Published in final edited form as:

Clin Sci (Lond). 2016 June 1; 130(12): 1005–1016. doi:10.1042/CS20160114.

GPER - novel membrane estrogen receptor

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Abstract

The recent discovery of the G protein-coupled estrogen receptor (GPER) presents new challenges and opportunities for understanding the physiology, pathophysiology, and pharmacology of many diseases. This review will focus on the expression and function of GPER in hypertension, kidney disease, atherosclerosis, vascular remodeling, heart failure, reproduction, metabolic disorders, cancer, environmental health, and menopause. Furthermore, this review will highlight the potential of GPER as a therapeutic target.

Keywords

GPR30; cardiovascular; reproduction; metabolism; cancer; menopause

Discovery of GPER

The complexity of estrogen signaling became apparent when some of its effects did not fit the time course for transcriptional signaling. Estrogen receptors (ER) ER α and ER β are classical steroid receptors that typically dimerize and translocate to the nucleus after ligand binding. Once in the nucleus, the receptors bind to estrogen response elements or interact with other transcription factors to alter gene expression. In 1967 Szego and Davis observed that intravenous administration of 17 β -estradiol more than doubles the amount of cyclic AMP in the uterus of ovariectomized female Sprague Dawley rats in less than one minute (1). Moreover, estradiol stimulates mitogen-activated protein kinases (MAPK) such as ERK-1 and ERK-2 within two minutes in MCF-7 breast cancer cells (2), and estradiol rapidly phosphorylates and activates nitric oxide synthase (NOS) in endothelial cells (3).

The presence of a plasma membrane-associated estrogen binding site was first demonstrated in 1977 by Pietras and colleagues using endometrial and liver cells (4). Estrogen also binds to the outer membrane of spermatozoa (5) and the plasma membrane of human umbilical vein endothelial cells (6). Furthermore, conjugated estrogen that is membrane-impermeable rapidly activates the MAPK/ERK pathways despite its inability to reach intracellular ER α or ER β (7). These studies provide evidence that an estrogen receptor is localized to the plasma membrane and initiates nongenomic effects, perhaps via a novel receptor.

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Declarations of interest None

In 1997, a cDNA screen identified an orphan G protein-coupled receptor named GPR30 in the ER positive breast cancer cell line MCF-7 that is not expressed in the ER negative breast cancer cell line MDA-MB-231 (8). Estradiol binds with nanomolar affinity to GPR30 in SKBR3 breast cancer cells that lack nuclear ERs and in HEK293 cells transfected with GPR30 (9). Activation by estrogen rapidly induces signaling molecules such as cyclic AMP (10), inositol trisphosphate (IP3), and Ca^{2+} (11) and activates the MAPK/ERK pathways (12). Because multiple studies showed that this seven transmembrane receptor is activated by estrogen, GPR30 was renamed G protein-coupled estrogen receptor (GPER) (13). Selective agonists (14) and antagonists (15, 16) for GPER are commercially available, allowing investigation of the actions of this receptor that are distinct from ER α and ER β .

GPER is ubiquitously expressed throughout the body, including the heart, brain, pancreas, skeletal muscle, kidney, vessels, and reproductive organs (17). The level of expression is influenced by tissue type, sex, disease, and age. For example, the placenta has an abundance of GPER while skeletal muscle has very low expression (8). In mesenteric resistance vessels, GPER protein is lower in age-matched males and 12 month-old females as compared with 3 month-old females (18). Interestingly, vascular GPER is not sensitive to hormonal status, as ovariectomized rats maintain receptor expression and function. However, renal GPER expression is dependent on the estrus cycle, with levels the highest during proestrus and estrus and lowest during metaestrus (19). Disease processes also regulate the expression of this receptor, as GPER is significantly upregulated in lung cancer (20), endometriosis (21), and in the kidneys of salt-loaded mRen2 females (22). Multiple GPER knockout models are now available to assist in determining a role for this receptor in physiology and pathophysiology (23).

GPER is not the only candidate for a membrane-bound estrogen receptor, as there is a truncated isoform of ER α that also induces acute signaling. ER α -36 is a novel variant of the full-length 66 kD ER α that lacks the transcriptional activation domains but maintains the ligand binding domain (24). ER α -36 activates the MAPK/ERK pathways (25) and is necessary for rapid activation of NOS by 17 β -estradiol in endothelial and airway epithelial cells (26). Therefore, there are most likely multiple membrane-bound receptors that mediate the acute actions of estrogen.

Hypertension and Kidney Disease

Women have a lower incidence of hypertension in comparison with age-matched men until menopause, suggesting that estrogen counteracts increases in blood pressure (27). The ability of estrogen to modulate blood pressure is observed in numerous experimental animal models of hypertension (28). We and others have recently investigated whether GPER mediates the anti-hypertensive actions of estrogen. In experimental animal studies, activation of GPER using the selective agonist G-1 decreases blood pressure acutely in male Sprague Dawley rats (29) and chronically in estrogen-deficient female mRen2 rats (30). However, GPER does not alter blood pressure in intact female mRen2 rats, indicating that the role of GPER is unmasked only when the endogenous ligand is absent. The contribution of GPER to blood pressure may also be dependent on age, as no difference in blood pressure is detected in female GPER knockout versus wildtype mice until 9 months of age (31). In

humans, a single nucleotide polymorphism of GPER produces a hypofunctional allele, and female but not male carriers of the GPER P16L variant have a significantly greater systolic blood pressure (32).

In many cases a cause or consequence of hypertension is vascular dysfunction. GPER is expressed in endothelial and smooth muscle cells of the carotid artery, middle cerebral arteries, and aorta (30, 33–36). In vascular preparations from rat, mouse, pig, and human, the selective agonist G-1 induces dose-dependent acute vasodilation that is not different from estradiol, however, the contribution of endothelium versus smooth muscle is dependent on the vascular bed being tested (29, 33, 37). In female mesenteric arteries, selective GPER activation promotes vasodilation via endothelial-derived nitric oxide and smooth muscle cell-initiated cAMP (34). However, in rat carotid arteries GPER-induced vasodilation is completely endothelium-dependent (35), and in coronary arteries the response is entirely endothelium-independent (38). These differing signaling pathways for GPER may result from alterations in smooth muscle versus endothelial expression levels of the receptor, which differ according to vascular bed (39). Regardless, the ability of GPER to directly induce vasodilation is well-established in animal models as well as in human arteries (40, 41).

GPER activation not only induces direct vasodilation but also counteracts the response to a variety of other vasoconstrictors, which may provide protection against hypertension. GPER-deficient mice have enhanced prostanoid production and vasoconstriction (42, 43) and elevated responses to endothelin-1 in carotid arteries (44). The GPER agonist G-1 attenuates coronary artery contractions to endothelin-1 to the same extent as the GPER agonist and ER α /ER β antagonist ICI 182,780 further demonstrating the independent actions of this membrane receptor in vessel function (37). In contrast, GPER is suggested to mediate the actions of aldosterone, a pro-hypertensive molecule of the RAS. Aldosterone-induced ERK phosphorylation in denuded aortic tissue from male Wistar-Kyoto rats is equally inhibited by a GPER or mineralocorticoid receptor antagonist (36), and aldosterone-mediated increases in vasoconstriction are mediated by GPER in mesenteric arteries from female mice (45). Since specific binding of aldosterone to GPER has yet to be demonstrated, it is unknown how these systems interact. Regardless, the influence of GPER in mediating the rapid effects of aldosterone introduces a novel pathway by which estrogen modulates RAS activation.

The kidney plays an important role in long term blood pressure control through volume homeostasis and is vulnerable to hypertension-induced damage. The loss of estrogen during menopause is associated with increased salt-sensitivity and reduced glomerular filtration rate (46). In mice, estradiol is protective against renal injury, and genetic deletion of ER α or ER β does not attenuate this protection suggesting an alternative receptor (47). While the initial report of renal GPER expression showed the highest levels in the renal pelvis (48), other studies show GPER expression predominantly in tubular epithelial cells (19, 22, 49). Chronic G-1 treatment in salt-sensitive female mRen2 rats increases estimated glomerular filtration rate and decreases renal hypertrophy and proteinuria, perhaps by increasing megalin expression and reducing reactive oxygen species in proximal tubules (22). GPER's renoprotective effects may also result from reductions in the permeability of glomerular endothelium (50) and buffering of elevated perfusion pressure (51). Taken together these

data suggest that GPER is protective during perturbations of the kidney and may prove to be an important drug target for kidney disease, especially in postmenopausal women.

Atherosclerosis and Vascular Remodeling

Female sex hormones are protective against atherosclerosis. Estrogen supplementation in ovariectomized monkeys reduces the formation of plaques independent of changes in total plasma cholesterol or HDL levels (52). In contrast, protection against the development of atherosclerotic plaques by estrogen in intact female monkeys is associated with a concomitant decrease in plasma HDL (53). Estradiol also reduces plasma cholesterol and lesion size in ovariectomized apolipoprotein E knockout mice but not in mice deficient in ER α (54). Despite this indication that ER α is the main mediator of atherosclerotic protection, estradiol still reduces advanced lesion characteristics suggesting non-ER α mechanisms. Moreover, mice deficient in ER α and fed an atherogenic diet maintain estradiol-induced protection (55). Lesion size is exacerbated in the aortas of intact and ovariectomized female GPER knockout mice, while treatment with the selective agonist G-1 reduces inhibits atherosclerosis in ovariectomized mice (56). The beneficial effects of GPER in this model are associated with a reduction in macrophage and T cell recruitment, indicating an anti-inflammatory mechanism.

Pathological vascular remodeling is most commonly induced by surgical interventions such as angioplasty and stent placement. Women have a decreased risk of death and restenosis after coronary stent placement compared to men, suggesting sex differences in neointima formation after vascular injury (57). Evidence from animal models suggests that these differences are, in part, mediated by endogenous estrogens. Female Sprague-Dawley rats are protected from carotid balloon injury compared with males, and this sex difference is abolished by ovariectomy but not castration (58). Local delivery of estradiol decreases coronary angioplasty-induced neointimal hyperplasia in pigs (59, 60). These studies led to the development of estrogen-eluting stents, which reduce neointimal proliferation and induce reendothelialization in rabbit aorta (61) and porcine coronary artery (62). This protective effect of estrogen on vascular injury is evident in both ER α (63) and ER β (63) knockout mice, indicating that another receptor may mediate this hormone's effects in the vasculature. While there are as to date no studies looking at the role of GPER in neointima formation, mesenteric arteries from female GPER knockout mice have an increased media-to-lumen ratio at nine months of age, an effect associated with increased blood pressure (31).

In vitro studies indicate that GPER has anti-proliferative, anti-inflammatory, and anti-oxidant actions which may contribute to vascular protection. The agonist G-1 attenuates serum-induced proliferation of human vascular smooth muscle cells (29) and promotes differentiation and suppresses proliferation of human and porcine coronary artery smooth muscle cells (64). G-1 reduces inflammatory mediators in macrophages and vascular smooth muscle cells from both young and old female C57BL/6 mice, despite the fact that estradiol is anti-inflammatory only in young cells (65). GPER is expressed in murine macrophages, and activation reduces the production of inflammatory cytokines (66). GPER also reduces inflammatory molecules in endothelial cells, an effect that is absent when the other ERs are activated (67). GPER decreases in NADPH-stimulated superoxide levels in carotid and

intracranial cerebral arteries, indicating anti-oxidant effects as well (35). Since estradiol's vascular anti-inflammatory effects are altered by aging and receptor subtype, selective GPER activation may provide an alternative pathway for protection against vascular remodeling and atherosclerosis.

Heart Failure

Adult men are more likely to have heart failure until age 80, when the incidence in women surpasses that of men (27). Interestingly, postmenopausal women are twice as likely to have diastolic heart failure, or heart failure with preserved ejection fraction (68). Sex differences are also observed in animal models of heart failure. For example, aortic banding induces heart failure to a greater extent in male versus female Wistar rats (69). In heart failure induced by arteriovenous shunt, mortality is 2.5% in female rats versus 24.5% in males (70). Moreover, ovariectomy increases the incidence of congestive heart failure while estrogen prevents disease progression (71). In a transverse aortic constriction model, female protection is lost in ER β but not ER α knockout mice (72), clearly supporting a role for membrane-initiated estrogen signaling in cardiac health (73).

GPER is expressed in cardiac myocytes (74) and fibroblasts (75). In intact female mRen2 rats, activation of GPER attenuates diastolic dysfunction induced by a high salt diet (76). G-1 treatment in ovariectomized mRen2 females on a normal salt diet reduces left ventricular filling pressure, left ventricular mass, wall thickness, and interstitial collagen deposition (74). The cardioprotective benefits of GPER extend beyond female animal models, as G-1 administration following ischemia reperfusion in male mice decreases myocardial infarct size and improves left ventricular pressure (77). G-1 administered prior to myocardial ischemia reperfusion also protects both male and female Sprague Dawley rats by improving contractile function and reducing infarct size (78). In contrast to these studies showing protective effects of GPER on both sexes, GPER deletion impairs left ventricular cardiac function in males but not females (79). Some cardioprotective effects of GPER may be centrally mediated, as G-1 microinjection into the nucleus ambiguus of male Wistar rats decreases cardiac vagal tone (80). Overall, the cardioprotective effects of the selective GPER agonist seem to be consistent with those of non-selective estrogen receptor stimulation.

Mechanisms for GPER-induced cardioprotection include decreased myocyte hypertrophy in salt-loaded mRen2 (76) and decreased natriuretic peptides and cardiac NADPH oxidase expression in ovariectomized mRen2 (74). G-1 treatment decreases the proliferation of cardiac fibroblasts and the expression of cell cycle proteins CDK1 and cyclin B1 (75). In female mRen2 rats, GPER activation attenuates ovariectomy-induced increases in CDK1 and cyclin B1, proliferation marker Ki-67, and fibroblast marker vimentin in the left ventricle (81). In ovariectomized female Sprague Dawley rats with isoproterenol-induced heart failure, G-1 administration increases left ventricular developed pressure and decreases left ventricular end diastolic pressure, an effect that was comparable to non-selective estrogen treatment (82). These benefits were associated with a concomitant decrease in cardiac fibrosis and enhanced myocardial contraction, suggesting that the cardioprotective effects of estrogen could be attributed to GPER.

Reproduction

Estrogen is an important regulator of reproductive physiology, but the role of GPER is less clear. GPER expression is considerably lower than ER α in the ovary, uterus, and mammary gland (83) and is absent in granulosa, mammary, and uterine epithelial cells of LacZ reporter mice (84). In female rhesus monkeys, GPER is expressed in gonadotropin-releasing hormone neurons suggesting a role for this receptor in central reproductive signaling (85). In humans, endometrial GPER gene expression varies across the menstrual cycle with lowest levels during the secretory phase, a pattern similar to that of ER α (21, 86). GPER expression is increased in uterine leiomyomas (87) and is also upregulated in endometriosis (21), suggesting that certain disease states regulate levels of this receptor.

GPER is not necessary for fetal development since genetic deletion is not embryonically lethal and does not impair fertility or sexual development (31, 83). Uterotrophic responses to estrogen are also unaltered between GPER-deficient and wildtype mice (88). Moreover, we and others have shown that chronic *in vivo* treatment with the selective agonist G-1 does not alter uterine wet weight (30, 56). In CD-1 mice, GPER activation alone does not alter uterine weight but antagonizes estrogenic effects on uterine cell proliferation by attenuating ER α phosphorylation (89). In contrast, G-1 administration in ovariectomized C57BL/6 mice produces a modest increase (3–4 fold) in the proliferative index of uterine epithelia as compared with estrogen (17-fold increase) (16), suggesting effects on uterine cell proliferation but not imbibition. In cultured endometrial cells, GPER activation increases while inhibition decreases cell proliferation and AKT phosphorylation (90). Overall, these results characterize a complex relationship between estrogen and its receptors and a gap in understanding their functions in uterine physiology.

The role of GPER in pregnancy is also not clear. GPER is expressed in the myometrium of female Sprague Dawley rats where it mediates depolarization and contraction (91). Estradiol-induced vasorelaxation in human placental and myometrial arteries is independent of GPER activation (92). GPER mRNA levels in human myometrium do not change during labor, however myometrial strips from non-laboring women treated with either G-1 or estradiol have enhanced contractility (93). In males, GPER is expressed in the apical membranes of epididymal epithelial cells (94) and localized to seminiferous tubules and Sertoli cells (95). Testicular biopsies from infertile men exhibit reduced or absent peritubular GPER expression compared to men with normal spermatogenesis, supporting a role for GPER in spermatogenesis and fertility (96). In adult male Sprague-Dawley rats, treatment with either the GPER agonist G-1 or estrogen in luteinizing hormone-stimulated Leydig cells dropped testosterone production by 20%, providing evidence that GPER may modulate steroidogenesis (97). There is still much to be learned about the function of GPER in normal reproduction and whether targeting this receptor could provide benefits during abnormal or disease states.

Metabolic Disorders

Estrogen improves the diabetic condition and plays an important role in metabolic regulation (98). Estrogen deficiency after menopause negatively influences adipose tissue distribution

and metabolism (99), while estradiol administration ameliorates weight gain and reduces adipocyte size in ovariectomized animal models (100). Estrogen also preserves beta cell survival and function in multiple animal models of diabetes (101). Interestingly, estradiol has protective effects in cultured islets even when ER α and ER β are absent, suggesting an alternative pathway for estrogen's actions (102). GPER is expressed in whole adipose tissue and isolated adipocytes of both male and female mice (103), in isolated mouse and human pancreatic islets (102), as well as cultured pancreatic beta cells (104).

Male and female GPER knockout mice display increased body weight, subcutaneous fat, and total adipose tissue compared to wildtype mice fed either standard chow (29) or a phytoestrogen-free diet (103). In contrast, Mårtensson *et al.* reported that genetic deletion of GPER reduces body weight in females with no alterations in males and correlates with decreased skeletal growth rather than alterations in adiposity (31). In Gpr30-lacZ mice fed either a normal or high fat diet, body weight, body mass composition, and glucose tolerance are not altered in males or females (79). However, these female knockout mice have reduced plasma high density lipoproteins and increased fat content in the liver compared to wildtype controls, with no alterations in males. In yet another GPER knockout strain, estradiol decreases fat mass even when GPER is deleted suggesting an ER α or ER β effect (88). Clearly, different conclusions about the role of GPER in adiposity can be drawn depending on the strain of GPER knockout mice utilized and are more thoroughly reviewed elsewhere (105).

Female GPER knockout mice have elevated plasma glucose levels, a diminished first-phase insulin response, and depressed glucagon levels (31). Isolated pancreatic islets from these same mice display decreased insulin expression and release. Similarly, male GPER knockout mice exhibit reduced glucose tolerance and insulin resistance (106). GPER-deficient males also have increased total and subcutaneous fat, circulating cholesterol and triglycerides, and fasting insulin levels compared with wildtype controls. The importance of GPER in mediating glucose homeostasis is further confirmed *in vitro*, as its agonist G-1 stimulates insulin and suppresses glucagon in isolated female islets (107). Moreover, these responses can be blocked by a GPER antagonist and are absent in islets isolated from female GPER knockout mice (108). In patients with type 1 diabetes mellitus, pancreatic islet transplantation has the potential to restore endogenous insulin production and glycemic stability (109). Interestingly, administration of the GPER agonist G-1 to male mice following islet transplantation improves blood glucose and islet engraftment (110). Overall, this work points to a critical role that estrogen and GPER play in maintaining body weight and glucose homeostasis, and suggests a more prominent role in females compared to males. Understanding the consequences of GPER dysfunction may be a potential target in treating metabolic disorders.

Cancer

The impact of GPER on cancer cell growth depends on the cancer type and tissue target. GPER stimulates the proliferation of ovarian and breast tumor cells by inducing the expression of c-fos (111) but inhibits the proliferation of both estrogen receptor-negative (112) and positive breast cancer cells (113). GPER-induced activation of c-jun and c-fos

attenuates the growth of prostate cancer cells (114), while in benign prostatic hyperplasia estrogen promotes disease progression via a non-genomic pathway (115). GPER also influences the tumor microenvironment, for example by reducing the levels of onco-suppressor Runx1 in cancer-associated fibroblasts (116). GPER may mediate hypoxic signaling, as its promoter region contains a hypoxia-inducible factor (HIF)-1 α responsive element (117). This mechanism may promote tumor growth as well as exacerbate other diseases characterized by a low oxygen environment (118). While GPER disrupts microtubule formation in normal endothelial cells (119), it promotes tube formation in endothelial cells conditioned with media from breast cancer cells (120). Therefore, the impact of GPER on cellular processes is extremely heterogeneous and must be individually evaluated for each type of cancer.

The role of GPER in breast cancer has been reviewed more thoroughly elsewhere (121). GPER-positive breast tumors occur at a higher frequency (68.5%) compared to ER-positive cells (43%) in inflammatory breast cancer, a rare but aggressive form of cancer characterized by rapid proliferation and resistance to endocrine therapy (122). Co-expression of ER and GPER is associated with better patient overall survival while the absence of ER or GPER worsen the outcome. Although GPER expression is unaltered during breast cancer progression, levels are reduced from normal tissue and correlate with overall survival, indicating that GPER may play an important role in tumor suppression (123). Approximately 80% of breast cancers are estrogen receptor positive, and anti-estrogenic compounds are standard treatment (124).

Tamoxifen (a selective ER modulator or SERM) is the most widely-used drug in premenopausal women, however some cancers are initially resistant or develop drug resistance (125). Complications with tamoxifen may arise because of its mixed agonist/antagonist activity at GPER versus ERs. For example, tamoxifen-resistant MCF-7 breast cancer cells maintain a proliferative response to estrogen by promoting translocation of GPER to the cell membrane and enhancing GPER signaling (126). GPER activation also increases aromatase expression, which may contribute to sustained growth and progression in tamoxifen-resistant breast cancer cells (127). These studies indicating a role for GPER in tamoxifen-resistance could be used to develop adjuvant therapies for this complex disease and are reviewed in greater depth elsewhere (128).

Environmental health

Since environmental estrogens such as bisphenol A (BPA), nonylphenol, dichlorodiphenyltrichloroethane (DDT), and genistein bind to GPER, these xenoestrogens may promote or antagonize GPER signaling (129, 130). BPA is a widely-used industrial compound that induces negative consequences on reproductive and developmental health (131). BPA also induces GPER gene and protein expression in Sertoli cells of male mice (132) and spermatogonial GC-1 cells (133). When GPER is silenced or inhibited, BPA no longer stimulates proliferation of murine ER-beta negative spermatogonial GC-1 cells (134) or mouse immature Sertoli (TM4) cells (132). DDT is a synthetic pesticide that disrupts estrogenic actions by binding to estrogen receptors (129). In cultured mouse hippocampal cells, DDT decreases GPER protein levels while G-1 attenuates DDT-stimulated caspase

activity (135). Genistein is an isoflavone derived from plants that is often used as a “naturally occurring” estrogen therapy and stimulates normal uterine proliferation (136). Genistein treatment of human periodontal ligament cells delays the ability of cytokine IL-1 β to induce MAPKs, while inhibition of GPER eliminates this effect (137).

GPER may be an important link between exposure to environmental chemicals and cancer. For example, atrazine is an herbicide associated with an increased risk for ovarian and breast cancer, and GPER mediates the negative effects of this chemical in both breast cancer cells and cancer-associated fibroblasts (138). Likewise, cadmium induces proliferation in SKBR3 breast cancer cells that contain GPER but lack ERs, and this effect is lost when GPER signaling is inhibited (139). BPA-induced cell proliferation of lung cancer cells is also mediated by GPER but not ER α /ER β (140). Genistein signals via GPER to promote endometrial cancer (141), and icariin and icaritin, which have a similar structure to genistein, are traditional Chinese herbs that stimulate proliferation of breast carcinoma cells via GPER (142).

Exposure to endocrine disruptors pose further concerns when considering the role of estrogen in cardiovascular health. Nonylphenol is an extensively used compound in both commercial and household products that also binds GPER and induces adenylyl cyclase activity (129). In the heart, nonylphenol has non-monotonic effects on myocardial contractility, and a GPER antagonist prevents low but not high dose effects (143). BPA also induces negative effects on the cardiac extracellular matrix and the baroreflex (144), but whether these effects occur via GPER is yet to be determined. These studies strongly indicate a role for GPER in the pathophysiological actions of environmental estrogens.

Menopause

Numerous animal studies demonstrate that estrogen generates positive effects in ovariectomized animal models, a result that is recapitulated in observational studies of postmenopausal women (145). However, when the Women's Health Initiative (WHI) conducted a large, randomized clinical trial to determine the impact of conjugated equine estrogens (CEE) alone or in combination with medroxyprogesterone acetate (MPA), an increased incidence of coronary heart disease, stroke, and breast cancer was found in women taking CEE plus MPA (146). In contrast, the 2004 WHI report on the use of CEE alone in women without a uterus shows no significant increase in coronary heart disease or breast cancer, highlighting the negative impact of MPA in combined therapy (147, 148). The WHI data also indicates significant changes in the hazard ratios when age is considered, with the most favorable outcomes in women aged 50–59 years (149). Nevertheless, the WHI study triggered a black box warning on menopausal hormones and a drastic reduction in the number of women taking these drugs (150).

Unopposed CEE is prescribed only to women with a complete hysterectomy due to the increased risk of endometrial cancer (151). However, the FDA recently approved the SERM bazedoxifene as a substitute for MPA in combined hormone therapy, which antagonizes the uterine effects of CEE while avoiding the negative effects of MPA (152). The impact of this new drug combination on long-term health is yet to be determined. Moreover, the

interpretation of the WHI results must now be expanded in light of the discovery of GPER. We and others have shown that activation of this receptor induces positive outcomes well past the time of ovariectomy in experimental models (30, 56, 153). Importantly, we also find that expression of this membrane estrogen receptor is maintained in resistance arteries well after the loss of endogenous estrogens but is reduced during normal aging (18). Similarly, expression of ER α is reduced in bone marrow-derived macrophages during aging (65). Alterations in GPER and ER expression may differ according to tissue type and may explain unexpected responses to menopausal hormone therapy. Over a decade after the WHI, the effects of menopausal hormone therapy on health are still unresolved, but recent discoveries in receptor pharmacology will hopefully advance our understanding and treatment of postmenopausal health and disease.

The Future of GPER

There are numerous areas where our knowledge of GPER and its function is lacking. In particular, there is little known about the location and signaling of this receptor in normal physiology. The debate on the subcellular location of GPER is ongoing, with varying reports showing cell surface (49), intracellular membrane (154), and even nuclear localization (155). Moreover, receptor location may influence pathologies such as breast cancer, where the receptor resides in the nucleus of MCF7 cells but the cytoplasm of T47D breast cancer cells (156). The signaling pathways activated by GPER are also varied and opposing. We and others have shown that GPER increases cAMP *in vitro* (34, 157), suggesting coupling of this GPCR to the G α s subunit. Indeed, recent data from our laboratory show that GPER and G α s co-localize in female rat vascular smooth muscle cells at both the plasma membrane and perinuclear region (Figure). However, others have demonstrated that GPER may couple to G α i and inhibit cAMP (158) or activate the G α q signaling cascade (159). It is important to recognize that there are most likely other hormones that influence GPER signaling, whether through direct binding or receptor-receptor interactions (160). Identification of these other endogenous ligands or binding partners may help to explain the diversity of GPER location and signaling. Finally, manuscripts that set out to disprove a role for GPER as a membrane estrogen receptor or minimize its role in comparison to the other ERs are merely obstructing and delaying the knowledge that can be gained by investigating the function of this novel estrogen binding site. GPER and the other ERs probably do not work independently but instead are part of a large integrated network of hormone signaling mechanism that tightly regulate intracellular responses under a variety of physiological and pathological stimuli.

In terms of human health, we still do not have a clear picture of the role of this receptor in pregnancy, labor, or childbirth. In general, this aspect has been overlooked merely because homozygous GPER knockout mice breed normally and uterine physiology is for the most part intact. However, GPER may be an important drug target for infertility, premature labor, or other reproductive problems. Another important challenge to be considered in light of GPER is the increasing life span of postmenopausal women. Women now live almost half of their lives after menopause, and whether quality of life could be augmented by selective estrogen receptor activation is an important area of research. Our laboratory is considering whether activation of GPER provides protection against cardiovascular disease, and others may find benefits in other pathologies as well. While this review has highlighted mostly

female health issues, data from GPER knockout mice provide strong evidence for a role in male health as well. How male sex and sex hormones influence GPER signaling, localization, and function is also pertinent to further our knowledge on this receptor.

Acknowledgements

None

Funding information This work was supported by the National Institutes of Health (HL103974 to S.H.L.), American Heart Association (POST25515E and GRNT25890021 to S.H.L.)

Abbreviations list

GPER	G protein-coupled estrogen receptor (formerly GPR30)
G-1	GPER agonist
G15	GPER antagonist
MAPK	mitogen-activated protein kinase
ER	estrogen receptor
SERM	selective estrogen receptor modulator
NOS	nitric oxide synthase
cAMP	cyclic adenosine monophosphate
GFR	glomerular filtration rate
CEE	conjugated equine estrogens
MPA	medroxyprogesterone acetate
WHI	Women's Health Initiative
BPA	bisphenol A
DDT	dichlorodiphenyltrichloroethane

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Figure.

(A) Vascular smooth muscle cells isolated from mesenteric arteries of female Lewis rats were cultured in charcoal-stripped and phenol red-free media for 48 hours. Cells were fixed with 2% paraformaldehyde, blocked with 3% normal donkey serum (NDS), and then incubated overnight at 4°C at a 1:100 dilution of both rabbit anti-GPER (H-300 Santa Cruz sc-134576) and goat anti-Gαs (Lifespan LS-C113002) in 3% NDS. Secondary antibodies were added at a 1:200 dilution in 3% NDS (Alexa Fluor 488 Goat Anti-Rabbit; Invitrogen A11008 and Alexa Fluor 647 Donkey Anti-Goat; Invitrogen A21447) before washing and applying mounting media with DAPI. GPER colocalized with Gαs on both the plasma membrane and the perinuclear region, indicating endoplasmic reticulum localization. (B) Primary antibodies were verified by Western blot and showed a major band corresponding to the appropriate molecular weight of each protein. (C) Proximity ligation assay (Duolink, Sigma-Aldrich) utilized the same primary antibodies but secondary antibodies conjugated to probes that generate a signal only when the proteins are within 40 nm. The appearance of red fluorescent spots signifies the close proximity of GPER and Gαs.