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REVIEW ARTICLE

GPCRs in context: sexual dimorphism in the cardiovascular system

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Cardiovascular disease (CVD) remains the largest cause of mortality worldwide, and there is a clear gender gap in disease occurrence, with men being predisposed to earlier onset of CVD, including atherosclerosis and hypertension, relative to women. Oestrogen may be a driving factor for female-specific cardioprotection, though androgens and sex chromosomes are also likely to contribute to sexual dimorphism in the cardiovascular system (CVS). Many GPCR-mediated processes are involved in cardiovascular homeostasis, and some exhibit clear sex divergence. Here, we focus on the G protein-coupled oestrogen receptor, endothelin receptors ET_A and ET_B and the eicosanoid G protein-coupled receptors (GPCRs), discussing the evidence and potential mechanisms leading to gender dimorphic responses in the vasculature. The use of animal models and pharmacological tools has been essential to understanding the role of these receptors in the CVS and will be key to further delineating their sex-specific effects. Ultimately, this may illuminate wider sex differences in cardiovascular pathology and physiology.

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Abbreviations

ASMC, aortic smooth muscle cell; CVD, cardiovascular disease; CVS, cardiovascular system; DHT, dihydrotestosterone; E_2, 17β-oestradiol; ECE, endothelin-converting enzyme; EETs, epoxyeicosatrienoic acids; EGFR, EGF receptor; ERα/ERβ, oestrogen receptor α or β; ERK1/2, extracellular-regulated kinases 1 and 2; ET-1/2/3, endothelin 1, 2 or 3; GPER, G protein-coupled oestrogen receptor; 20-HETE, 20-hydroxyeicosatetraenoic acid; LV, left ventricle/ventricular; MAP, mean arterial pressure; mPEGES1, microsomal PGE synthase 1; OVX, ovariectomized; PTX, *Pertussis* toxin; SHR, spontaneously hypertensive rat; VSMC, vascular smooth muscle cell

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Introduction

Cardiovascular disease (CVD) is collectively responsible for one in four deaths and is currently the leading cause of death worldwide (WHO, 2014). Premenopausal women have lower blood pressure (BP) and a reduced incidence of CVD and related mortality than age-matched men (Pilote *et al.*, 2007). During menopause, the ratio of sex hormones changes dramatically: circulating **oestrogen (17β-oestradiol)** levels decrease by >90%, **oestrone** by 70% and **testosterone** by 40% (Rothman *et al.*, 2011) (see Figure 1A for sex hormone synthesis). Despite this decline in oestrogen abundance, the female cardiovascular death rate does not increase at the age of menopause, suggesting that oestrogen is not the only cause of female cardioprotection (Liu *et al.*, 2003). Further, hormone replacement therapy with conjugated equine oestrogens plus **medroxyprogesterone** in postmenopausal women does not reduce primary and secondary cardiovascular events to premenopausal levels (Hulley *et al.*, 1998). Therefore, it is likely that the balance of sex hormones in addition to sex chromosome complement contributes to sexual dimorphism in the cardiovascular system (CVS).

Genetic models of hypertension, including oestrogen-sensitive mRen2.Lewis (Chappell *et al.*, 2008), Dahl salt-sensitive (Hinojosa-Laborde *et al.*, 2000) and spontaneously hypertensive rats (SHRs) (Reckelhoff *et al.*, 2000), display exacerbated hypertension in males compared with females, an effect that appears to be partially mediated by sex hormones, either *via* oestrogenic protection or exacerbation by testosterone. 17β-Oestradiol (E_2) is the principal endogenous oestrogen in females of reproductive age and is typically more physiologically potent than the other endogenous oestrogens, oestrone and **oestriol**. While E_2 is primarily produced by the ovaries of premenopausal women, oestradiol is also produced locally by aromatase conversion of androgens in other tissues including the vasculature, adipose tissue and the brain of both males and females (Nelson and Bulun, 2001), consistent with roles of oestrogen not only in female reproduction but also in various physiological systems. In the CVS, E_2 is generally regarded as cardioprotective, with effects including vasodilation, reduction of vascular inflammation and inhibition of vascular smooth muscle cell (VSMC) proliferation. These effects of E_2 are both direct, through activation of cognate oestrogen receptors, and indirect, through modulation of other important receptor systems involved in cardiovascular homeostasis. When discussing differences between males and females, consistency is needed in the use of terms *sex* and *gender*. Based on definitions recommended by the Institute of Medicine (Pardue and Wizemann, 2001), the term 'gender' as used here refers to differences between men and women based on self-identity, while 'sex' is used to describe differences in animals based on chromosomal complement and reproductive organs.

In this review, we discuss the role of **GPCRs** in sexually dimorphic cardiovascular physiology and pathophysiology. GPCRs are the most successful family of drug targets to date (Rask-Andersen *et al.*, 2011) and are intimately involved in many physiological systems, positioning them as a research area of interest as novel therapeutic targets for disease. Sex differences have been noted in a number of key GPCR mediators in the CVS, most notably the angiotensin II **AT_1** and **AT_2** receptors of the renin–angiotensin system (Sullivan, 2008) and also vasopressin receptors (Bankir, 2001), relaxin receptors (Samuel *et al.*, 2017) and the adrenoceptors (Luzier *et al.*, 1998). For brevity, we focus on three key receptor systems that exhibit sexual dimorphism in their cardiovascular effects: G protein-coupled oestrogen receptor (GPER), endothelin (ET) receptors and eicosanoid receptors.

G protein-coupled oestrogen receptor

The classical nuclear oestrogen receptors **ERα** and **ERβ** act as ligand-activated transcription factors and are responsible for the long-term effects of oestrogens (Prossnitz and Arterburn, 2015). Contrastingly, rapid oestrogenic signalling occurs *via*

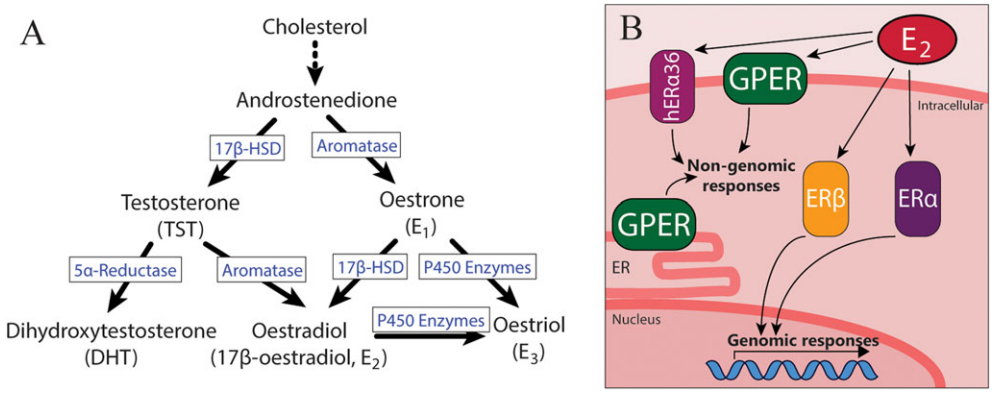

Figure 1

Diagram of (A) sex hormone biosynthetic pathways and (B) oestrogen receptor localization within the cell. Oestrogens and androgens are both produced by sequential steps from cholesterol. Importantly, E_2 can be produced from both oestrogenic and androgenic precursors. E_2 can act on intracellular ERα and ERβ and their splice variants, including membrane localized human ER α-36 (hERα36). Additionally, the GPERs also respond to E_2 and is localized to either the plasma membrane or intracellular membranes depending on cell type. 17β-HSD, 17β-hydroxysteroid dehydrogenase.

membrane-associated oestrogen receptors (ERs), which include splice variants of ERα and a third member of the family, the GPER (Prossnitz and Arterburn, 2015) (Figure 1B).

GPER is expressed widely in vascular, lung, kidney, gastrointestinal, heart, adrenal, brain and nervous system tissues (Isensee *et al.*, 2008; Uhlen *et al.*, 2015) and is involved in the immune, nervous and cardiovascular systems, as well as cancer development, though is less important than the nuclear ERs for reproduction. The broader involvement of GPER in physiology has been reviewed extensively by Prossnitz and Arterburn (2015); here, we focus on the gender-specific effects of GPER in the CVS.

GPER ligands and signalling

GPER (previously known as GPR30) was relatively recently deorphanized as an oestrogen receptor, with high affinity for 17β-oestradiol (pK_i = 8.2–8.5 nM) and low affinity for other endogenous oestrogens (Revankar *et al.*, 2005; Thomas *et al.*, 2005). GPER exhibits >1000-fold selectivity for E_2 over testosterone, **cortisol** and **progesterone** (Thomas *et al.*, 2005). In addition to endogenous estrogenic compounds, a number of phytoestrogens and xenoestrogens, including **resveratrol**, **quercetin** and **bisphenol A**, have also been shown to have agonist activity at GPER (Prossnitz and Arterburn, 2015). Ligands with high selectivity for GPER over ERα and ERβ have been developed to probe GPER physiology *in vivo* and *in vitro* and include agonist **G-1** (Bologa *et al.*, 2006) and antagonists **G15** (Dennis *et al.*, 2009) and **G36** (Dennis *et al.*, 2011). Interestingly, two compounds that are used clinically as negative modulators of nuclear ER activity, **tamoxifen** and **fulvestrant (ICI 182 780)**, are both full agonists at GPER (Thomas *et al.*, 2005).

Understanding the pharmacology of GPER has been complicated by seemingly discrepant results, and indeed, subcellular localization and cell-type specific signalling of GPER remain controversial. While GPER was initially identified as a membrane-associated oestrogen receptor mediating rapid signalling in response to E_2 (Filardo *et al.*, 2000; Thomas *et al.*, 2005), there is also evidence for the localization of GPER to intracellular sites, such as the Golgi and endoplasmic reticulum (Revankar *et al.*, 2005; Otto *et al.*, 2008). Additionally, changes in subcellular localization of GPER were observed throughout the oestrous cycle in mice (Cheng *et al.*, 2014); hence, sex hormone abundance may account for the discrepancies in cellular GPER distribution.

To add further complexity, GPER appears to signal through both $G\alpha_s$ and $G\alpha_{i/o}$ G proteins, sometimes in the same cell type. Filardo *et al.* (2000) initially postulated GPER to be $G\alpha_{i/o}$ coupled, leading to transactivation of the **EGF receptor (EGFR)**, initiation of **extracellular-regulated kinase (ERK)** phosphorylation, **PI3K/Akt** signalling and calcium mobilization. Subsequently, the same group reported $G\alpha_s$ signalling, causing cAMP generation that quenched activation of ERK1/2 generated by the $G\alpha_{i/o}$ pathway (Filardo *et al.*, 2002). A GPER-mediated increase in intracellular cAMP was also reported by Thomas *et al.* (2005). However, while an independent group did confirm that EGFR transactivation is a downstream effect of GPER stimulation and that it occurs in both COS-7 and the MDA-MB-231 cells used by Filardo *et al.* (2000), they found no evidence of EGFR activation being sensitive to the $G\alpha_{i/o}$ inhibitor, *Pertussis* toxin (PTX), suggesting that this element of GPER signalling does not involve coupling to $G\alpha_{i/o}$ (Revankar *et al.*, 2005). Interestingly, this report also showed that GPER-mediated calcium mobilization is partly inhibited by PTX and is also partly dependent on EGFR activation (Revankar *et al.*, 2005), suggesting multiplicity of signalling modes for GPER. These discordant results highlight the complexity of GPER pharmacology and the likelihood of cell type-specific signalling pathways.

Tissue expression and role in the cardiovascular system

Direct vasodilatory effects of oestrogen in the vasculature are likely to be mediated by GPER. In isolated aortae of female mRen2.Lewis rats preconstricted with phenylephrine, the magnitude of vasodilation produced by E_2 was identical to that produced by the agonist G-1 (Lindsey *et al.*, 2011a). Additionally, acute i.v. infusion of G-1 also caused a dose-dependent decrease in BP in normotensive male Sprague–Dawley rats of up to 15% within minutes (Haas *et al.*, 2009), while chronic infusion of G-1 for 2 weeks lowers mean arterial pressure (MAP) by 20% in the hypertensive ovariectomized (OVX) mRen2.Lewis rats (Lindsey *et al.*, 2009). However, in this study, G-1 infusion failed to reduce the BP of normotensive intact female and hypertensive male mRen2.Lewis rats (Lindsey *et al.*, 2009), potentially implicating GPER in BP control in males at baseline and in females during chronic hypertension. Furthermore, GPER plays an important role in maintaining basal tone in arteries, as shown by enhanced vasoconstriction and impaired vasodilation in response to the GPER antagonist G15 (Meyer *et al.*, 2012a). In addition to acute effects on the circulatory system, abundance of GPER in kidney (Kurt and Buyukafsar, 2013) and the CNS, particularly in the autonomic nuclei of the brainstem and the hypothalamic–pituitary axis (Brailoiu *et al.*, 2007), suggests a role for GPER in long-term maintenance of BP homeostasis. As such, GPER is important for both acute and chronic cardiovascular responses to oestrogen.

Despite uncertainty surrounding the coupling of GPER, as discussed above, the downstream effectors that mediate vasodilation are generally agreed upon. Within the vasculature, GPER-positive immunostaining was reported in both endothelial and VSMCs in rat thoracic aorta (Lindsey *et al.*, 2009), as well as rat carotid and middle cerebral arteries of both sexes (Broughton *et al.*, 2010), in agreement with a GPER-lacZ reporter mouse model (Isensee *et al.*, 2008) and consistent with both endothelium-dependent and endothelium-independent actions of GPER agonists. GPER-dependent vasodilation is mediated both by the endothelium and VSMCs, as endothelial denudation reduces, but does not completely abolish, G-1-stimulated vasodilation (Lindsey *et al.*, 2009). Activation of endothelial GPER triggers calcium mobilization and PI3K/Akt activation to produce nitric oxide (**NO**), as well as potassium efflux and membrane hyperpolarization, both of which lead to VSMC relaxation (Meyer *et al.*, 2012a; Lindsey *et al.*, 2014). G-1 also acts directly on VSMCs to induce vasodilation *via* cAMP accumulation and modulation of large conductance potassium channels (Lindsey *et al.*, 2014). Interestingly, GPER agonists produce similar degrees of vasorelaxation in isolated carotid arteries from male and female rats (Broughton *et al.*, 2010). Additionally, there

is evidence for antioxidant effects of GPER agonism with G-1 in the kidney (Lindsey *et al.*, 2011b), and G-1 itself may act directly as a scavenger of superoxide anions, further increasing its vasculoprotective actions (Broughton *et al.*, 2010).

There is evidence for regulation of GPER expression by female sex hormones, with higher protein abundance in the kidney during oestrus and pro-oestrus (Cheng *et al.*, 2014) and increased mRNA and protein expression in SKBR3 cells following prolonged treatment with progesterone or E_2 (Thomas *et al.*, 2005). However, GPER protein is expressed in rodent heart tissue at similar levels in both sexes (Deschamps and Murphy, 2009).

Genetic deletion studies have given further clues to the metabolic and cardiovascular effects of GPER, although debate remains due to discordant results. Female mice of the GPER knockout mouse line described by Martensson *et al.* (2009) had 23% higher MAP at 9 months, due to changes in resistance artery structure. The female GPER null mice also had impaired metabolism, with reduced body weight due to impaired skeletal growth (though no alterations in white adipose tissue relative to body weight) and hyperglycaemia due to glucose intolerance. No such metabolic or cardiovascular effects were seen in males. Contrastingly, Haas *et al.* (2009) observed increased body weight and adiposity in $GPER^{-/-}$ mice of both sexes. In further contrast to Martensson *et al.* (2009), no BP difference between genotypes was seen by Isensee *et al.* (2008), though this may be attributable to the animals being studied at a younger age or the mixed-strain background used by Martensson *et al.* (2009), or differing gene targeting strategies (cre/lox system used by Martensson *et al.* (2009), Neo^r/LacZ cassette insertion into exon 3 used by Isensee *et al.* (2008)). Intriguingly, Wang *et al.* (2016) report that cardiomyocyte-specific deletion of GPER causes left ventricular (LV) dysfunction and remodelling in both sexes but a male-specific inflammatory response. This result is difficult to reconcile with the above studies, which show no deleterious cardiovascular effects in male mice with global GPER gene deletion, especially considering the similar expression of cardiac GPER between the sexes (Deschamps and Murphy, 2009). It is unlikely that these discrepancies are due to compensatory increases in the expression of classical ERs, as both Wang *et al.* (2016) and Martensson *et al.* (2009) reported no change in mRNA abundance of ERα and ERβ. Thus, despite GPER being a receptor for a primarily female sex hormone, there is surprisingly little conclusive evidence for sex differences in effects of this receptor, exacerbated by the fact that many initial BP characterization studies in rodents did not compare both sexes simultaneously (Haas *et al.*, 2009; Lindsey *et al.*, 2011a; Meyer *et al.*, 2012a).

Interactions of GPER with the angiotensin system

Oestrogen is known to decrease expression of the pro-hypertensive AT_1 receptor, and it is likely that actions of GPER contribute to this. Treatment with GPER agonist, G-1, in OVX mRen2.Lewis rats reduced mRNA for AT_1 receptors in the aorta (Lindsey *et al.*, 2009) but had no effect on the abundance of AT_2 receptor transcript. In addition, the oestradiol metabolite, 2-methoxyestradiol, exerts similar effects on AT_1 receptor expression in a GPER-dependent and EGFR-dependent manner (Koganti *et al.*, 2014), which may partially explain the female-specific cardioprotective effects of **CYP450 1B1**, which produces 2-methoxyestradiol (Pingili *et al.*, 2017).

Interactions of GPER with the endothelin system

GPER is known to inhibit **ET-1**-induced contractions in isolated porcine coronary arteries (Meyer *et al.*, 2010). Similarly, isolated carotid arteries from GPER null mice had augmented contractile responses to ET-1, indicating that endogenous GPER dulls the vasoconstrictive response to ET-1, an effect that may be due to decreased sensitivity of contractile machinery to Ca^{2+} release in VSMCs (Meyer *et al.*, 2012b). Additionally, $GPER^{-/-}$ mice do not exhibit an increase in **ET_B** receptors and **endothelin-converting enzyme (ECE)-2** in the ageing myocardium, ET system components, which may be associated with heart failure and hypertrophy in older animals (Meyer *et al.*, 2016). Interestingly, there is also evidence that ET-1 increases mRNA expression of GPER and that GPER is necessary for some downstream actions of ET-1 in SKBR3 and HepG2 cell lines (Bartella *et al.*, 2016).

Potential for GPER–mineralocorticoid receptor crosstalk

GPER appears to be necessary for some rapid actions of **aldosterone** (Gros *et al.*, 2013) which may be more efficacious than oestrogen to activate GPER-dependent downstream signalling (Gros *et al.*, 2011). However, competition binding assays indicate that aldosterone does not directly bind to GPER, at least not at the same site as E_2, and does not cause [^{35}S]GTPγS binding as E_2 does (Cheng *et al.*, 2014). This excludes GPER as a receptor for aldosterone but may imply functional crosstalk between GPER and the **mineralocorticoid receptor (NR3C2)**, the cognate receptor for aldosterone.

Clinical relevance

The GPER maps to human chromosome 7p22.3, a locus that has been associated with hypertension due to familial hyperaldosteronism-II (Lafferty *et al.*, 2000), suggesting a role in BP homeostasis. A single nucleotide polymorphism (rs11544331) in the gene is associated with elevated BP in women but not men. Furthermore, in patients with resistant hypertension, females are almost twice as likely to carry the variant, with an allele frequency of 31% compared with 16% (Feldman *et al.*, 2014), suggesting that, particularly in women, GPER plays a role in BP regulation. In rat aortic VSMCs, this Phe16Leu variant showed hyporesponsiveness and a reduced ability to stimulate ERK phosphorylation and apoptosis in response to G-1 compared with animals expressing wild-type GPER (Feldman *et al.*, 2014).

The therapeutic potential of GPER in the CVS is fourfold. Firstly, GPER itself may be a useful target for hypertension, considering that G-1 has both acute and chronic antihypertensive actions in males and females (Haas *et al.*, 2009; Lindsey *et al.*, 2009), as well as protective effects on LV function and remodelling (Wang *et al.*, 2012), and will not activate potentially deleterious classical ER signalling in postmenopausal women (Hulley *et al.*, 1998). Secondly, GPER agonism has protective effects following myocardial

ischaemia/reperfusion injury, as prior treatment with G-1 reduces infarction size and improves recovery of contractile function in a rat model of ischaemic injury (Deschamps and Murphy, 2009). Thirdly, GPER antagonism by centrally administered G15 reduces stroke size and neurological deficit in male mice following a 1 h period of middle cerebral artery occlusion, even when administered up to 3 h after the ischaemic event (Broughton *et al.*, 2014). Lastly, GPER may also be an attractive candidate in atherosclerosis treatment and prevention; anti-inflammatory effects of oestrogen are likely partially mediated by GPER, and it is known that **raloxifene** (a selective oestrogen receptor modulator and GPER agonist) provides protection against atherosclerotic processes (Prossnitz and Barton, 2009). Agonism of GPER also has anti-inflammatory and anti-proliferative effects; in umbilical vein endothelial cells, G-1 abolished the pro-inflammatory effects of TNF-α, which is known to be involved in atherosclerosis and other inflammatory processes (Chakrabarti and Davidge, 2012). Similarly, G-1 and ICI 182780 inhibited serum-stimulated proliferation of human VSMCs (Haas *et al.*, 2009), which might be a valuable therapeutic approach for preventing excessive VSMC proliferation, as seen in atherosclerosis and following coronary stent implantation.

Endothelins and endothelin receptors

ET-1 is well known as one of the most important biological vasoconstrictors, with an unusually potent and prolonged action (Yanagisawa *et al.*, 1988). ET-1, along with two other isoforms, **ET-2** and **ET-3**, comprise the family of biologically active ET peptides. ET-1 and ET-2 have equal affinity for both ET receptors, **ET_A** and ET_B, while ET-3 has higher affinity for ET_B over ET_A receptors (see Figure 2 for a schematic representation of ET receptor ligands mentioned in this review; for a more comprehensive list, please refer to Davenport *et al.*, 2016). All are produced from inactive precursors by the action of ECEs. There are established sex differences in the ET system that may partially explain sex differences in the development of hypertension and other CVD, previously reviewed by Gillis *et al.* (2016) but briefly summarized here.

Female gonadal hormones have depressant effects on circulating ET-1 levels. Ovariectomy of Sprague–Dawley rats increases plasma concentration of ET-1 and aortic expression of prepro-ET-1 (Tan *et al.*, 2003), indicating that female hormones favourably decrease pro-hypertensive ET system components. As well as E_2, oestrogen metabolites, 2-hydroxyestradiol and 2-methoxyestradiol, inhibited ET-1 release from porcine coronary artery endothelial cells, which was not blocked by the ERα/ERβ antagonist, ICI 182780, indicating a mechanism that is independent of nuclear ERs (Dubey *et al.*, 2001). Interestingly, there is evidence for involvement of GPER in the response to ET-1; GPER inhibits vasoconstrictor responses to ET-1, while in isolated arteries from $GPER^{-/-}$ mice, ET-1-induced vasoconstriction is enhanced due to sensitization of myofilaments to calcium release (Meyer *et al.*, 2012b).

ET_A receptors

ET_A, a $G\alpha_{q/11}$-coupled GPCR, is expressed ubiquitously in VSMCs and promotes potent vasoconstriction through intracellular calcium release. The ET_A receptor is the predominant subtype expressed in cardiomyocytes, where it mediates positive inotropic effects (Davenport *et al.*, 2016). It is known that female sex hormones influence expression of ET_A receptors. Hormone replacement with E_2 or conjugated equine oestrogens decreases ET_A receptor mRNA expression in the aorta of OVX New Zealand white rabbits (Pedersen *et al.*, 2009). Similarly, E_2 treatment reduces ET_A receptor mRNA expression in lungs of OVX Sprague–Dawley rats (Gohar *et al.*, 2016).

Intriguingly, women retain differential expression of ET receptor subtypes even after menopause. Saphenous veins isolated from men have 4–7 times higher ET receptor density than those from women, as well as a higher ratio of ET_A to ET_B receptors than women (approximately 3:1 compared with 1:1), favouring the constrictive actions of ET-1. This is reflected by the maximal vessel constriction to 1 μM ET-1

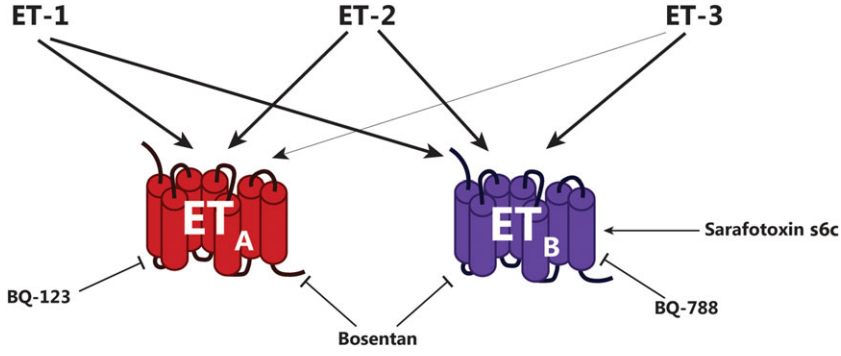

Figure 2

Endothelin receptors and ligands. Relative potency of ET isoforms for respective receptors is indicated by thickness of arrows. Potency order of endogenous agonists for ET_A receptors: ET-1 = ET-2 > > ET-3. Potency order of endogenous agonists for ET_B receptors: ET-1 = ET-2 = ET-3. Compounds mentioned in the text as pharmacological modulators for the ET_A and ET_B receptors are shown: BQ-123 (selective ET_A receptor antagonist), bosentan (unbiased ET_A/ET_B receptor antagonist), BQ-788 (selective ET_B receptor antagonist) and sarafotoxin s6c (selective ET_B receptor agonist).

being nearly doubled in men (Ergul *et al.*, 1998). Consistent with higher abundance of vasoconstrictive ET_A receptors in male blood vessels, selective blockade of ET_A receptors (with **BQ-123**) increases forearm blood flow in males more than females, while dual blockade of ET_A and ET_B receptors (BQ-123 and **BQ-788**) produced equal increases in blood flow between the genders, suggesting ET_B receptors have a more significant role in women than men (Stauffer *et al.*, 2010). Both studies involved women of postmenopausal age that were not taking hormone replacement therapy, suggesting that oestrogen is not the sole contributor to sex-specific ET_A receptor expression and that sex chromosomes or other developmental factors may play a role.

ET_B receptors

The ET_B receptor is functionally distinct from the ET_A receptor, mediating effects that generally oppose the pro-hypertensive actions of the ET_A receptors. ET_B receptors are expressed abundantly in lung and kidney tissue, where they function as clearance receptors to remove excess ET-1 from the circulation, preventing unnecessary activation of ET_A receptors (Fukuroda *et al.*, 1994). The ET_B receptor also promotes natriuresis and diuresis by direct actions on renal tubules (Nakano *et al.*, 2008; Kohan *et al.*, 2011). In the vasculature, ET_B receptors are localized to the endothelium, where they promote NO production and release of vasorelaxant COX metabolites to induce endothelium-dependent vasodilation. VSMCs in certain vessel types also express the ET_B receptor, which mediates sex-specific and tissue-specific vasoconstriction (Kellogg *et al.*, 2001; Schneider *et al.*, 2007).

Regarding the sex differences in the effects of this receptor, initial studies suggested that ET_B receptor deficiency causes high BP only in males at baseline, while female rats deficient in ET_B receptors develop more severe hypertension than males following a high-salt diet (Taylor *et al.*, 2003). However, this was later determined to be an artefact of the tail cuff method of BP measurement. Radiotelemetry measurements revealed that salt-induced hypertension in ET_B receptor-deficient rats is comparable between the sexes, though ET_B receptor-deficient females are more sensitive than controls to acute stress on both normal and high-salt diets, explaining the higher BP observed when measured by tail cuff (Speed *et al.*, 2015). The greater production of ROS in the female ET_B receptor-deficient mice is a potential contributor to this more severe response (Sullivan *et al.*, 2006), indicating that ET_B receptors are involved in protection against BP increase in females (Kittikulsuth *et al.*, 2013).

A possible explanation for these sex-specific effects of ET_B receptors is that females have lower expression ratios of ET_A to ET_B receptors, compared with males, as mentioned above (Ergul *et al.*, 1998), and vascular mRNA abundance of ET_B receptors is increased in male but not female DOCA–salt hypertensive rats (David *et al.*, 2002). This may be due to direct effects of female sex hormones, as E_2 treatment in OVX rabbits increased the mRNA for ET_B receptors in coronary vessels and attenuated vasoconstriction by ET-1 (Pedersen *et al.*, 2008). Contrastingly, other studies have shown ET_B receptor transcript abundance to be increased by loss of female hormones (OVX) and decreased LV and renal inner medulla following E_2 replacement (Nuedling *et al.*, 2003; Gohar *et al.*, 2016). While exogenous oestrogen may decrease ET_B receptor transcript levels in the inner medulla of females, receptor density between the sexes is comparable at baseline (Jin *et al.*, 2013), maintaining the lower ET_A : ET_B ratio of females. These disparate results demonstrate that ET_B receptor expression and trafficking are likely mediated by sex hormones in a complex, tissue-specific manner and at present cannot explain the sexually divergent roles of this receptor.

Interactions between the endothelin and angiotensin systems

In the **angiotensin II** infusion model of hypertension, renal ET_B receptor density is decreased in male hypertensive Sprague–Dawley rats compared with saline-infused controls, while density is preserved in females (Kittikulsuth *et al.*, 2011). As shown in this study and others, the angiotensin II hypertension model produces more profound increases in MAP in males than in females (Kittikulsuth *et al.*, 2011; Xue *et al.*, 2013), and the reduction in ET_B receptor abundance in males specifically may partly explain this.

Clinical relevance

Bosentan, a dual ET_A/ET_B receptor antagonist and the first clinically used drug to target the ET receptors, is one of several ET antagonists approved for the treatment of pulmonary hypertension, which primarily affects women (Seeland and Regitz-Zagrosek, 2012). A variant (Gly5565Thr) in the prepro-ET-1 gene previously shown to be associated with higher BP also affects patient outcomes to antihypertensive treatment in a gender-specific manner. In patients given either an angiotensin receptor blocker (**irbesartan**) or a beta-blocker (**atenolol**), male carriers of the T allele have greater reductions in systolic BP compared with those with GG genotype, while systolic BP reduction in females was not different between genotypes (Hallberg *et al.*, 2004).

In addition to this, elevated plasma ET-1 is associated with endothelial dysfunction and may be a clinical predictor of cardiovascular risk, particularly in women (Daka *et al.*, 2015). Higher circulating ET-1 levels are associated with coronary heart disease events in women but not men, independent of other risk factors and levels of circulating oestradiol (Daka *et al.*, 2015). Even in a young cohort of 2160 men and women aged between 25 and 41 without diabetes or body mass index scores above 35 $kg{\cdot}m^{-2}$, higher plasma ET-1 concentration was associated with elevated systolic BP and higher cardiovascular risk estimated by the Prospective Cardiovascular Münster and Framingham scores (Bossard *et al.*, 2015), though this was not reported by gender.

Eicosanoids and eicosanoid GPCRs

Arachidonic acid is the metabolic precursor for eicosanoids, including prostanoids, epoxyeicosatrienoic acids (EETs) and leukotrienes. COX activity at arachidonic acid produces **PGH_2** from which the primary bioactive prostanoids, **PGD_2**, **PGE_2** and **$PGF_{2\alpha}$**, **PGI_2** and **TXA_2** are derived. These act at their cognate GPCRs; $DP_{1–2}$, $EP_{1–4}$, **FP**, **IP** and **TP** receptors, as summarized in Figure 3.

Oestrogens have broad effects on the biosynthesis of prostanoids, including decreasing activity of **COX-1** and **COX-2**. There is evidence that many of these actions are

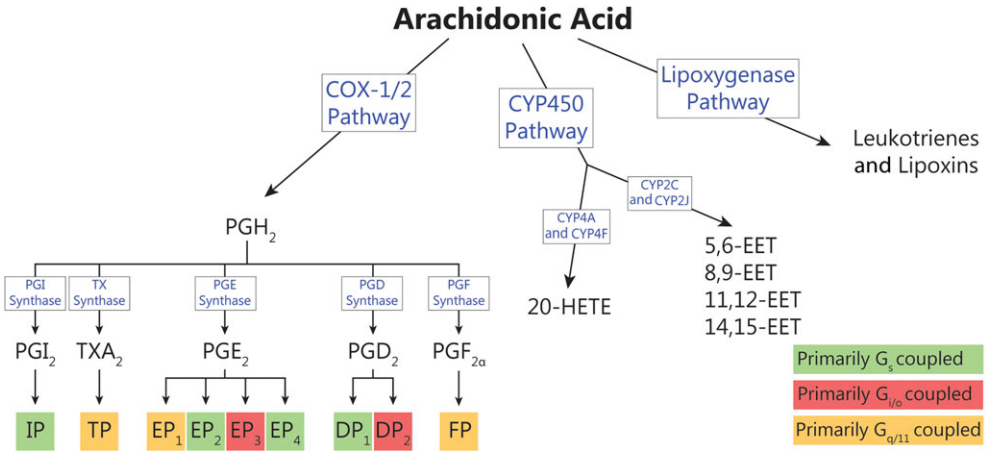

Figure 3

Metabolic pathways of arachidonic acid. COX-1/2 metabolism of arachidonic acid produces bioactive eicosanoid hormones; PGI_2, TXA_2, PGE_2, PGD_2 and $PGF_{2\alpha}$. These prostanoids then act at nine cognate GPCRs: IP, TP, EP_{1-4}, $DP_{1,2}$ and FP receptors. Actions of CYP450 enzymes on arachidonic acid produce 20-HETE and EETs, which may act through GPCRs. The final metabolic pathway of arachidonic acid produces leukotrienes and lipoxins *via* lipoxygenase activity, which are not discussed in this review.

mediated by GPER, as it inhibits prostanoid production and activity (Meyer *et al.*, 2015), while loss of GPER is associated with increased endothelial prostanoid-mediated vasoconstriction and increases in TP receptor-mediated contractions (Meyer *et al.*, 2012a). In the CVS, the prostanoid receptors producing significant sexually dimorphic phenotypes are the TP, IP and EP receptors, discussed below.

TXA_2 (TP) receptors

The prostanoid TXA_2 is a potent activator of platelet aggregation, and inducer of vasoconstriction and vascular cell proliferation. This prostanoid is the primary agonist of the TP receptors, though other prostanoids including PGH_2 are also TP receptor agonists. This receptor is known to couple to $G\alpha_{q/11}$ proteins and activates Ca^{2+}/DAG signalling (Kinsella *et al.*, 1997).

It has been long established that there are gender differences in response to TP receptor agonists. Following administration of **U46619**, a synthetic and stable TP receptor agonist, there was a 25% greater contraction in isolated aortae from male rats than from female, with no sex differences in contractile responses after **5-HT** and **noradrenaline** treatment (Karanian *et al.*, 1981). Furthermore, TP receptor activation increases synthesis of the vasoconstrictor **20-hydroxyeicosatetraenoic acid (20-HETE)** to three times that of basal levels in small porcine coronary arteries, potentially exacerbating effects of androgen-regulated 20-HETE synthesis in males (Randriamboavonjy *et al.*, 2005). *In vivo*, U46619 increases MAP by approximately 25 mmHg in male SHRs, without any significant effect on MAP of females (Schirner and Taube, 1993).

Sex differences in sensitivity to TP receptor agonists may be due to changes in receptor abundance driven by the hormonal milieu. Testosterone increases TP receptor density in cultured male rat aortic smooth muscle cells (ASMCs) and male guinea pig coronary artery smooth muscle cells (Higashiura *et al.*, 1997). The more active testosterone metabolite, **dihydrotestosterone (DHT)**, also increased receptor density in male rat ASMCs; though in females ASMCs, the increase was comparatively minor (Higashiura *et al.*, 1997). Acute administration of testosterone (two doses of 200 mg) to healthy men increased platelet TP receptor density and platelet aggregation response (Ajayi *et al.*, 1995). Androgens thus increase expression of TP receptors, and this effect appears to be augmented in males. Contrastingly, E_2 has no effect on TP receptor density in cultured rat ASMCs (Masuda *et al.*, 1991). However, pretreatment with E_2 desensitizes arteries to agonists of the TP receptors, causing decreased calcium influx and intracellular calcium release in response to U46619 in isolated porcine coronary arteries from both sexes in a dose-dependent manner (Han *et al.*, 1995), which may be attributable to changes in receptor trafficking mechanisms rather than changes in expression. The sensitizing effects of male gonadal hormones and converse effects of oestrogens partly explains why male arteries are more sensitive to contractile effects of TXA_2 and mimetics.

Prostacyclin (IP) receptors

PGI_2 is produced in vascular endothelium and VSMCs from arachidonic acid *via* COX-1/2 and subsequent PGI_2 synthase activity. PGI_2 opposes the pro-thrombotic effects of TXA_2 to prevent platelet aggregation and reduce vascular proliferation, as well as acting as a potent vasodilator and promoting repair following vascular injury (Miggin and Kinsella, 2002). PGI_2 is the main endogenous ligand for the IP receptor, which primarily couples to $G\alpha_s$ and **adenylate cyclase**, though is also capable of signalling *via* $G\alpha_{i/o}$ and $G\alpha_{q/11}$ to inhibit adenylate cyclase and stimulate phospholipase C (**PLC**) respectively (Miggin and Kinsella, 2002). Studies in IP receptor-deficient mice indicate that perturbations in receptor function cause vulnerability to CVD such as ischaemic heart disease due to enhanced thrombosis, without changes in BP (Murata *et al.*, 1997), suggesting that the IP receptor is involved in acute responses rather than regulation of basal BP. In the LDL receptor$^{-/-}$ mouse model of atherosclerosis, female mice lacking the IP receptor developed more severe

lesions than controls, an effect that was gene dose-dependent and not seen in males. In addition to this, platelet activation associated with early atherogenesis was more severe in IP receptor-deficient females than males (Egan *et al.*, 2004). As such, atheroprotective actions of IP receptors are pertinent to both sexes, though loss of IP receptors is additionally deleterious in females.

Both the IP receptor and its ligand, PGI_2, are regulated by sex hormones. Prostacyclin production in human endothelial cells was stimulated in a dose-dependent manner by E_2 and inhibited by selective oestrogen receptor modulator, tamoxifen (Mikkola *et al.*, 1995). This was reproduced in rat cerebral blood vessels, in which E_2 replacement after ovariectomy increases protein expression of COX-1 by five times and PGI_2 synthase by six times that of basal levels (Ospina *et al.*, 2002). Interestingly, both E_2 and DHT increased the abundance of IP receptor mRNA and protein *in vitro*, as a result of oestrogen and androgen response elements in the IP receptor gene promotor region (Eivers and Kinsella, 2016). The finding that the testosterone metabolite, DHT, can mediate expression of IP receptors may account for some of the cardioprotective effects of androgens in the CVS.

PGE_2 receptors (EP_1, EP_2, EP_3 and EP_4)

PGE_2 is the major COX-2 metabolite in the kidney and is important for renal function through its vasodilatory and natriuretic functions (Breyer and Breyer, 2001). It is the principal endogenous ligand for the **EP_1**, **EP_2**, **EP_3** and **EP_4** receptors, though each have distinct affinity profiles for other prostanoids. EP_1 receptors couple to $G\alpha_{q/11}$ causing increased cellular calcium, EP_2 and EP_4 receptors signal through $G\alpha_s$ to raise cAMP, while EP_3 receptors are $G\alpha_{i/o}$ coupled and inhibit cAMP production. EP_4 and EP_2 receptors are involved in vasorelaxation in response to PGE_2, while EP_1 receptors are responsible for transient vasoconstriction (Purdy and Arendshorst, 2000).

Delineating the individual contributions of PGE receptor subtypes to male and female BP homeostasis has proven difficult and is currently understudied. One group investigated the roles of each subtype in male and female mice in response to acute i.v. administration of a bolus dose of PGE_2, which caused an immediate reduction in MAP of both sexes, though this was greater in females (−30 mmHg compared with −23 mmHg in males) (Audoly *et al.*, 1999). Additionally, the maximal MAP reduction is blunted by EP_2 or EP_4 receptor knockout in females but not males, suggesting that EP_2 and EP_4 receptors, both $G\alpha_s$ coupled, are the primary subtypes mediating acute vasodilatory action of PGE_2 in females. Correspondingly, knockout of the $G\alpha_{i/o}$-coupled EP_3 receptors augmented MAP reduction in response to PGE_2 in males only (Audoly *et al.*, 1999). Interestingly, the genetic deletion of EP_1 receptors results in a systolic BP reduction of approximately 10 mmHg in males only and was associated with compensatory effects including increased heart rate and a doubling of renin–angiotensin activity compared with controls (Audoly *et al.*, 1999). This suggests a role for EP_1 receptors in long-term BP homeostasis in males (Stock *et al.*, 2001).

A further point of sexual dimorphism in PGE receptor mechanisms may be the synthesis of the ligand, PGE_2, produced by the sequential actions of COX-1/2 and PGE synthases on PGH_2. COX-2 is the main producer of PGE_2 in the kidney and shown to be higher in female DBA/1 mouse whole kidney homogenate, while ablation of the **microsomal PGE synthase 1 (mPGES1)** in mice causes a number of sex-specific effects on prostanoid synthesis and metabolism (Francois *et al.*, 2007). Further, females have higher expression of mPGES1 mRNA in the kidney at baseline and nearly two-fold higher PGE_2 levels, as indicated by urinary excretion of metabolites (Francois *et al.*, 2007). Higher PGE_2 production in the kidney of females may increase its beneficial natriuretic functions and provides further evidence for female-specific cardiovascular advantages.

CYP450 metabolites of arachidonic acid (EETs and 20-HETE)

The cytochrome P450 arm of arachidonic acid metabolism produces EETs and 20-HETE. Both EETs and 20-HETE are vasoactive and have roles in regulation of renal tubular sodium transport and pressure–natriuresis (Fan *et al.*, 2015). **CYP2C** and **CYP2J epoxygenases** are primarily responsible for producing EETs, which have anti-inflammatory and vasodilator capacity. These effects may be *via* a cognate receptor, with some evidence for EETs binding to a $G\alpha_s$ coupled receptor with 11,12-EET increasing the binding of [^{35}S]-GTPγS to $G\alpha_s$ but not $G\alpha_i$ proteins (Node *et al.*, 2001). However, a screen of 79 orphan receptors in HEK293 cells with a photoaffinity-labelled 14,15-EET analogue showed no evidence of binding (Chen *et al.*, 2011). An alternative possibility for the mechanistic basis for effects of EETs is their antagonist activity at TP receptors, relaxing arteries preconstricted with TXA_2 and preventing binding of a specific TP receptor antagonist (Behm *et al.*, 2009). EETs may also act as secondary mediators of anti-inflammatory effects of the stimulation of AT_2 receptors (Rompe *et al.*, 2010). There is some evidence for gonadal hormone involvement in biosynthesis of EETs, as DHT treatment reduced renal mRNA for the primary epoxygenase CYP2C23 (Singh and Schwartzman, 2008).

Contrastingly, 20-HETE stimulates VSMC contraction and endothelial cell dysfunction and is produced by androgen-stimulated ω-hydroxylases of the CYP4A and CYP4F subfamilies. 20-HETE may contribute to the higher basal BP seen in males by androgen-induced ω-hydroxylase expression (Holla *et al.*, 2001; Wu and Schwartzman, 2011). Recent evidence suggests that a current orphan GPCR, **GPR75**, may be a receptor for 20-HETE (Garcia *et al.*, 2017). GPR75 had previously been reported as a receptor for the chemokine CCL5 (Ignatov *et al.*, 2006), although this pairing has not been confirmed (Southern *et al.*, 2013; Garcia *et al.*, 2017). In cultured human microvascular endothelial cells, 20-HETE induced inositol phosphate accumulation, consistent with GPR75 acting as a $G\alpha_{q/11}$-coupled GPCR, and phosphorylation of EGFR, which is known to be activated by GPR75. Importantly, siRNA knockdown of GPR75 in these cells prevents induction of the mRNA for **angiotensin-converting enzyme**, which is a major effect of 20-HETE on the vasculature. Furthermore, GPR75 shRNA knockdown in mice completely attenuates hypertension and vascular remodelling caused by doxycycline-induced expression of CYP4A12 (a murine 20-HETE

synthase) (Garcia *et al.*, 2017). Alternatively, 20-HETE may exert its vascular effects by acting at TP receptors, as flow-induced constriction of rat and human arteries was abolished by an inhibitor of 20-HETE synthesis (HET0016) or a TP receptor antagonist (**SQ 29548**) but not COX inhibitor (**indomethacin**) or a specific TXA_2 synthase inhibitor (ozagrel) (Toth *et al.*, 2011), though direct binding of 20-HETE to the TP receptor has not been demonstrated.

There are significant effects of gender on levels of 20-HETE. The male ratio of 20-HETE to EETs in interlobar arteries is almost twice as high as females, and treatment with DHT increased this ratio in both sexes and eliminated sex difference (Singh and Schwartzman, 2008). DHT treatment also increased mRNA expression of CYP4A8, the primary hydroxylase responsible for 20-HETE production in the kidney (Singh and Schwartzman, 2008). Reciprocally, inhibitors of CYP4A activity and 20-HETE production reduce hypertension induced by androgen treatment (Wu and Schwartzman, 2011). Genetic deletion of CYP4A14 (a murine 20-HETE synthase) causes hypertension more prominently in males, which was attenuated by castration and rescued by androgen administration. $CYP4A14^{-/-}$ males also have associated higher serum androgen (testosterone and DHT) levels, suggesting a positive regulatory loop for sex hormones and CYP4A hydroxylase activity (Holla *et al.*, 2001) that may lead to higher BP in males.

Eicosanoids and the angiotensin system

Angiotensin-(1–7), a peptide ligand reportedly active at the **Mas** GPCR, is known for having generally opposing actions to the pro-hypertensive peptide, angiotensin II. Angiotensin-(1–7) acts as a vasodilator but additionally causes release of arachidonic acid and the production of prostanoids, including PGI_2 (Muthalif *et al.*, 1998). Furthermore, s.c. angiotensin-(1–7) infusion attenuates BP increase in both sexes of Dahl salt-sensitive rats following high salt diet and this effect was seen in females for up to 2 weeks (Eatman *et al.*, 2001).

Clinical relevance

Aspirin, an irreversible inhibitor of COX-1 and a modulator of COX-2 function, is used as a primary and secondary preventative treatment against CVD. Sex bias in the actions of aspirin is well documented and is covered in a recent review by Pace *et al.* (2017). The first report of gender-divergent responses noted reduced antithrombotic effects in female rabbits compared with males (Kelton *et al.*, 1978), and multiple clinical trials have since noted gender differences in the efficacy of aspirin as a primary prevention for CVD, though men and women reap similar benefits from low-dose aspirin as a secondary prevention for CVD. In a randomized, placebo-controlled primary prevention trial of low-dose aspirin in 39 876 women, no effect was seen in reducing total cardiovascular risk, though there was a significant reduction in overall and ischaemic stroke rates of 26 and 33%, respectively, with no effect on risk of myocardial infarction (Ridker *et al.*, 2005). In the associated meta-analysis of randomized controlled trials, low-dose aspirin caused a 32% reduction in the risk of myocardial infarction for men with no effect on rate of stroke (Ridker *et al.*, 2005). In economic terms, aspirin treatment for the primary prevention of CVD is only cost-effective in men with a greater than 10% 10 year CVD risk and women with greater than 15% risk, which occurs much later in life (Greving *et al.*, 2008).

In conclusion, this review has discussed three key GPCR-mediated systems that exhibit sexual dimorphism in the CVS and produce meaningful differences in homeostatic function and pathological dysfunction between the sexes. Acute sex-specific effects of GPER on the CVS are likely to be mediated through vascular action of GPER rather than effects on the heart, as males and females have similar cardiac GPER abundance. GPER in the CNS and the kidneys may also play a role in cardioprotection in females (Kurt and Buyukafsar, 2013), though these actions have not been extensively investigated for both sexes at present. Rodent knockout studies show sex-dependent cardiovascular and metabolic phenotypes due to genetic deletion of GPER, though controversy remains and more studies investigating both sexes are needed to confirm acute effects on the vasculature. This positions GPER as a potential therapeutic target, though further insight into the physiological mechanism of GPER is needed, specifically in relation to sex differences.

In the ET system, notable sex differences exist in receptor density and ligand abundance. Men have higher overall ET receptor density and a higher ratio of ET_A to ET_B receptors than women, encouraging the vasoconstrictive actions of ET-1. On the other hand, oestrogens suppress the release of ET-1, and females are more resistant to ET-1-mediated rises in BP, but it remains unclear how female sex steroids affect abundance of the generally anti-hypertensive ET_B receptor, as effects appear to be tissue-specific and species-related.

Eicosanoid receptors, most importantly the TP and IP receptors in female, play a significant role in gender divergence of the CVS. Male sex hormones increase expression of the pro-aggregatory, pro-hypertensive TP receptor. Interestingly, expression of the IP receptor is increased by both male and female sex hormones, potentially having implications for hormonal changes during ageing. Importantly, aspirin, a common preventative therapy for cardiovascular events, is not as effective in women as in men, and an alternative method for reducing incidence of CVD may be necessary for low-risk females.

Considering these functional differences in the CVS between the sexes, it is imperative that animal studies incorporate both sexes in order to delineate sex-dependent mechanisms of cardiovascular homeostasis (Blenck *et al.*, 2016). Looking forward, improving our knowledge of sex hormone contribution to cardiovascular sexual dimorphism will inform recommendations for clinical treatment of CVD between the genders.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b,c,d).

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Conflict of interest

The authors declare no conflicts of interest.

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