar concentration in the organ chamber.
Thromboxane A$_2$ production in human endothelial cells

Human endothelial cells of a hTERT-immortalized umbilical vein endothelial (TIVE) cell line, which expresses GPER (Meyer et al. 2014a), were kindly provided by Rolf Renne (University of Florida, Gainesville, FL, USA). Cells were isolated from a male donor as confirmed by fluorescence in situ hybridization (FISH) analysis (TriCore Reference Laboratories, Albuquerque, NM, USA), generated as described (An et al. 2006), and cultured in M199 basal medium supplemented with 20% FBS bovine endothelial cell growth factor (6 µg/ml) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamycin). TIVE cells express endothelial cell-specific markers at passage 12 that are identical to expression patterns observed in primary human umbilical vein endothelial cells at passage 2 (An et al. 2006) and therefore were used up to passage 12. Their endothelial nature was confirmed by assessing expression of von Willebrand factor and endothelial NO synthase, as well as acetylcholine-mediated NO production. After replacing with phenol red-free, charcoal-stripped medium, TIVE cells were incubated with 17β-estradiol (100 nmol/l), the GPER-selective antagonist G36 (1 µmol/l) (Dennis et al. 2011), or solvent (DMSO 0.01%) in the presence and absence of TNF-α (1 ng/ml) for 24 h. The supernatant was collected and analyzed for thromboxane A$_2$ production by determining the concentration of its stable hydrolyzed metabolite, thromboxane B$_2$, using a competitive enzyme immunoassay (Cayman Chemical) according to the manufacturer’s instructions. Thromboxane A$_2$ production was normalized to cell number.

Animals

Female Gper$^{-/-}$ mice (Proctor & Gamble, Cincinnati, OH, USA, provided by Jan S Rosenbaum) were generated and backcrossed onto the C57BL/6j background as described (Meyer et al. 2014a). Gper$^{-/-}$ and WT littermates (Harlan Laboratories, Indianapolis, IN, USA) were housed at the University of New Mexico Animal Resources Facility with a 12 h light:12 h darkness cycle and unlimited access to chow and water. All procedures were approved by the University of New Mexico Institutional Animal Care and Use Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

To study the role of endogenous estrogens, animals underwent ovariectomy or sham surgery using isoflurane anesthesia at 4 weeks of age. Successful ovariectomy was confirmed after sacrifice by a profound reduction in uterus weight (data not shown). At 6 weeks of age, animals were changed from standard rodent chow to a pro-inflammatory, phytoestrogen-free diet containing 15.8% w/w fat (representing 37% of total calories), 1.25% w/w cholesterol, and 0.5% w/w sodium cholate (Teklad TD.90221, Harlan Laboratories, Madison, WI, USA) for 16 weeks (Paigen et al. 1987, Lin et al. 2007, Chen et al. 2010, Denes et al. 2012, Meyer et al. 2014a).

Carotid artery ring preparation and myography setup

After sacrifice by i.p. injection of sodium pentobarbital (2.2 mg/g body weight), common carotid arteries were immediately excised, carefully cleaned of perivascular adipose and connective tissue, and cut into 2 mm long rings in cold (4 °C) PSS. Rings were mounted in organ chambers of a Mulvany–Halpern myograph (620 M Multi Wire Myograph, Danish Myo Technology, Aarhus, Denmark). A PowerLab 8/35 data acquisition system and LabChart Pro software (AD Instruments, Colorado Springs, CO, USA) were used for recording isometric tension.

Vascular reactivity studies

Experiments to measure vascular reactivity of carotid arteries were performed as described (Meyer et al. 2014b, 2015). Briefly, rings were equilibrated in warmed (37 °C) PSS bubbled with 21% O$_2$, 5% CO$_2$, and balanced N$_2$ (pH 7.4) before they were stretched stepwise to the optimal level of passive tension for force generation. The functional integrity of vascular smooth muscle was confirmed by repeated exposure to KCl (PSS with substitution of 60 mmol/l potassium for sodium; Table 1). Functional integrity of the endothelium was assumed if arteries precontracted with phenylephrine (1 µmol/l or 10 µmol/l) dilated ≥85% in response to acetylcholine (100 nmol/l). Acetylcholine initiates two distinct endothelium-dependent responses in murine carotid arteries, with NO-mediated relaxations at lower concentrations (1–100 nmol/l), followed by contractions mediated by COX-derived prostанoids at concentrations ≥100 nmol/l (Traupe et al. 2002b, Zhou et al. 2005, Meyer et al. 2015). To study the full biphasic response to acetylcholine, rings in protocol 1 were precontracted with phenylephrine to a stable plateau at 40% of KCl (60 mmol/l)-induced contractions, and concentration-dependent effects of acetylcholine (0.1 nmol/l–10 µmol/l) were recorded. These experiments were repeated in the presence of the COX inhibitor meclofenamate (1 µmol/l,
pretreatment for 30 min). The contractile response to acetylcholine is more potent and can be observed in quiescent arteries when endothelial NO synthase is inhibited (Kauser & Rubanyi 1995, Traupe et al. 2002b, Zhang & Kosaka 2002, Zhou et al. 2005, Feletou & Vanhoutte 2006). Therefore and to determine whether NO affects acetylcholine-induced, prostanoid-mediated contractions, rings in protocol 2 were incubated with the NO synthase inhibitor l-NAME (DeLean et al. 1978). Area under the curve is expressed as arbitrary units (AU). All data (n = 5–16 per group) are mean ± S.E.M.

### Results

**GPER mediates estrogen-dependent inhibition of endothelial prostanoid production under pro-inflammatory conditions in vitro**

We first studied production of the major vasoconstrictor thromboxane A₂ in human endothelial cells, which was unaffected by 17β-estradiol or the GPER-selective antagonist G36 (Dennis et al. 2011) in quiescent cells under basal conditions (Fig. 1), which was prevented by 17β-estradiol (n = 3, P < 0.05 vs solvent; Fig. 1). Inhibition of thromboxane A₂ production by 17β-estradiol under pro-inflammatory conditions was blocked by G36 (n = 3, P < 0.05; Fig. 1), indicating that the estrogen-dependent effect is mediated by GPER.

**GPER reduces endothelium-dependent contractions to vasoconstrictor prostanoids under pro-inflammatory conditions in vivo**

Given that GPER-mediated, estrogen-dependent inhibition of cellular prostanoid production was only detectable under pro-inflammatory conditions (Fig. 1), we next studied acetylcholine-stimulated vasoconstrictor prostanoid activity in intact arteries from animals fed an inflammation-inducing diet (Paigen et al. 1987, Lin et al. 2007, Chen et al. 2010, Denes et al. 2012, Meyer et al. 2014a).
Contractile responses to acetylcholine are potentiated and can be observed in quiescent vascular rings when endothelial NO synthase is blocked (Kauser & Rubanyi 1995, Traupe et al. 2002b, Zhang & Kosaka 2002, Zhou et al. 2005, Feletou & Vanhoutte 2006). In carotid arteries from ovary-intact animals, upon NO synthase blockade using i-NAME, endothelium-dependent, prostanoid-mediated contractions increased by 1.9-fold in mice lacking Gper (n=5–6, P<0.05 vs WT; Fig. 3 and Table 1). Ovariectomy of WT mice potentiated responses to vasoconstrictor prostanooids by 2.3-fold (n=5–6, P<0.05 vs ovary-intact; Fig. 3 and Table 1). Furthermore, no additional effect of ovariectomy was observed in Gper−/− mice, indicating that GPER mediates the inhibitory effects of endogenous estrogens on prostanoid-mediated vasoconstriction, which do not depend on the bioavailability of estrogens.

GPER mediates estrogen-dependent inhibition of endothelium-dependent vasoconstrictr prostanoid activity

We next compared responses in arteries from ovary-intact to responses in arteries from ovariecotomized animals to determine the role of endogenous estrogens. In WT mice, ovariectomy increased prostanoid-mediated contractions (n=5–9, P<0.05 vs ovary-intact; Fig. 2A), with no additional effect of ovariectomy in Gper−/− mice, indicating that in the setting of diet-induced vascular inflammation, inhibitory effects of endogenous estrogens on vasoconstrictr prostanoid activity are mediated by GPER.
of NO. In addition, neither the sensitivity to acetylcholine (pD2 values, Table 1) nor responses to exogenous TP receptor activation by equal concentrations of the synthetic agonist U46619 (Table 1) were different between groups, suggesting that estrogen modulates endogenous prostanoid production rather than TP receptor signaling in vascular smooth muscle.

**Endothelin-1-dependent contractions in female mice are unaffected by GPER deletion or ovariectomy**

To determine whether the effects observed with prostanoid-mediated contractions were related to general changes in contractility to G protein-coupled receptor activation, we evaluated concentration-dependent contractions to endothelin-1, an endothelium-derived vasoconstrictor also implicated in acetylcholine-dependent vasoconstriction (Traupe et al. 2002b). Neither Gper deficiency nor ovariectomy had any effect on vasoconstriction to endothelin-1 in arteries from mice fed a pro-inflammatory, high-fat diet (Fig. 4 and Table 1), further confirming that estrogen modulates contractility through GPER-mediated inhibition of prostanoid activity.

**Discussion**

COX-derived prostanoids are important modulators of vascular tone and inflammation (Feletou & Vanhoutte 2006, Nakahata 2008, Ricciotti & FitzGerald 2011). Here, we show that GPER mediates estrogen-dependent inhibitory effects on the production and activity of endothelium-derived vasoconstrictor prostanoids under pro-inflammatory conditions. These findings provide evidence for a novel mechanism through which GPER inhibits vascular tone and inflammation.

Epidemiological and experimental data provide strong evidence that endogenous estrogens, such as 17β-estradiol, contribute to the inhibition of vasoconstriction, vascular inflammation, and atherosclerosis (Murphy 2011, Schenck-Gustafsson et al. 2011, Barrett-Connor 2013); however, because 17β-estradiol is a nonselective agonist of GPER as well as estrogen receptors α and β (Murphy 2011), identifying the specific target is critical to understanding the mechanisms mediating estrogen’s salutary effects in the vascular wall. Although we and others (Sobrino et al. 2010) found that 17β-estradiol does not affect prostanoid production in quiescent endothelial cells, the present study is the first demonstration that under pro-inflammatory conditions, estrogen-dependent inhibition of thromboxane A2 production requires the presence of functional GPER. This finding is in line with previous studies demonstrating that Gper deficiency leads to a pro-inflammatory state (Sharma et al. 2013) and that activation of GPER inhibits the expression of pro-inflammatory proteins in endothelial cells (Chakrabarti & Davidge 2012), induces the expression of anti-inflammatory cytokines in inflammatory T cells (Brunsing & Prossnitz 2011) while inhibiting TNF-α and IL6 production by macrophages (Blasko et al. 2009), and inhibits the infiltration of immune cells into atherosclerotic plaques.
in vivo (Meyer et al. 2014a). However, given that arachidonic acid is converted into various additional metabolites besides thromboxane A2, such as prostaglandin G2, prostaglandin H2, prostaglandin D2, prostaglandin E2, prostaglandin F2α, and prostaglandin I2, future studies may identify specific components of the prostanoid biosynthetic pathway that are regulated by GPER.

Given that GPER activation inhibits vascular prostanoid production in TNFα-stimulated endothelial cells, we examined estrogen-dependent functional effects of endothelium-derived vasoconstrictor prostanoids in animals with diet-induced vascular inflammation (Paigen et al. 1987, Lin et al. 2007, Chen et al. 2010, Denes et al. 2012, Meyer et al. 2014a) that is associated with increased COX expression and activity (Lin et al. 2007, Chen et al. 2010). In these animals, estrogen-dependent inhibitory effects on prostanoid production and activity depend on GPER, which extends the previous observation that GPER inhibits responses to vasoconstrictor prostanoids in arteries of male animals (Meyer et al. 2012a). Furthermore, given that we observed estrogen-dependent, GPER-mediated inhibitory effects on vasoconstrictor prostanoids in male human endothelial cells as well as in arteries from female mice, these findings confirm previous reports that GPER is capable of regulating vascular homeostasis independent of sex (Haas et al. 2009, Lindsey et al. 2009, Meyer et al. 2010, 2012a, 2014a).

Although different vascular cell types such as endothelial cells, vascular smooth muscle cells, and adipocytes are all known to synthesize prostanoids, GPER-dependent, estrogen-mediated effects specifically involve the inhibition of prostanoid production in the endothelium, because TP receptor signaling in the underlying smooth muscle was unaffected by the deletion of Gper or ovariectomy. Accordingly, endothelial release of the prostanoid prostaglandin F2α in mesenteric arteries of female spontaneously hypertensive rats (SHR) increases following ovariectomy (Dantas et al. 1999). Consistent with the regulation of endothelial prostanoid production and in line with previous reports (Kauser & Rubanyi 1995, Zhang & Kosaka 2002), estrogen-dependent, GPER-mediated effects on acetylcholine-induced contractions are independent of NO, although GPER is capable of modulating NO bioactivity (Meyer et al. 2010, 2012a, 2014a).

The present study provides the first mechanistic explanation for the observed inhibitory effect of 17β-estradiol on acetylcholine-induced, prostanoid-mediated contractions in female animals (Kauser & Rubanyi 1995, Davidge & Zhang 1998, Dantas et al. 1999, Zhang & Kosaka 2002) and in postmenopausal women (Gilligan et al. 1994). Such experimental evidence was largely obtained in studies using arteries from the SHR (Kauser & Rubanyi 1995), a model in which immune mechanisms are involved in vascular changes (Schiffrin 2013). In the SHR, responses to vasoconstrictor prostanoids are greater in males than in ovary-intact females (Kauser & Rubanyi 1995) and increased following ovariectomy (Dantas et al. 1999). It is intriguing to speculate that in the female SHR, GPER mediates inhibitory effects of ovarian estrogens on vasoconstrictor prostanoid activity that contribute at least partly to the lower blood pressure compared to male or ovariectomized female littermates (Kauser & Rubanyi 1995, Dantas et al. 1999). In addition, blood pressure lowering effects of the GPER-selective agonist G-1 in ovariectomized hypertensive rats (Lindsey et al. 2009) may be partly mediated by the reduced activity of vasoconstrictor prostanoids.

Although 17β-estradiol (Sudhir et al. 1997, Teoh et al. 2000) and G-1 (Meyer et al. 2010) acutely improve vasodilation by inhibiting contractions to endothelin-1, we found no effect of estrogen withdrawal due to ovariectomy or the deletion of the Gper gene on responses to endothelin-1 in the present study. The current findings are also in contrast to previous observations of enhanced contractions to endothelin-1 in carotid arteries from healthy male Gper−/− mice (Meyer et al. 2012b), suggesting sex differences or effects of the pro-inflammatory diet used in the present study on vascular contractility. However, given that endothelin-1 and endothelium-derived prostanoids are vasoconstrictors that exhibit similar properties with regard to their endothelial origin and their involvement in vascular inflammation (Trape et al. 2002b, Feletou & Vanhoutte 2006, Nakahata 2008, Ricciotti & FitzGerald 2011), the absence of functional changes to endothelin-1 reinforces a specific role for prostanoids in the enhanced vasoconstrictor responses following estrogen withdrawal.

In summary, we have identified GPER as a novel mediator underlying estrogen-dependent inhibition of endothelium-derived vasoconstrictor prostanoid production and thus vascular tone. Intra-arterial infusion of acetylcholine causes vasoconstriction in atherosclerotic human coronary arteries but not in individuals with structurally normal coronary arteries (Horio et al. 1986, Ludmer et al. 1986). Moreover, increased production of COX-derived thromboxane A2 has been observed in the aorta from animals with atherosclerosis compared to vessels from healthy littermates (Mehta et al. 1988). Together, these data support the notion that vasoconstrictor prostanoids are important modulators of vascular
inflammation and thus involved in the propagation of atherosclerosis (Nakahata 2008, Ricciotti & FitzGerald 2011). Although endogenous estrogens inhibit coronary artery inflammation (Burke et al. 2001) and 17β-estradiol therapy has been found to slow atherosclerosis progression (Hodis et al. 2001), it is currently not a therapeutic option in postmenopausal women based on the results of large, randomized, placebo-controlled trials using conjugated equine estrogen therapy (Rossouw et al. 2002, Schenck-Gustafsson et al. 2011, Barrett-Connor 2013).

A receptor-targeted approach using the GPER-selective agonist G-1 was recently demonstrated to inhibit atherosclerosis, while displaying no uterotrophic activity, in mice after ovariectomy (Meyer et al. 2014a). Whether selective GPER activation also represents a novel approach to inhibit prostanoid-dependent increased vasomotor tone or vascular inflammation in postmenopausal women remains to be determined.

Declarations of interest
E R P is an inventor on United States patent number 7 875 721. M R M, M B, and E R P are inventors on a United States patent application on the use of GPER-targeting compounds. N C F declares no conflict of interest.

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Author contribution statement
M R M, M B, and E R P were involved in the conception and design of research; M R M and N C F performed experiments; M R M analyzed data; M R M, M B, and E R P interpreted the results of experiments; and M R M, M B, and E R P prepared figures and wrote the manuscript. All authors approved the final version of the manuscript.

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