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

Matthias R. Meyer^{1,2}, Natalie C. Fredette¹, Matthias Barton³, and Eric R. Prossnitz¹

¹Department of Internal Medicine, University of New Mexico Health Sciences Center, Albuquerque, NM, United States ²Department of Cardiology, Cantonal Hospital, Aarau, Switzerland ³Molecular Internal Medicine, University of Zürich, Zürich, Switzerland

Abstract

Complications of atherosclerotic vascular disease, such as myocardial infarction and stroke, are the most common cause of death in postmenopausal women. Endogenous estrogens inhibit vascular inflammation-driven atherogenesis, a process that involves cyclooxygenase-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 TNF- α . Moreover, *Gper*-deficient (*Gper*^{-/-}) and wild-type mice were fed a pro-inflammatory diet and underwent ovariectomy or sham surgery to unmask the role of endogenous estrogens. Thereafter, endothelium-dependent contractions to acetylcholine-stimulated 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 2-fold. Ovariectomy also augmented prostanoid-dependent contractions by 2-fold in wild-type mice, but had no additional effect in *Gper*^{-/-} mice. These contractions were blocked by the cyclooxygenase inhibitor meclofenamate and unaffected by the nitric oxide synthase inhibitor L-NAME. 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

Correspondence should be addressed to: Matthias R. Meyer, M.D., Department of Cardiology, Cantonal Hospital, Tellstrasse, 5001 Aarau, Switzerland, Tel: +41 62 838 4483, Fax: +41 62 838 4299, matthias.meyer@ksa.ch, eprossnitz@unm.edu or Eric R. Prossnitz, Ph.D., University of New Mexico Health Sciences Center, Department of Internal Medicine, 915 Camino de Salud NE, Albuquerque, NM 87131, United States, Tel: +1 505 272 5647, Fax: +1 505 272 1421.

Declaration 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.

Author contribution statement

M.R.M., M.B., and E.R.P. were involved in 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 results of experiments; M.R.M., M.B. and E.R.P. prepared figures and wrote the manuscript; all authors approved the final version of the manuscript.

novel strategy to treat increased prostanoid-dependent vasomotor tone or vascular disease in postmenopausal women.

Keywords

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 towards potent inhibition of atherogenesis by endogenous estrogens such as 17 β -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 since 17 β -estradiol non-selectively 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 (Isensee *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 and Davidge 2012; Meyer *et al.* 2012a; Meyer *et al.* 2012b; Meyer *et al.* 2014b), although the mechanisms involved are only partially understood.

The vascular 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 prostanoids and endothelin-1 (Feletou and 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 prostanoids at high concentrations (100 nmol/L) (Kauser and Rubanyi 1995; Traupe *et al.* 2002b; Zhang and Kosaka 2002; Zhou *et al.* 2005; Feletou and 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 and 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 release of prostanoids in humans modulates vasoconstriction. Since COX-derived prostanoids are also important modulators of vascular inflammation involved in atherogenesis (Ricciotti and 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 the reduction of

vasoconstrictor prostanoid production and activity (Kauser and Rubanyi 1995; Davidge and Zhang 1998; Dantas *et al.* 1999; Zhang and Kosaka 2002), the specific estrogen receptor that modulates these responses is unclear. Given that GPER activation inhibits vascular inflammation in mice (Meyer *et al.* 2014b), 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 17 β -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 ovary-intact and ovariectomized wild-type 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.* 2014b).

Materials and methods

Materials

L-N^G-nitroarginine methyl ester (L-NAME), 2-[(2,6-dichloro-3-methylphenyl)amino]-benzoic acid (meclofenamate), and 9,11-dideoxy-9 α ,11 α -methanoepoxy prostaglandin F_{2 α} (U46619) were from Cayman Chemical (Ann Arbor, MI, USA). Endothelin-1 was from American Peptide (Sunnyvale, CA, USA), and 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 A₂ production in human endothelial cells

Human endothelial cells of a *hTERT*-immortalized umbilical vein endothelial (TIVE) cell line, which expresses GPER (Meyer *et al.* 2014b), were kindly provided by Rolf Renne (University of Florida, 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 μ g/mL) and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin and 50 μ g/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 17 β -estradiol (100 nmol/L), the GPER-selective antagonist G36 (1 μ mol/L) (Dennis *et al.* 2011), or solvent (DMSO 0.01%) in the presence and absence of TNF- α (1 ng/mL) for 24 hours. The supernatant was

collected and analyzed for thromboxane A₂ production by determining the concentration of its stable hydrolyzed metabolite, thromboxane B₂, using a competitive enzyme immunoassay (Cayman Chemical) according to the manufacturer's instructions. Thromboxane A₂ 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.* 2014b). *Gper*^{-/-} and wild-type littermates (Harlan Laboratories, Indianapolis, IN, USA) were housed at the University of New Mexico Animal Resources Facility with a 12 hour light-dark 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, high-fat, 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.* 2014b).

Carotid artery ring preparation and myography set-up

After sacrifice by intraperitoneal 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 of isometric tension.

Vascular reactivity studies

Experiments to measure vascular reactivity of carotid arteries were performed as described (Meyer *et al.* 2014a; Meyer *et al.* 2015). Briefly, rings were equilibrated in warmed (37 °C) PSS bubbled with 21% O₂, 5% CO₂, and balanced N₂ (pH 7.4) before they were stretched step-wise to the optimal level of passive tension for force generation. 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 contracted 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 prostanoids at concentrations 100 nmol/L (Traupe *et al.* 2002a; Zhou *et al.* 2005; Meyer *et*

al. 2015). To study the full biphasic response to acetylcholine, rings in protocol 1 were contracted 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 μ mol/L) were recorded. These experiments were repeated in the presence of the COX inhibitor meclofenamate (1 μ mol/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 (Kausar and Rubanyi 1995; Traupe *et al.* 2002a; Zhang and Kosaka 2002; Zhou *et al.* 2005; Feletou and Vanhoutte 2006). Therefore and to determine whether NO affects acetylcholine-induced, prostanoid-mediated contractions, rings in protocol 2 were incubated with the nitric oxide (NO) synthase inhibitor L- NAME (300 μ mol/L for 30 min), and concentration-dependent contractions to acetylcholine (10 nmol/L – 10 μ mol/L) were measured. In protocol 3, responses to the TP receptor agonist U46619 (10 nmol/L, a concentration that yields responses similar to acetylcholine-stimulated vasoconstrictor prostanoids) were determined. In protocol 4, concentration-dependent responses to endothelin-1 (0.1 nmol/L – 100 nmol/L), an endothelium-derived vasoconstrictor implicated in acetylcholine-dependent responses (Traupe *et al.* 2002a), were measured. These rings were pretreated with the nitric oxide (NO) synthase inhibitor L- NAME (300 μ mol/L for 30 min) to unmask the weak contractions to endothelin-1 in murine carotid arteries (Traupe *et al.* 2002b; Meyer *et al.* 2014a).

Calculations and statistical analyses

Contractions are given relative to KCl-induced responses, and relaxations relative to precontraction by phenylephrine. Maximal effects, area under the curve and EC₅₀ values (as negative logarithm: pD₂) were calculated by fitting of dose-response curves as described (DeLean *et al.* 1978). Data was analyzed by one-way or two-way ANOVA followed by Bonferroni's post-hoc test or the unpaired Student's *t*-test as appropriate (Prism version 5.0 for Macintosh, GraphPad Software, San Diego, CA, USA). Values are expressed as mean \pm s.e.m.; n equals the number of animals or cell preparations used. Statistical significance was accepted at a p value <0.05.

Results

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

We first studied production of the major vasoconstrictor prostanoid thromboxane A₂ in human endothelial cells, which was unaffected by 17 β -estradiol or the GPER-selective antagonist G36 (Dennis *et al.* 2011) in quiescent cells under basal conditions (Figure 1). TNF- α , a pro-inflammatory cytokine, increased thromboxane A₂ production by 160% (n=3, p<0.05 vs. basal conditions, Figure 1), which was prevented by 17 β -estradiol (n=3, p<0.05 vs. solvent, Figure 1). Inhibition of thromboxane A₂ production by 17 β -estradiol under pro-inflammatory conditions was blocked by G36 (n=3, p<0.05, Figure 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 (Figure 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.* 2014b). Acetylcholine as a pharmacological agonist initiates two distinct endothelium-dependent responses, stimulating NO-mediated vasodilation at low concentrations (1–100 nmol/L), followed by prostanoid-mediated vasoconstriction at concentrations 100 nmol/L (Kauser and Rubanyi 1995; Zhang and Kosaka 2002; Zhou *et al.* 2005; Feletou and Vanhoutte 2006). We found that, whereas NO-mediated vasodilation to acetylcholine was not different between groups, prostanoid-mediated contractions were increased by *Gper* deficiency in ovary-intact mice (n=5–6, p<0.05 vs. wild-type, Figure 2A). Consistent with a response to vasoconstrictor prostanoids, acetylcholine-induced vasodilation was restored in both wild-type and *Gper*^{-/-} mice by the COX inhibitor meclofenamate (n=5–12, p<0.05 vs. untreated rings, Figure 2B).

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

We next compared responses in arteries from ovary-intact to responses in arteries from ovariectomized animals in order to determine the role of endogenous estrogens. In wild-type mice, ovariectomy increased prostanoid-mediated contractions (n=5–9, p<0.05 vs. ovary-intact, Figure 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 vasoconstrictor prostanoid activity are mediated by GPER.

Contractile responses to acetylcholine are potentiated and can be observed in quiescent vascular rings when endothelial NO synthase is blocked (Kauser and Rubanyi 1995; Traupe *et al.* 2002a; Zhang and Kosaka 2002; Zhou *et al.* 2005; Feletou and Vanhoutte 2006). In carotid arteries from ovary-intact animals, upon NO synthase blockade using L-NAME, endothelium-dependent, prostanoid-mediated contractions increased by 1.9-fold in mice lacking *Gper* (n=5–6, p<0.05 vs. wild-type, Figure 3 and Table 1). Ovariectomy of wild-type mice potentiated responses to vasoconstrictor prostanoids by 2.3-fold (n=5–6, p<0.05 vs. ovary-intact, Figure 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 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.* 2002a). Neither *Gper* deficiency nor OVX had any effect on vasoconstriction to endothelin-1 in arteries from mice fed a pro-inflammatory, high-fat diet (Figure 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 and Vanhoutte 2006; Nakahata 2008; Ricciotti and 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 17 β -estradiol, contribute to inhibition of vasoconstriction, vascular inflammation, and atherosclerosis (Murphy 2011; Schenck-Gustafsson *et al.* 2011; Barrett-Connor 2013); however, since 17 β -estradiol is a non-selective 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 17 β -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 expression of pro-inflammatory proteins in endothelial cells (Chakrabarti and Davidge 2012), induces expression of anti-inflammatory cytokines in inflammatory T cells (Brunsing and Prossnitz 2011) while inhibiting TNF- α and IL-6 production by macrophages (Blasko *et al.* 2009), and inhibits infiltration of immune cells into atherosclerotic plaques *in vivo* (Meyer *et al.* 2014b). 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 F_{2 α} , 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 TNF α -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.* 2014b) 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 extend previous observations that GPER is capable of regulating vascular homeostasis independent of sex (Haas *et al.* 2009; Lindsey *et al.* 2009; Meyer *et al.* 2010; Meyer *et al.* 2012a; Meyer *et al.* 2014b).

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 inhibition of prostanoid production in the endothelium, since TP receptor signaling in the underlying smooth muscle was unaffected by deletion of *Gper* or ovariectomy. Accordingly, endothelial release of the prostanoid prostaglandin F_{2α} 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 (Kausar and Rubanyi 1995; Zhang and 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; Meyer *et al.* 2012a; Meyer *et al.* 2014b).

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 (Kausar and Rubanyi 1995; Davidge and Zhang 1998; Dantas *et al.* 1999; Zhang and Kosaka 2002) and in postmenopausal women (Gilligan *et al.* 1994). Such experimental evidence was largely obtained in studies using arteries from the SHR (Kausar and 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 (Kausar and 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 (Kausar and 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 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 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 (Traupe *et al.* 2002a; Feletou and Vanhoutte 2006; Nakahata 2008; Ricciotti and 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 cyclooxygenase-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 and FitzGerald 2011). Although endogenous estrogens inhibit coronary artery inflammation (Burke *et al.* 2001) and 17 β -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.* 2014b). 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.

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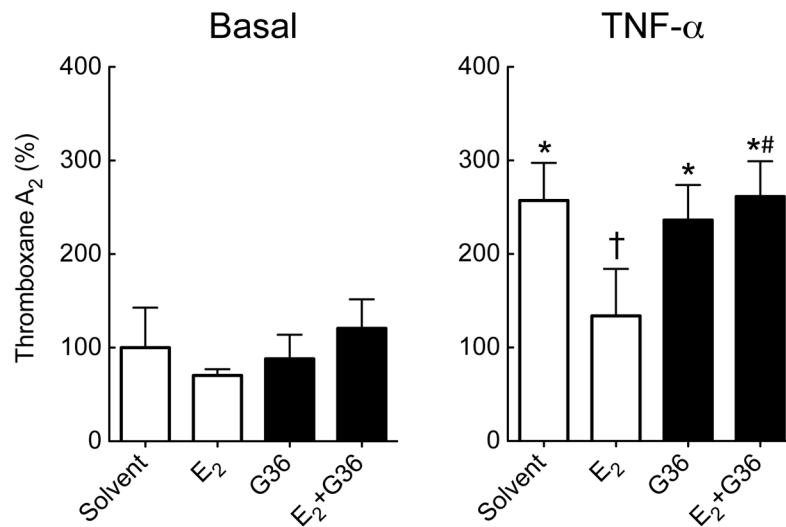


Figure 1. Role of GPER in estrogen-dependent inhibition of thromboxane A₂ production in human endothelial cells. Endothelial cells were treated with 17β-estradiol (E₂, 100 nmol/L), the GPER-selective antagonist G36 (1 μmol/L), or solvent (DMSO 0.01%) for 24 hours, and thromboxane A₂ production was measured under basal conditions or after concomitant stimulation with the pro-inflammatory cytokine TNF-α (1 ng/mL). *P<0.05 vs. basal; †P<0.05 vs. solvent; #P<0.05 vs. 17β-estradiol. All data (n=3 independent experiments per group) are mean±s.e.m.

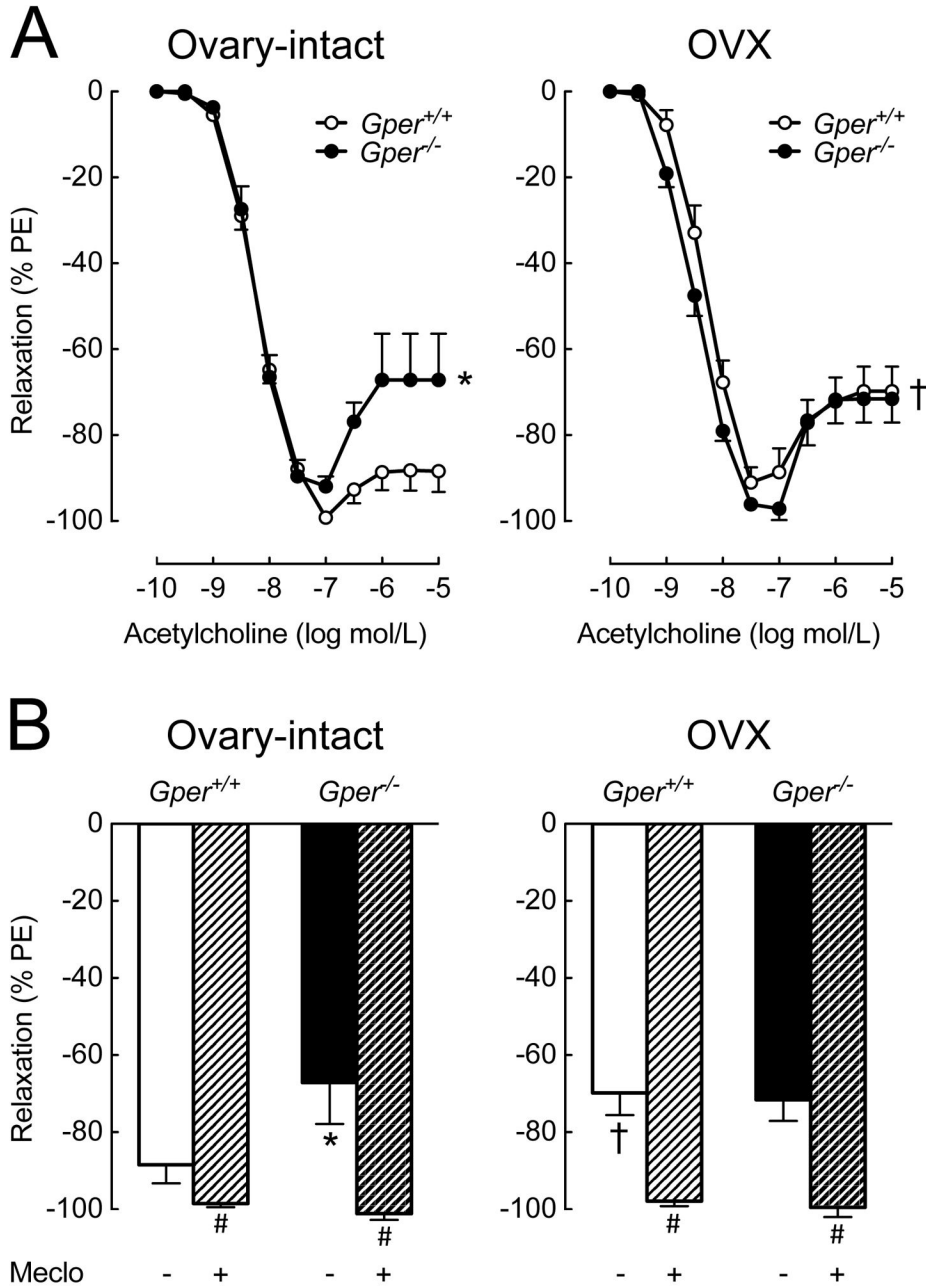


Figure 2. Effect of GPER and endogenous estrogens on cyclooxygenase-dependent, prostanoid-mediated vasoconstriction. A, Concentration-dependent dilations and contractions were induced by acetylcholine in arteries constricted with phenylephrine (PE). B, Responses to acetylcholine (10 μ mol/L) were obtained in the absence (-) and presence (+) of the cyclooxygenase inhibitor meclufenamate (Mecl, 1 μ mol/L). Arteries were isolated from ovary-intact and ovariectomized (OVX) wild-type ($Gper^{+/+}$) and $Gper^{-/-}$ mice fed a pro-inflammatory, high-fat diet. * $P < 0.05$ vs. wild-type; † $P < 0.05$ vs. ovary-intact; # $P < 0.05$ vs.

matched arteries in the absence of meclofenamate. All data (n=5–12 per group) are mean \pm s.e.m.

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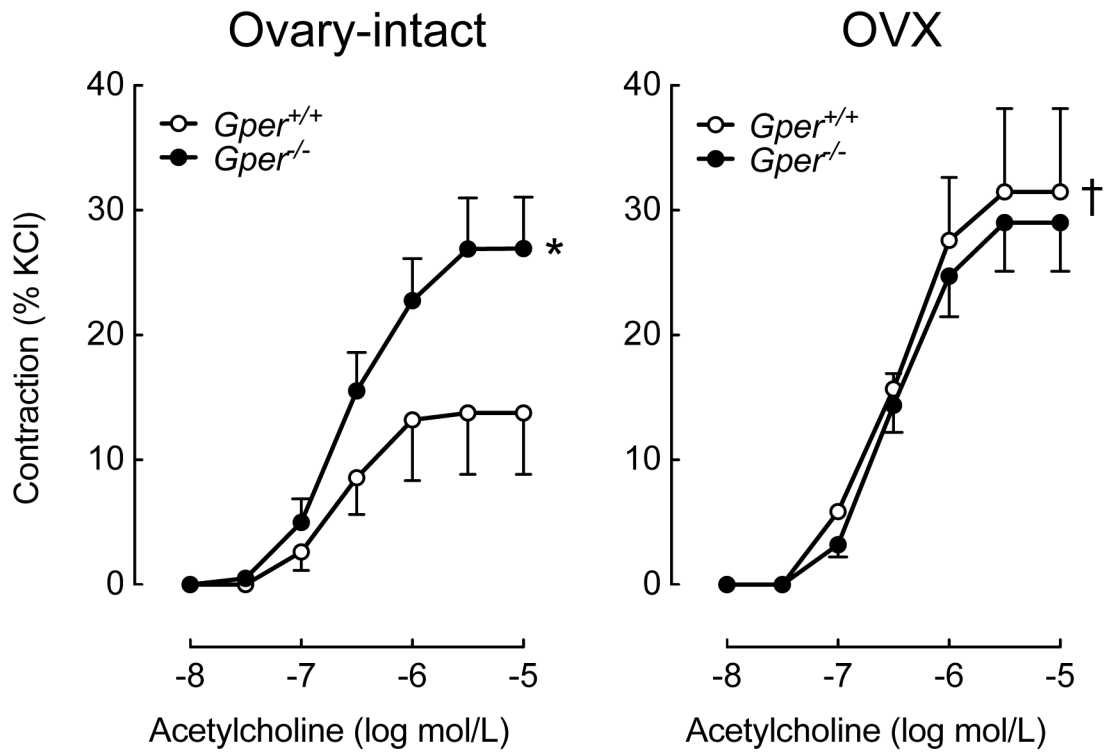


Figure 3.

NO-independent contractions to acetylcholine in arteries from ovary-intact and ovariectomized (OVX) wild-type (*Gper*^{+/+}) and *Gper*^{-/-} mice. Acetylcholine-dependent, prostanoid-mediated contractions were induced in the presence of the NO synthase inhibitor L-NAME (300 μ mol/L). Mice were fed a pro-inflammatory, high-fat diet. * $P < 0.05$ vs. wild-type; † $P < 0.05$ vs. ovary-intact. All data (n=5–8 per group) are mean \pm s.e.m.

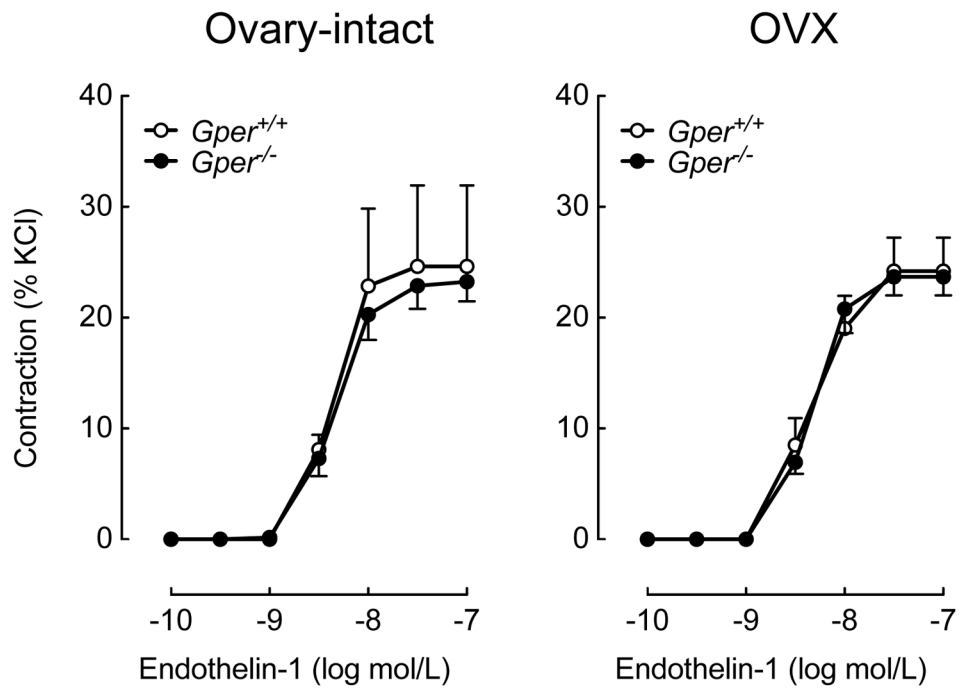


Figure 4. Endothelin-1-dependent vasoconstriction in ovary-intact and ovariectomized (OVX) mice fed a pro-inflammatory, high-fat diet. Responses were recorded in arteries from wild-type (*Gper*^{+/+}) and *Gper*^{-/-} mice in the presence of the NO synthase inhibitor L-NAME (300 μ mol/L). All data (n=5–8 per group) are mean \pm s.e.m.

Table 1

Vasoconstrictor responses to KCl, acetylcholine, the thromboxane-prostanoid receptor agonist U46619, and endothelin-1. Responses were determined in carotid arteries from ovary-intact and ovariectomized (OVX) wild-type (*Gper*^{+/+}) and *Gper*^{-/-} mice. Maximal responses, area under the curve, and pD₂ values were calculated based on fitting of dose-response curves to acetylcholine (10 nmol/L – 10 μmol/L) and endothelin-1 (0.1 nmol/L – 100 nmol/L) in the presence of the NO synthase inhibitor L-NAME (DeLean, *et al.* 1978). Area under the curve is expressed as arbitrary units (AU).

Stimulus	Ovary-intact		OVX	
	<i>Gper</i> ^{+/+}	<i>Gper</i> ^{-/-}	<i>Gper</i> ^{+/+}	<i>Gper</i> ^{-/-}
<i>KCl (60 mmol/L)</i>				
Response (mN)	5.8±0.3	5.5±0.3	5.8±0.3	6.2±0.4
<i>Acetylcholine</i>				
Maximal responses (% KCl)	14±4	27±4*	32±6 [†]	29±4
Area under the curve (AU)	22±6	43±6*	48±8 [†]	43±6
pD ₂ values (-log mol/L)	6.5±0.2	6.6±0.1	6.6±0.1	6.5±0.1
<i>U46619 (10 nmol/L)</i>				
Response (% KCl)	14±2	14±4	12±3	11±2
<i>Endothelin-1</i>				
Maximal responses (% KCl)	25±7	23±2	25±3	24±2
Area under the curve (AU)	34±10	31±3	32±4	32±2
pD ₂ values (-log mol/L)	8.3±0.1	8.3±0.1	8.3±0.1	8.3±0.1

* P<0.05 vs. *Gper*^{+/+};

[†] P<0.05 vs. ovary-intact.

All data (n=5–16 per group) are mean±s.e.m.