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

Matthias R Meyer[1,2], Natalie C Fredette[1], Matthias Barton[3] and Eric R Prossnitz[1]

[1]Department of Internal Medicine, University of New Mexico Health Sciences Center, 915 Camino de Salud NE, Albuquerque, New Mexico 87131, USA
[2]Department of Cardiology, Cantonal Hospital, Tellstrasse, 5001 Aarau, Switzerland
[3]Molecular Internal Medicine, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland

Correspondence should be addressed to M R Meyer or E R Prossnitz
Emails
matthias.meyer@ksa.ch or eprossnitz@salud.unm.edu

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 A_2. 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 A_2 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 A_2 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-N^G-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

Journal of Endocrinology (2015) **227**, 61–69

Journal of Endocrinology

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-15-0257

Published by Bioscientifica Ltd.

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 (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 & Davidge 2012, Meyer *et al.* 2012*a*,*b*, 2014*a*), 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 prostanoids 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 prostanoids at high concentrations (≥100 nmol/l) (Kauser & Rubanyi 1995, Traupe *et al.* 2002*a*, Zhang & Kosaka 2002, Zhou *et al.* 2005, Feletou & Vanhoutte 2006). Prostanoids, such as thromboxane A_2, 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 A_2 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 (Kauser & 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.* 2014*a*), 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 ovary-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.* 2014*a*).

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-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (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 $MgSO_4$, 0.43 NaH_2PO_4, 19 $NaHCO_3$, 1.8 $CaCl_2$, and 5.5 glucose; pH 7.4) to the required concentrations before use. Concentrations are expressed as final molar concentration in the organ chamber.

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-15-0257

Published by Bioscientifica Ltd.

Thromboxane A_2 production in human endothelial cells

Human endothelial cells of a h*TERT*-immortalized umbilical vein endothelial (TIVE) cell line, which expresses GPER (Meyer *et al*. 2014*a*), 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 mg/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*. 2014*a*). *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*. 2014*a*).

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*. 2014*b*, 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 prostanoids at concentrations ≥100 nmol/l (Traupe *et al*. 2002*b*, 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,

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-15-0257

Published by Bioscientifica Ltd.

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) WT (*Gper*$^{+/+}$) and *Gper*$^{-/-}$ mice. Maximal responses, area under the curve, and pD_2 values were calculated based on the fitting of dose-response curves to acetylcholine (10 nmol/l–10 μmol/l) and endothelin-1 (0.1–100 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). All data (n=5–16 per group) are mean±S.E.M.

	Ovary-intact		OVX	
Stimulus	WT	*Gper*$^{-/-}$	WT	*Gper*$^{-/-}$
KCl (60 mmol/l)				
Response (mN)	5.8±0.3	5.5±0.3	5.8±0.3	6.2±0.4
Acetylcholine				
Maximal response (% KCl)	14±4	27±4*	32±6†	29±4
Area under the curve (AU)	22±6	43±6*	48±8†	43±6
pD_2 value (−log mol/l)	6.5±0.2	6.6±0.1	6.6±0.1	6.5±0.1
U46619 (10 nmol/l)				
Response (% KCl)	14±2	14±4	12±3	11±2
Endothelin-1				
Maximal response (% KCl)	25±7	23±2	25±3	24±2
Area under the curve (AU)	34±10	31±3	32±4	32±2
pD_2 value (−log mol/l)	8.3±0.1	8.3±0.1	8.3±0.1	8.3±0.1

*P<0.05 vs WT; $^{\dagger}P$<0.05 vs ovary-intact.

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.* 2002*b*, 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 (300 μmol/l for 30 min), and concentration-dependent contractions to acetylcholine (10 nmol/l–10 μmol/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 (0.1–100 nmol/l), an endothelium-derived vasoconstrictor implicated in acetylcholine-dependent responses (Traupe *et al.* 2002*b*), were measured. These rings were pretreated with the NO synthase inhibitor L-NAME (300 μmol/l for 30 min) to unmask the weak contractions to endothelin-1 in murine carotid arteries (Traupe *et al.* 2002*a*, Meyer *et al.* 2014*b*).

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 EC_{50} values (as negative logarithm: pD_2) were calculated by the 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±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 A_2 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). TNF-α, a pro-inflammatory cytokine, increased thromboxane A_2 production by 160% (n=3, P<0.05 vs basal conditions; Fig. 1), which was prevented by 17β-estradiol (n=3, P<0.05 vs solvent; Fig. 1). Inhibition of thromboxane A_2 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.* 2014*a*).

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-15-0257

Published by Bioscientifica Ltd.

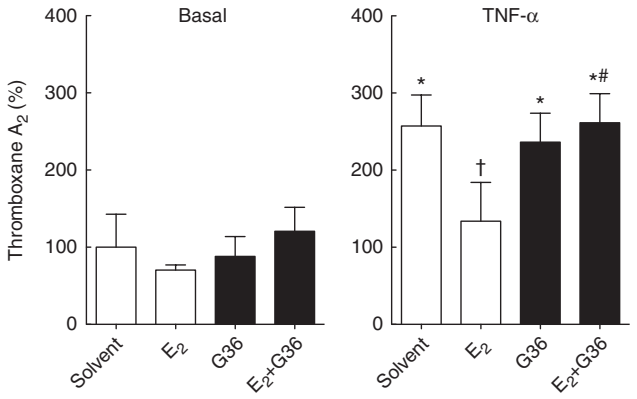

Figure 1
Role of GPER in estrogen-dependent inhibition of thromboxane A_2 production in human endothelial cells. Endothelial cells were treated with 17β-estradiol (E_2, 100 nmol/l), the GPER-selective antagonist G36 (1 μmol/l), or solvent (DMSO 0.01%) for 24 h, and thromboxane A_2 production was measured 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.

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 & Rubanyi 1995, Zhang & Kosaka 2002, Zhou *et al.* 2005, Feletou & 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 WT, Fig. 2A). Consistent with a response to vasoconstrictor prostanoids, acetylcholine-induced vasodilation was restored in both WT and $Gper^{-/-}$ mice by the COX inhibitor meclofenamate ($n=5$–12, $P<0.05$ vs untreated rings, Fig. 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 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 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 & Rubanyi 1995, Traupe *et al.* 2002*b*, Zhang & Kosaka 2002, Zhou *et al.* 2005, Feletou & 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 WT; Fig. 3 and Table 1). Ovariectomy of WT mice potentiated responses to vasoconstrictor prostanoids 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

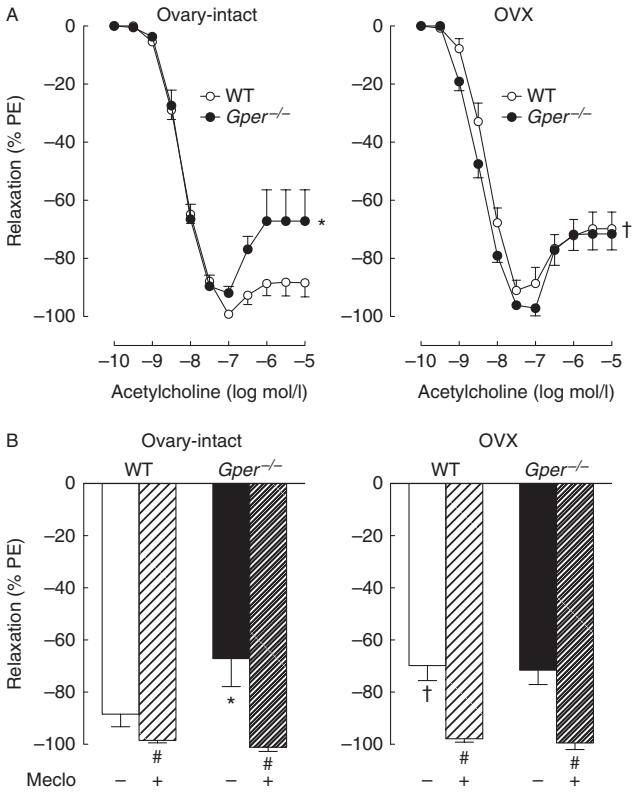

Figure 2
Effect of GPER and endogenous estrogens on cyclooxygenase-dependent, prostanoid-mediated vasoconstriction. (A) Concentration-dependent dilations and contractions were induced by acetylcholine in arteries precontracted with phenylephrine (PE). (B) Responses to acetylcholine (10 μmol/l) were obtained in the absence (−) and presence (+) of the cyclooxygenase inhibitor meclofenamate (Meclo, 1 μmol/l). Arteries were isolated from ovary-intact and ovariectomized (OVX) WT and $Gper^{-/-}$ mice fed a pro-inflammatory, high-fat diet. *$P<0.05$ vs WT; †$P<0.05$ vs ovary-intact; #$P<0.05$ vs matched arteries in the absence of meclofenamate. All data ($n=5$–12 per group) are mean ± S.E.M.

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-15-0257

Published by Bioscientifica Ltd.

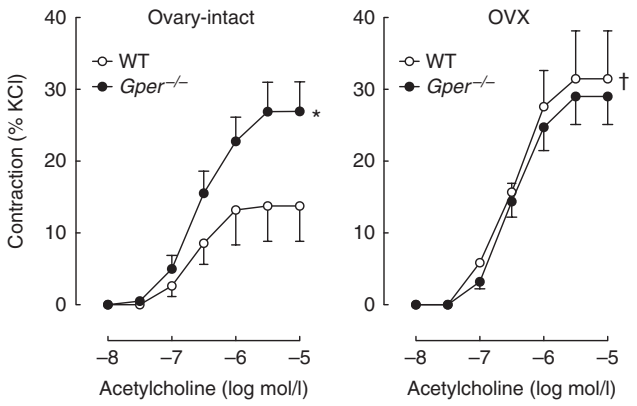

Figure 3
NO-independent contractions to acetylcholine in arteries from ovary-intact and ovariectomized (OVX) WT and *Gper*$^{-/-}$ mice. Acetylcholine-dependent, prostanoid-mediated contractions were induced in the presence of the NO synthase inhibitor L-NAME (300 μmol/l). Mice were fed a pro-inflammatory, high-fat diet. *$P<0.05$ vs WT; †$P<0.05$ vs ovary-intact. All data ($n=5$–8 per group) are mean±S.E.M.

of NO. In addition, neither the sensitivity to acetylcholine (pD_2 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.* 2002*b*). 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 A_2 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

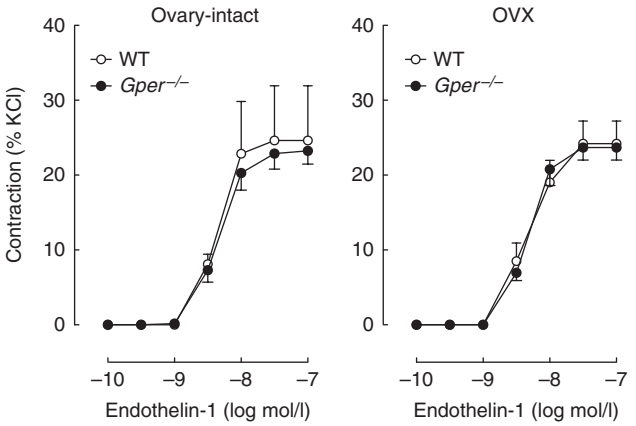

Figure 4
Endothelin-1-dependent vasoconstriction in ovary-intact and ovariectomized (OVX) mice fed a pro-inflammatory, high-fat diet. Responses were recorded in arteries from WT and *Gper*$^{-/-}$ mice in the presence of the NO synthase inhibitor L-NAME (300 μmol/l). All data ($n=5$–8 per group) are mean±S.E.M.

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-15-0257

Published by Bioscientifica Ltd.

in vivo (Meyer *et al.* 2014*a*). However, given that arachidonic acid is converted into various additional metabolites besides thromboxane A_2, such as prostaglandin G_2, prostaglandin H_2, prostaglandin D_2, prostaglandin E_2, prostaglandin $F_{2\alpha}$, and prostaglandin I_2, 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.* 2014*a*) 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.* 2012*a*). 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, 2012*a*, 2014*a*).

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 $F_{2\alpha}$ 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, 2012*a*, 2014*a*).

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.* 2012*b*), 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.* 2002*b*, 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 A_2 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

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-15-0257

Published by Bioscientifica Ltd.

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*. 2014*a*). 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.

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.

Funding

This work was supported by the National Institutes of Health (grant numbers R01 CA127731 and CA163890 to E R P); dedicated Health Research Funds from the University of New Mexico School of Medicine allocated to the Signature Program in Cardiovascular and Metabolic Diseases (to E R P); and the Swiss National Science Foundation (grant numbers 135874 and 141501 to M R M and grant numbers 108258 and 122504 to M B). N C F was supported by the National Institutes of Health (training grant number HL07736).

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.

Acknowledgements

We thank Dr Chelin Hu for expert technical assistance.

References

An FQ, Folarin HM, Compitello N, Roth J, Gerson SL, McCrae KR, Fakhari FD, Dittmer DP & Renne R 2006 Long-term-infected telomerase-immortalized endothelial cells: a model for Kaposi's sarcoma-associated herpesvirus latency *in vitro* and *in vivo*. *Journal of Virology* **80** 4833–4846. (doi:10.1128/JVI.80.10.4833-4846.2006)

Barrett-Connor E 2013 Menopause, atherosclerosis, and coronary artery disease. *Current Opinion in Pharmacology* **13** 186–191. (doi:10.1016/j.coph.2013.01.005)

Blasko E, Haskell CA, Leung S, Gualtieri G, Halks-Miller M, Mahmoudi M, Dennis MK, Prossnitz ER, Karpus WJ & Horuk R 2009 Beneficial role of the GPR30 agonist G-1 in an animal model of multiple sclerosis. *Journal of Neuroimmunology* **214** 67–77. (doi:10.1016/j.jneuroim.2009.06.023)

Brunsing RL & Prossnitz ER 2011 Induction of interleukin-10 in the T helper type 17 effector population by the G protein coupled estrogen receptor (GPER) agonist G-1. *Immunology* **134** 93–106. (doi:10.1111/j.1365-2567.2011.03471.x)

Burai R, Ramesh C, Shorty M, Curpan R, Bologa C, Sklar LA, Oprea T, Prossnitz ER & Arterburn JB 2010 Highly efficient synthesis and characterization of the GPR30-selective agonist G-1 and related tetrahydroquinoline analogs. *Organic & Biomolecular Chemistry* **8** 2252–2259. (doi:10.1039/c001307b)

Burke AP, Farb A, Malcom G & Virmani R 2001 Effect of menopause on plaque morphologic characteristics in coronary atherosclerosis. *American Heart Journal* **141** S58–S62. (doi:10.1067/mhj.2001.109946)

Chakrabarti S & Davidge ST 2012 G-protein coupled receptor 30 (GPR30): a novel regulator of endothelial inflammation. *PLoS ONE* **7** e52357. (doi:10.1371/journal.pone.0052357)

Chen S, Shimada K, Zhang W, Huang G, Crother TR & Arditi M 2010 IL-17A is proatherogenic in high-fat diet-induced and Chlamydia pneumoniae infection-accelerated atherosclerosis in mice. *Journal of Immunology* **185** 5619–5627. (doi:10.4049/jimmunol.1001879)

Dantas AP, Scivoletto R, Fortes ZB, Nigro D & Carvalho MH 1999 Influence of female sex hormones on endothelium-derived vasoconstrictor prostanoid generation in microvessels of spontaneously hypertensive rats. *Hypertension* **34** 914–919. (doi:10.1161/01.HYP.34.4.914)

Davidge ST & Zhang Y 1998 Estrogen replacement suppresses a prostaglandin H synthase-dependent vasoconstrictor in rat mesenteric arteries. *Circulation Research* **83** 388–395. (doi:10.1161/01.RES.83.4.388)

DeLean A, Munson PJ & Rodbard D 1978 Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *American Journal of Physiology* **235** E97–E102.

Denes A, Drake C, Stordy J, Chamberlain J, McColl BW, Gram H, Crossman D, Francis S, Allan SM & Rothwell NJ 2012 Interleukin-1 mediates neuroinflammatory changes associated with diet-induced atherosclerosis. *Journal of the American Heart Association* **1** e002006. (doi:10.1161/JAHA.112.002006)

Dennis MK, Field AS, Burai R, Ramesh C, Petrie WK, Bologa CG, Oprea TI, Yamaguchi Y, Hayashi SI, Sklar LA *et al*. 2011 Identification of a GPER/GPR30 antagonist with improved estrogen receptor counter-selectivity. *Journal of Steroid Biochemistry and Molecular Biology* **127** 358–366. (doi:10.1016/j.jsbmb.2011.07.002)

Feletou M & Vanhoutte PM 2006 Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture). *American Journal of Physiology. Heart and Circulatory Physiology* **291** H985–H1002. (doi:10.1152/ajpheart.00292.2006)

Gilligan DM, Quyyumi AA & Cannon RO III 1994 Effects of physiological levels of estrogen on coronary vasomotor function in postmenopausal women. *Circulation* **89** 2545–2551. (doi:10.1161/01.CIR.89.6.2545)

Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P & Chambon P 1986 Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* **320** 134–139. (doi:10.1038/320134a0)

Greene GL, Gilna P, Waterfield M, Baker A, Hort Y & Shine J 1986 Sequence and expression of human estrogen receptor complementary DNA. *Science* **231** 1150–1154. (doi:10.1126/science.3753802)

Haas E, Bhattacharya I, Brailoiu E, Damjanovic M, Brailoiu GC, Gao X, Mueller-Guerre L, Marjon NA, Gut A, Minotti R *et al*. 2009 Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity. *Circulation Research* **104** 288–291. (doi:10.1161/CIRCRESAHA.108.190892)

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-15-0257

Published by Bioscientifica Ltd.

Hodis HN, Mack WJ, Lobo RA, Shoupe D, Sevanian A, Mahrer PR, Selzer RH, Liu CR, Liu CH & Azen SP 2001 Estrogen in the prevention of atherosclerosis. A randomized, double-blind, placebo-controlled trial. *Annals of Internal Medicine* **135** 939–953. (doi:10.7326/0003-4819-135-11-200112040-00005)

Horio Y, Yasue H, Rokutanda M, Nakamura N, Ogawa H, Takaoka K, Matsuyama K & Kimura T 1986 Effects of intracoronary injection of acetylcholine on coronary arterial diameter. *American Journal of Cardiology* **57** 984–989. (doi:10.1016/0002-9149(86)90743-5)

Isensee J, Meoli L, Zazzu V, Nabzdyk C, Witt H, Soewarto D, Effertz K, Fuchs H, Gailus-Durner V, Busch D *et al*. 2009 Expression pattern of G protein-coupled receptor 30 in LacZ reporter mice. *Endocrinology* **150** 1722–1730. (doi:10.1210/en.2008-1488)

Kauser K & Rubanyi GM 1995 Gender difference in endothelial dysfunction in the aorta of spontaneously hypertensive rats. *Hypertension* **25** 517–523. (doi:10.1161/01.HYP.25.4.517)

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S & Gustafsson JA 1996 Cloning of a novel receptor expressed in rat prostate and ovary. *PNAS* **93** 5925–5930. (doi:10.1073/pnas.93.12.5925)

Lin JA, Watanabe J, Rozengurt N, Narasimha A, Martin MG, Wang J, Braun J, Langenbach R & Reddy ST 2007 Atherogenic diet causes lethal ileo-ceco-colitis in cyclooxygenase-2 deficient mice. *Prostaglandins & Other Lipid Mediators* **84** 98–107. (doi:10.1016/j.prostaglandins.2007.04.004)

Lindsey SH, Cohen JA, Brosnihan KB, Gallagher PE & Chappell MC 2009 Chronic treatment with the G protein-coupled receptor 30 agonist G-1 decreases blood pressure in ovariectomized mRen2.Lewis rats. *Endocrinology* **150** 3753–3758. (doi:10.1210/en.2008-1664)

Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW & Ganz P 1986 Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *New England Journal of Medicine* **315** 1046–1051. (doi:10.1056/NEJM198610233151702)

Mehta JL, Lawson D, Mehta P & Saldeen T 1988 Increased prostacyclin and thromboxane A2 biosynthesis in atherosclerosis. *PNAS* **85** 4511–4515. (doi:10.1073/pnas.85.12.4511)

Meyer MR, Baretella O, Prossnitz ER & Barton M 2010 Dilation of epicardial coronary arteries by the G protein-coupled estrogen receptor agonists G-1 and ICI 182,780. *Pharmacology* **86** 58–64. (doi:10.1159/000315497)

Meyer MR, Amann K, Field AS, Hu C, Hathaway HJ, Kanagy NL, Walker MK, Barton M & Prossnitz ER 2012*a* Deletion of G protein-coupled estrogen receptor increases endothelial vasoconstriction. *Hypertension* **59** 507–512. (doi:10.1161/HYPERTENSIONAHA.111.184606)

Meyer MR, Field AS, Kanagy NL, Barton M & Prossnitz ER 2012*b* GPER regulates endothelin-dependent vascular tone and intracellular calcium. *Life Sciences* **91** 623–627. (doi:10.1016/j.lfs.2012.01.007)

Meyer MR, Fredette NC, Howard TA, Hu C, Ramesh C, Daniel C, Amann K, Arterburn JB, Barton M & Prossnitz ER 2014*a* G protein-coupled estrogen receptor protects from atherosclerosis. *Scientific Reports* **4** 7564. (doi:10.1038/srep07564)

Meyer MR, Fredette N, Barton M & Prossnitz ER 2014*b* Endothelin-1 but not angiotensin II contributes to functional aging in murine carotid arteries. *Life Sciences* **118** 213–218. (doi:10.1016/j.lfs.2014.02.027)

Meyer MR, Fredette NC, Barton M & Prossnitz ER 2015 Prostanoid-mediated contractions of the carotid artery become Nox2-independent with aging. *Age* **37** 9806. (doi:10.1007/s11357-015-9806-9)

Murphy E 2011 Estrogen signaling and cardiovascular disease. *Circulation Research* **109** 687–696. (doi:10.1161/CIRCRESAHA.110.236687)

Nakahata N 2008 Thromboxane A2: physiology/pathophysiology, cellular signal transduction and pharmacology. *Pharmacology & Therapeutics* **118** 18–35. (doi:10.1016/j.pharmthera.2008.01.001)

Paigen B, Morrow A, Holmes PA, Mitchell D & Williams RA 1987 Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* **68** 231–240. (doi:10.1016/0021-9150(87)90202-4)

Revankar CM, Cimino DF, Sklar LA, Arterburn JB & Prossnitz ER 2005 A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* **307** 1625–1630. (doi:10.1126/science.1106943)

Ricciotti E & FitzGerald GA 2011 Prostaglandins and inflammation. *Arteriosclerosis, Thrombosis, and Vascular Biology* **31** 986–1000. (doi:10.1161/ATVBAHA.110.207449)

Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC *et al*. 2002 Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *Journal of the American Medical Association* **288** 321–333. (doi:10.1001/jama.288.3.321)

Schenck-Gustafsson K, Brincat M, Erel CT, Gambacciani M, Lambrinoudaki I, Moen MH, Tremollieres F, Vujovic S, Rozenberg S, Rees M *et al*. 2011 EMAS position statement: managing the menopause in the context of coronary heart disease. *Maturitas* **68** 94–97. (doi:10.1016/j.maturitas.2010.10.005)

Schiffrin EL 2013 The immune system: role in hypertension. *Canadian Journal of Cardiology* **29** 543–548. (doi:10.1016/j.cjca.2012.06.009)

Sharma G, Hu C, Brigman JL, Zhu G, Hathaway HJ & Prossnitz ER 2013 GPER deficiency in male mice results in insulin resistance, dyslipidemia, and a proinflammatory state. *Endocrinology* **154** 4136–4145. (doi:10.1210/en.2013-1357)

Sobrino A, Oviedo PJ, Novella S, Laguna-Fernandez A, Bueno C, Garcia-Perez MA, Tarin JJ, Cano A & Hermenegildo C 2010 Estradiol selectively stimulates endothelial prostacyclin production through estrogen receptor-α. *Journal of Molecular Endocrinology* **44** 237–246. (doi:10.1677/JME-09-0112)

Sudhir K, Ko E, Zellner C, Wong HE, Hutchison SJ, Chou TM & Chatterjee K 1997 Physiological concentrations of estradiol attenuate endothelin 1-induced coronary vasoconstriction *in vivo*. *Circulation* **96** 3626–3632. (doi:10.1161/01.CIR.96.10.3626)

Teoh H, Quan A, Leung SW & Man RY 2000 Differential effects of 17β-estradiol and testosterone on the contractile responses of porcine coronary arteries. *British Journal of Pharmacology* **129** 1301–1308. (doi:10.1038/sj.bjp.0703164)

Thomas P, Pang Y, Filardo EJ & Dong J 2005 Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* **146** 624–632. (doi:10.1210/en.2004-1064)

Traupe T, Lang M, Goettsch W, Munter K, Morawietz H, Vetter W & Barton M 2002*a* Obesity increases prostanoid-mediated vasoconstriction and vascular thromboxane receptor gene expression. *Journal of Hypertension* **20** 2239–2245. (doi:10.1097/00004872-200211000-00024)

Traupe T, D'Uscio LV, Muenter K, Morawietz H, Vetter W & Barton M 2002*b* Effects of obesity on endothelium-dependent reactivity during acute nitric oxide synthase inhibition: modulatory role of endothelin. *Clinical Science* **103**(Suppl 48) 13S–15S. (doi:10.1042/CS103S013S)

Zhang L & Kosaka H 2002 Sex-specific acute effect of estrogen on endothelium-derived contracting factor in the renal artery of hypertensive Dahl rats. *Journal of Hypertension* **20** 237–246. (doi:10.1097/00004872-200202000-00013)

Zhou Y, Varadharaj S, Zhao X, Parinandi N, Flavahan NA & Zweier JL 2005 Acetylcholine causes endothelium-dependent contraction of mouse arteries. *American Journal of Physiology. Heart and Circulatory Physiology* **289** H1027–H1032. (doi:10.1152/ajpheart.00226.2005)

Received in final form 17 August 2015
Accepted 24 August 2015
Accepted Preprint published online 24 August 2015

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-15-0257

Published by Bioscientifica Ltd.