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Estrogen Biology: New Insights into GPER Function and Clinical Opportunities

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Abstract

Estrogens play an important role in the regulation of normal physiology, aging and many disease states. Although the nuclear estrogen receptors have classically been described to function as ligand-activated transcription factors mediating genomic effects in hormonally regulated tissues, more recent studies reveal that estrogens also mediate rapid signaling events traditionally associated with G protein-coupled receptors. The G protein-coupled estrogen receptor GPER (formerly GPR30) has now become recognized as a major mediator of estrogen's rapid cellular effects throughout the body. With the discovery of selective synthetic ligands for GPER, both agonists and antagonists, as well as the use of GPER knockout mice, significant advances have been made in our understanding of GPER function at the cellular, tissue and organismal levels. In many instances, the protective/beneficial effects of estrogen are mimicked by selective GPER agonism and are absent or reduced in GPER knockout mice, suggesting an essential or at least parallel role for GPER in the actions of estrogen. In this review, we will discuss recent advances and our current understanding of the role of GPER and certain drugs such as SERMs and SERDs in physiology and disease. We will also highlight novel opportunities for clinical development towards GPER-targeted therapeutics, for molecular imaging, as well as for theranostic approaches and personalized medicine.

Keywords

17 -estradiol; Cardioprotection; Cardiovascular; Endothelium; endoplasmic reticulum, Endothelin, eNOS; Endothelial Nitric Oxide Synthase, epidermal growth factor receptor, EGFR, extracellular signal-regulated kinase, ERK; Endothelium; endothelial cells; ER; ER; ER; ERE; Estradiol;

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Estrogen; Estrogen Receptor; Genomic Signaling; GPCR; GPER; GPR30; MAPK; Non-Genomic Signaling; Personalized Medicine; Phosphatidylinositol-3-OH Kinase; PI3K; Plasma Membrane; Raloxifene; SERM; SERD; Tamoxifen; Theranostics; Vascular Smooth Muscle Cell

1. Introduction

Historically, cellular responses to estrogens and estrogenic compounds, as well as other steroids, have been described in terms of the “classical” nuclear receptors and in the case of estrogen, the estrogen receptors α (ER α) (King and Greene, 1984; Green et al., 1986; Greene et al., 1986) and β (ER β) (Kuiper et al., 1996). These receptors mediate cellular effects on gene expression known as “genomic” signaling, through the formation of receptor homo- or heterodimers, and binding to estrogen response elements (ERE) in the promoter and regulatory regions of target genes (Edwards, 2005). Nuclear ERs (even in the absence of ligand binding or direct binding to DNA) also interact with other classes of transcription factors and DNA modifying proteins through complex protein-protein interactions, thus regulating gene expression and cell function (Schultz-Norton et al., 2011). Such mechanisms are the topic of many of the articles in this special issue.

In addition to genomic cellular effects, estrogen, as well as other steroids, mediates a variety of “rapid” cellular responses to physiological concentrations of estrogens, responses that occur on a time frame of seconds to minutes, inconsistent with *de novo* transcription and protein synthesis (Falkenstein et al., 2000). In fact, some of the earliest cellular effects of estrogen were rapid effects on cAMP synthesis (Szego and Davis, 1967) and calcium mobilization (Pietras and Szego, 1975). These rapid estrogen-mediated effects are transmitted via enzymatic pathways and ion channels through the activation of what are generically denoted as membrane-associated ERs (mER), and are referred to as “non-genomic” or “extra-nuclear” pathways (Fu and Simoncini, 2008; Levin, 2009). It should however be noted that any absolute distinction between genomic and non-genomic effects is rather arbitrary as many intracellular signaling pathways result in the modulation of gene expression (Ho et al., 2009). As a result, the combination of these multiple cellular actions allows for the fine-tuning of estrogen-mediated regulation of gene expression (Bjornstrom and Sjoberg, 2005). In addition, ERs also undergo extensive post-translational modifications including phosphorylation, acetylation, sumoylation and palmitoylation that modulate their function (Anbalagan et al., 2012). Thus, the ultimate cellular response to estrogen stimulation results from a complex interplay of transcriptional and non-transcriptional events.

In addition to the classical nuclear estrogen receptors, a now extensive body of literature over the last ~10 years has identified and characterized the functions of a 7-transmembrane spanning G protein-coupled receptor, GPER (previously named GPR30), predominantly in the rapid actions of estrogen (Filardo et al., 2000; Prossnitz et al., 2008a; Prossnitz et al., 2008b; Prossnitz and Barton, 2011; Filardo and Thomas, 2012), although effects on gene expression have also been described (Prossnitz and Maggiolini, 2009; Vivacqua et al., 2012). GPER was identified by a number of laboratories between 1996-1998 as an orphan receptor with no known ligand, and thus named GPR30, belonging to the family of 7-transmembrane spanning G protein-coupled receptors. The receptor cDNA was identified

from multiple sources including B lymphocytes (Owman et al., 1996; Kvingedal and Smeland, 1997), ER-positive breast cancer cells (Carmeci et al., 1997), human endothelial cells exposed to fluid shear stress (Takada et al., 1997) as well as database mining (O'Dowd et al., 1998) and degenerate oligonucleotide screening of genomic DNA (Feng and Gregor, 1997). However, in 2000, pioneering studies by Filardo and colleagues demonstrated that the expression of GPER was required for the rapid estrogen-mediated activation of ERK1/2 (Filardo et al., 2000) and subsequently in 2002 cAMP generation (Filardo et al., 2002). In 2005, estrogen binding to GPER was demonstrated by multiple groups (Revankar et al., 2005; Thomas et al., 2005) and in 2006, the first GPER-selective agonist was described (Bologa et al., 2006). This and the subsequent identification of GPER-selective antagonists (Dennis et al., 2009; Dennis et al., 2011) led to an increasing number of studies addressing the potential cellular and physiological functions of GPER. To date, functions for GPER have been described in almost every physiological system, including reproductive, endocrine, urinary, nervous, immune, musculoskeletal and cardiovascular (Prossnitz and Barton, 2011). Thus, combined with the actions of estrogen through the classical ERs, GPER serves to add to the complexity of mechanisms involved in the physiological responses to estrogen.

Endogenous estrogens are protective for multiple diseases prior to menopause (Rettberg et al., 2013), not the least of which are cardiovascular disease and atherosclerosis, based in part on the beneficial effects of estrogen on blood pressure and cholesterol profiles (Meyer et al., 2011b). In addition to beneficial metabolic effects (e.g. cholesterol regulation (Faulds et al., 2012)), estrogens exert multiple direct beneficial effects on the heart and arterial wall, including vasodilation, inhibition of smooth muscle cell proliferation, inhibition of inflammation, antioxidant effects, and endothelial/cardiac cell survival following injury (Meyer et al., 2006; Meyer and Barton, 2009; Meyer et al., 2009; Knowlton and Lee, 2012). Although nuclear ERs contribute to several of these effects, presumably by regulating ERE-containing genes, the actions of non-nuclear ER α have also been demonstrated (Chambliss et al., 2010; Wu et al., 2011; Banerjee et al., 2013). However, more recent studies have demonstrated that GPER also activates multiple signaling pathways in cardiovascular and immune cells that either acutely regulate cellular function, or possibly modulate gene expression through ERE-independent mechanisms (Lindsey and Chappell, 2011; Prossnitz and Barton, 2011; Filardo and Thomas, 2012; Han et al., 2013). In this review, we will discuss recent advances in our understanding of how GPER contributes to the beneficial effects of estrogen in the cardiovascular and immune systems with an outlook to possible clinical applications.

2. GPER Ligands and Signaling Mechanisms

2.1 GPER Ligands

Following the seminal report of rapid estrogen signaling requiring the expression of GPER in 2000 (Filardo et al., 2000), many studies have demonstrated specific estrogen binding to cells (or membranes from cells) expressing GPER. In 2005, Thomas *et al.* reported selective binding of tritiated estradiol to membrane preparations of cells endogenously expressing or transfected to express GPER, with an affinity of ~3 nM (Thomas et al., 2005). In that same

year, Revankar *et al.* utilized a fluorescent estrogen derivative to demonstrate specific estrogen binding to various cancer cell lines and GPER-transfected COS cells with an affinity of ~6 nM, which was directly proportional to the level of receptor expression (Revankar *et al.*, 2005). It should be noted that since GPER, like most GPCRs, has not yet been purified to homogeneity, such studies provide a correlation between ligand binding and presence of GPER, although imaging studies with fluorescent estrogen derivatives demonstrate strong colocalization of receptor and fluorescent estrogen, suggesting GPER likely represents the site of estrogen binding. Since these original studies, many other groups have similarly reported estrogen binding to GPER-expressing cells or membrane preparations (Wang *et al.*, 2008b; Liu *et al.*, 2009; Lappano *et al.*, 2010). Importantly, within the steroid family, estrogen binding to GPER exhibits high selectivity (>1000×) over testosterone, cortisol, and progesterone (Thomas *et al.*, 2005), and although studies have suggested a requirement for GPER expression in certain rapid actions of aldosterone (Ding *et al.*, 2009; Gros *et al.*, 2011), a recent study was unable to detect aldosterone binding in membranes when estrogen binding was readily demonstrable (Cheng *et al.*, 2013). Furthermore, although estrone and 17 α -estradiol show poor binding activity towards GPER (Thomas *et al.*, 2005), estriol at μ M concentrations has been reported to act as an antagonist (Lappano *et al.*, 2010), and 2-methoxy-estradiol has recently been reported to bind to GPER (Koganti *et al.*, 2013).

With the wealth of synthetic and natural estrogen substances in existence, it is perhaps not surprising that a large number have been shown to interact with GPER. Of the therapeutic anti-estrogens, ICI182,780 (a selective estrogen receptor downregulator, SERD) was first demonstrated to interact with GPER, but surprisingly, as opposed to its antagonistic action towards ER α / β , ICI182,780 acted as an agonist towards GPER (Filardo *et al.*, 2000), with subsequent binding studies revealing an affinity of ~30 nM (Fig. 1) (Thomas and Dong, 2006). Similarly, 4-hydroxytamoxifen (the active metabolite of tamoxifen, a selective estrogen receptor modulator, SERM) also acts as a GPER agonist (Revankar *et al.*, 2005; Vivacqua *et al.*, 2006b), and recently raloxifene has also been demonstrated to activate GPER in cells deficient for ER α (Petrie *et al.*, 2013), consistent with the actions of a series of benzothiophene SERMs in neuroprotection (Abdelhamid *et al.*, 2011). Many synthetic compounds from the pesticide and plastics industries known to have estrogenic effects have also been demonstrated to bind and/or activate GPER, including atrazine (Albanito *et al.*, 2008), bisphenol A (Dong *et al.*, 2011; Chevalier *et al.*, 2012; Pupo *et al.*, 2012; Sheng *et al.*, 2013), daidzein (Kajta *et al.*, 2013), zearalalone, nonphenol, kepone, *p,p'*-DDT, *o,p'*-DDE and 2,2',5',-PCB-4-OH (Thomas and Dong, 2006). Finally, a number of phytoestrogens display agonist activity towards GPER, including genistein (Maggiolini *et al.*, 2004; Thomas and Dong, 2006; Vivacqua *et al.*, 2006a), quercetin (Maggiolini *et al.*, 2004), equol (Rowlands *et al.*, 2011), resveratrol (Dong *et al.*, 2013), oleuropein, and hydroxytyrosol (Chimento *et al.*, 2013).

New synthetic and screening approaches have identified a number of compounds that display selectivity among the various estrogen receptors (ER α , ER β , and GPER). The first GPER-selective ligand identified was G-1, which displays an affinity for GPER of ~10 nM with no significant binding or function at either ER α or ER β at concentrations as high as 10

μM (Bologa et al., 2006; Dennis et al., 2011). Subsequent studies identified a related compound lacking the ethanone moiety, G15, which functioned as a highly selective GPER antagonist (Dennis et al., 2009). As a result of low levels of activity against ER α or ER β at concentrations of $\sim 10 \mu\text{M}$, a derivative (G36) was synthesized that restored the steric bulk of G-1, maintaining antagonism while restoring the ER counterselectivity to levels similar to those of G-1 (Dennis et al., 2011). Together these three compounds have now been used in over 150 publications to study the effects of GPER in systems from cells to tissues and organs to live animals and have served as the basis of GPER-selective radioimaging agents (Nayak et al., 2010; Ramesh et al., 2010; Burai et al., 2012). STX, a diphenylacrylamide analog of tamoxifen, had been demonstrated to exert rapid neurological effects through a Gq protein-coupled mechanism unrelated to classical ERs (Qiu et al., 2003), suggesting GPER might be involved. However, although the effects of STX persist in a GPER knockout mouse (as well as in ER α /ER β double knockout mice) (Qiu et al., 2008), STX has been demonstrated to mimic estrogen-mediated effects on the SF-1 transcription factor by GPER (Lin et al., 2009). MIBE (ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyl)-1-methyl-1H-indol-3-yl] but-2-enoate) has been described as an antagonist of both ER α and GPER, although the affinity for both receptors is greater than $10 \mu\text{M}$ (Lappano et al., 2012b). Two additional compounds, GPER-L1 and GPER-L2 have been reported to function as GPER-selective agonists, with binding affinities of $\sim 100 \text{ nM}$ (Lappano et al., 2012a). Interestingly, the widely used ER α -selective (410-fold over ER β) agonist PPT has recently been shown also to be capable of acting as an agonist of GPER (at concentrations of $10\text{--}100 \text{ nM}$), while the ER β -selective agonist DPN displayed no corresponding activity towards GPER (up to concentrations of $10 \mu\text{M}$) (Petrie et al., 2013). Thus although PPT remains selective for ER α over both ER β and GPER, care needed in the interpretation of results employing receptor-selective ligands, particularly with respect to the concentrations and doses of these compounds used (Washburn et al., 2013). Nevertheless, the increasing spectrum of GPER/ER-modulating compounds suggests that many new opportunities exist in their experimental use and perhaps clinical development.

Although the amino acid sequence of GPER bears homology to the chemokine receptor subfamily of GPCRs (Owman et al., 1996; Feng and Gregor, 1997), early studies found no activation in response to a panel of chemokines (Owman et al., 1996). However, there is evidence to suggest that a chemokine (CCL-18) modulates GPER activity, possibly through direct binding to GPER (Feng and Gregor, 1997). Thus, CCL-18 could act as an endogenous inhibitor of GPER, similar to the actions of IL-1ra, which attenuates the activation of IL-1R by IL-1 β (Dripps et al., 1991).

2.2 GPER-mediated Signaling Mechanisms

In the original report of estrogen-mediated rapid signaling by GPER, Filardo et al. (Filardo et al., 2000) described a mechanism of ERK1/2 activation by GPER employing transactivation of the EGFR (Fig. 2) (Daub et al., 1996). Consistent with early events of G protein-coupled receptor-mediated signaling, GPER acted via a G $\beta\gamma$ -dependent, pertussis toxin-sensitive pathway, implying the involvement of G $\alpha_{i/o}$ proteins. However, inhibition of EGFR kinase activity or downregulating HB-EGF with CRM-197 or scavenging free HB-EGF with antibodies also prevented estrogen-mediated signaling, suggesting the

involvement of a transactivation of the EGFR by metalloproteinase-mediated release of HB-EGF, a mechanism subsequently confirmed by many studies (Revankar et al., 2005; Peyton and Thomas, 2011; Li et al., 2013b). Furthermore, a role for fibronectin-recruited $\alpha 5 \beta 1$ integrin has been implicated in the transactivation of the EGFR (Quinn et al., 2009) as has a role for sphingosine kinase (Sukocheva et al., 2006). In addition to these pathways, GPER has also been demonstrated to mediate $G\alpha_s$ protein activation (Thomas et al., 2005) leading to adenylyl cyclase activation and cAMP accumulation (Filardo et al., 2002), which further leads to PKA activation (Zucchetti et al., 2013) and transcriptional activation of CREB (Kanda and Watanabe, 2004) as well as vascular effects (Lindsey et al., 2013b) among other sequelae. Additional downstream pathways reported to be activated by GPER include PI3K (Revankar et al., 2005; Petrie et al., 2013), PKC (Goswami et al., 2011), calcium mobilization (Revankar et al., 2005; Tica et al., 2011), and other ion channels (Fraser et al., 2010; Goswami et al., 2011). Interestingly, many studies have observed that a vast majority of GPER is expressed in intracellular membranes (Revankar et al., 2005; Otto et al., 2008; Filardo and Thomas, 2012), with little expressed at the cell surface in most cells and cell lines likely due to both inefficient export mechanisms, which involve receptor activity-modifying protein 3 (RAMP3) (Bouschet et al., 2012; Lenhart et al., 2013), as well as constitutive GPER internalization (Cheng et al., 2011a; Cheng et al., 2011b). Furthermore, differentially permeable estrogen derivatives reveal that membrane permeability of the ligand is required for rapid signaling by GPER, suggesting the intracellular pool is functional (Revankar et al., 2007). A pathological role for RAMP3 (Receptor Activity-modifying Protein 3) in the trafficking of GPER has been demonstrated through the G-1-mediated attenuation of cardiac hypertrophy and perivascular fibrosis in a mouse model prone to heart disease (Lenhart et al., 2013). Finally, analyses of serum-stimulated human endothelial cells identified PALS1-associated tight junction protein and FUN14 domain containing 2 as GPER-interacting partners, with functional or trafficking roles yet to be described (Zazzu, 2011).

Beyond rapid signaling effects, GPER also regulates gene expression, as demonstrated by some of the earliest studies on genomic signaling by GPER (Kanda and Watanabe, 2003a; Kanda and Watanabe, 2003b; Kanda and Watanabe, 2004; Maggiolini et al., 2004; Ylikomi et al., 2004). These and subsequent studies revealed the upregulation of the immediate early gene *c-fos* (and other genes) by estrogen (O'Brien et al., 2006; Vivacqua et al., 2006b; Albanito et al., 2007; Prossnitz and Maggiolini, 2009), as had been demonstrated almost 20 years earlier in the rat uterus (Weisz and Bresciani, 1988) and rat aortic vascular smooth muscle (Orimo et al., 1993). In addition, estrogen and the GPER-selective agonist have been demonstrated to upregulate the expression of cell cycle regulators such as cyclins A, D1 and E (Albanito et al., 2007), connective tissue growth factor (Pandey et al., 2009), Early growth response-1 (Vivacqua et al., 2012), and VEGF (De Francesco et al., 2013), in many cases through EGFR- and ERK-dependent mechanisms. The latter mechanism is also involved in the cleavage of cyclin E, associated with tumor progression and resistance to anti-estrogens, and induced by tamoxifen and G-1 in GPER-expressing cells (Li et al., 2013b). Interestingly, recent studies by Maggiolini and colleagues have suggested that GPER localization to the nucleus via an importin-dependent mechanism is involved in its transcriptional activity (Pupo et al., 2013). In addition, GPER in a complex with EGFR has

been localized to the cyclin D1 promoter (Madeo and Maggiolini, 2010). How two integral membrane proteins are localized to DNA within the nucleus is an intriguing question and in need of further investigation.

3. Interactions of GPER with Other Receptors

3.1 Lipid Signaling via Nuclear Steroid Hormone Receptors: Interactions of GPER

Estrogen Receptor (ER)—Co-expression of ER α and ER β with GPER suggests the possibility of interactions between these receptors and their signaling pathways (Prossnitz and Barton, 2011). Functional cross-talk has been reported, where GPER expression is required along with ER α for estrogen-mediated activity in cancer cells (Albanito et al., 2007) or for inhibiting ER α -mediated functions in uterine epithelial cells (Gao et al., 2011). Functional cross-talk between ER α and GPER is also evident from functional vascular studies in porcine coronaries, where acute NO-dependent vasodilation is observed only with ER α -selective agonists such as PPT, but is completely abrogated when ER α , ER β , and GPER are activated simultaneously by estrogen (Traupe et al., 2007). The recent discovery that responses to PPT, which is often employed as an ER α -selective agonist (relative to ER β), also activated GPER complicates our understanding of PPT-mediated responses (Petrie et al., 2013), as they may, at least in part, involve GPER, depending on the receptors expressed and concentrations used. A redundancy for the receptors mediating effects of estrogen is also suggested by work by Mauvais-Jarvis and colleagues (Tiano et al., 2011), who reported that the protective effects of estrogen on pancreatic islets are comparable and independent of selective activation of ER α , ER β , or GPER (Tiano et al., 2011). Thus, cross-talk between GPER and estrogen receptors (and possibly other steroid receptors) appears to be likely and should be considered when assessing possible pathogenic and therapeutic questions related to activation or inhibition of estrogen receptors (Barton, 2012). Receptor cross-talk and functional redundancies are likely to be important for understanding the actions of drugs targeting either one or several estrogen receptors. ER α -36, a membrane-localized splice variant of the human ER α identified in breast cancer cells as a dominant negative effector of full length ER α (Wang et al., 2005), has also been proposed as an effector of GPER-dependent signaling (Wang et al., 2005; Wang et al., 2006). Its function remains controversial and *in vivo* data in particular are scarce. Lindsey and coworkers reported renoprotective effects of G-1 but did not detect ER α -36 (whereas ER α -46 and ER α -66 were abundantly expressed) (Lindsey et al., 2011). Thus, *in vivo* evidence for a direct role of ER α -36 in mediating the effects of GPER is still lacking. Whether ER α -36 has indirect effects such as cross-talk involving GPER remains to be determined. Finally, the orphan nuclear receptor estrogen receptor-related receptor α (ERR α) has been implicated in GPER-mediated signaling as GPER activation causes transcriptional activation of ERR α protein synthesis and regulates ERR α -mediated downstream effects and cell proliferation (Li et al., 2010).

Glucocorticoid Receptor (GR)—Glucocorticoids are important regulators of energy and bone metabolism (Tisdale, 2002; Meyer et al., 2011a) and a role for GPER in maintaining metabolism has been suggested as GPER activation reduces food intake (Washburn et al., 2013) and stimulates insulin secretion (Sharma and Prossnitz, 2011), whereas GPER-

deficient mice are obese and insulin-resistant (Haas et al., 2009; Ford et al., 2011; Meyer et al., 2011a; Sharma et al., 2013). GPER has also been demonstrated to negatively regulate the transcriptional activity of glucocorticoid receptor (Ylikomi et al., 2004) and may thereby also indirectly affect energy metabolism, expenditure, and overall energy homeostasis and body weight through GR activity.

Mineralocorticoid Receptor (MR)—Aldosterone, a potent vasoconstrictor and mitogen synthesized by the adrenal gland and by vascular smooth muscle cells (Matsuzawa et al., 2013), has been proposed to act through GPER for certain functions (Gros et al., 2011; Gros et al., 2013). Although aldosterone does not bind to GPER (Cheng et al., 2013), thus appearing to exclude the possibility of GPER being a true aldosterone receptor, it is possible that, similar to what has been reported for estrogen receptors, a functional cross-talk between MR and GPER exists. This likely also underlies the recently reported vagal tone-activating effect of central aldosterone infusion (Brailoiu et al., 2013). On the other hand, since both deletion of vascular MR (McCurley et al., 2012; Schiffrin, 2013; Pruthi et al., 2014) and pharmacological activation of GPER (Haas et al., 2009) lower blood pressure, whereas aldosterone in most studies has the contrary effect by causing vasoconstriction and thereby increasing blood pressure, these effects would argue against a role for aldosterone as a main co-factor mediating GPER-dependent effects. Finally, since aldosterone is produced via AT₁ receptor-dependent mechanisms (Volpe et al., 1997), the inhibitory effect of GPER activation on AT₁ receptor expression (Koganti et al., 2013) may represent a novel mechanism for how aldosterone and GPER interact.

Vitamin D Receptor (VDR)—As a secosteroid, vitamin D (cholecalciferol) maintains bone function and growth through activation of the vitamin D receptor (VDR) (Norman and Powell, 2014). GPER expression can be detected in human bone (Heino et al., 2008) and GPER-dependent regulation of bone growth has been reported (Heino et al., 2008; Ford et al., 2011; Ren and Wu, 2012). An inflammation-dependent interaction between vitamin D receptor signaling and GPER is strongly suggested by the estrogen-dependent inhibitory effects of vitamin D on autoimmune encephalitis, an animal model of multiple sclerosis, where the effect of vitamin D is completely abrogated in female mice lacking GPER (Subramanian et al., 2012). GPER/VDR interactions are also likely to be involved in the beneficial effects of vitamin D on bone structure in postmenopausal women, which have been shown to be estrogen-dependent (Durusu Tanriover et al., 2010).

3.2 Other Receptors Interacting with GPER

In the ovary, GPER function is dependent on the transmembrane GPCR mediating the effects of luteinizing hormone, a heterodimeric glycoprotein, which shares the same α -subunit as FSH, TSH, TSH, and hCG (Dufau, 1998). Luteinizing hormone was recently shown to regulate GPER expression at the mRNA and protein level in human granulosa cells (Pavlik et al., 2011). Interactions and cross-talk between GPER and growth factor receptors, particularly EGFR and IGFR, have been recently summarized by Maggiolini and colleagues (Bartella et al., 2012; Lappano et al., 2013). In addition, functions of GPER may be affected, both in health and disease, by transcription factor-induced reprogramming of its

expression pattern, which has been recently described for ER α in breast cancer (Ross-Innes et al., 2012).

4. New Aspects of GPER Function in the Cardiovascular System

The vascular physiology of GPER, including the initial studies demonstrating a role in the regulation of vascular tone and protection from myocardial reperfusion injury, has been recently reviewed in a number of articles (Fig. 3) (Meyer et al., 2011b; Chakrabarti et al., 2013; Han et al., 2013; Holm and Nilsson, 2013). GPER was originally cloned as an orphan GPCR after induction by fluid shear stress in human vascular endothelial cells, as well as from different cancer cell lines (Prossnitz and Barton, 2011). Shear stress is a strong inducer of expression of the enzyme responsible for production of the vasodilator nitric oxide (NO) (Fleming, 2010), suggesting that GPER function might be linked to the L-arginine/NO pathway (Meyer et al., 2011b). Indeed, numerous laboratories have demonstrated NO- and endothelium-dependent vasodilation via activation of GPER, effects that are abrogated by endothelial denudation or treatment with NO synthase inhibitors such as L-NAME (Haynes et al., 2002; Moriarty et al., 2006; Meyer et al., 2011b; Barton et al., 2013; Lindsey et al., 2013b). Several recent studies have provided further insights into how GPER functions in vascular endothelial cells. In both cerebrovascular as well as acute kidney injury, estrogen-mediated protection is in part mediated via GPER in both males and females (Hutchens et al., 2012; Murata et al., 2013). In mesenteric resistance arteries, which dilate partly via endothelium-dependent hyperpolarization, vasodilator responses via GPER involve endothelium-derived NO (Lindsey et al., 2013b). Finally, GPER has been identified as an inhibitory regulator of pro-inflammatory proteins in endothelial cells (Chakrabarti and Davidge, 2012) and consistent with this, chronic G-1 treatment improves endothelium-dependent vasomotion in disease conditions associated with vascular inflammation, such as diabetes mellitus (Li et al., 2012).

Rapid, non-genomic effects of estrogen, an agonist for ER α , ER β , and GPER, have been demonstrated in human coronary and internal mammary arteries of male and female patients (Mugge et al., 1993; Haas et al., 2007), suggesting a possible role for GPER in regulating vascular and organ function of males. Indeed, vascular smooth muscle internal mammary arteries of male patients express GPER (Haas et al., 2007) and GPER-dependent effects in arteries and organs of animals of both sexes have been demonstrated (Prossnitz and Barton, 2011). Pharmacological stimulation of GPER utilizing G-1 has no effect in intact arteries or renal cells derived from GPER null mice (Haas et al., 2009; Hofmeister et al., 2012), confirming the specificity of this ligand-receptor interaction. Pharmacological inhibition of GPER utilizing antagonists such as G15 results in vasoconstriction (Meyer et al., 2012a); (Lindsey et al., 2009; Yu et al., 2011), suggesting that endogenous GPER has vasodilatory actions. However, the lack of hypertension in GPER knockout mice suggests (yet unidentified) counter-regulatory mechanisms facilitating vasodilation, possibly involving central (Brailoiu et al., 2012), cardiac, or renal effects or alternatively modulating responses in the autonomous nervous system.

Using mice deficient for GPER, several recent studies performed in male mice have revealed that endogenous GPER functions as an endogenous inhibitor of the activity of

endothelium-dependent contracting factor (EDCF) (Meyer et al., 2012a), as well as the newly discovered *adipose-derived contracting factor* (ADCF) formed in perivascular fat (Meyer et al., 2013), with both the EDCF and the ADCF vasoconstrictor pathways being entirely dependent on cyclooxygenase activity. As cyclooxygenase-derived vasoconstrictor prostanoids have been shown to accelerate progression of experimental atherosclerosis (Belton and Fitzgerald, 2003), endogenous GPER might represent a novel mechanism protecting from atherosclerosis. Atheroprotective mechanisms via endogenous GPER might also involve interference with adipogenesis, inflammation, and dyslipidemia, three of the key factors strongly implicated in atherogenesis (Barton, 2013), since male GPER-deficient mice exhibit increased adipogenesis, inflammatory activation, and increased circulating lipid levels (Sharma et al., 2013). GPER deficiency results not only in visceral obesity but also in perivascular adipose formation (Meyer et al., 2013), potentially involving a direct inhibitory effect of GPER on mitotic clonal expansion of adipocytes (Zhu et al., 2013). Unlike activation of ER α , which *stimulates* endothelial cell proliferation, angiogenesis, and wound healing (Haynes et al., 2002; Kim and Bender, 2009), selective agonists for GPER *inhibit* endothelial cell proliferation, effects that have also been demonstrated in studies using ICI 182,780 and genistein, agonists of GPER (Filardo et al., 2000; Petrie et al., 2013), to interfere with endothelial cell proliferation (Holm et al., 2011).

GPER is highly expressed in vascular smooth muscle cells (Haas et al., 2009), a key cell required for the development of atherogenesis (Barton et al., 2007). In human arteries, as well as cultured human vascular smooth muscle cells, expression of GPER can be detected (Haas et al., 2007; Haas et al., 2009) and its activation results in cell growth inhibition (Haas et al., 2009), with comparable effects demonstrated in porcine coronary vascular smooth muscle cells (Li et al., 2013a). In rat vascular smooth muscle cells, GPER is expressed at a comparable level in both males and females, and at a much higher level than ER β (Ma et al., 2010). Activation of vascular smooth muscle cell GPER also results in rapid ERK phosphorylation (Haas et al., 2009; Gros et al., 2011), an effect enhanced by adenoviral overexpression of GPER (Gros et al., 2011). GPER expression also interferes with the contractile response to agonists for other GPCRs, including endothelin receptors (Meyer et al., 2010) and in intact arteries, endogenous GPER suppresses endothelin-mediated changes in intracellular calcium and endothelin-mediated vasoconstriction, as the response is enhanced in GPER knockout mice (Meyer et al., 2012b). The role of GPER in the regulation of intracellular calcium has now been confirmed in cultured vascular smooth muscle cells, demonstrating an involvement of voltage-sensitive calcium changes (Holm et al., 2013). Moreover, a role for estrogen-dependent GPER-mediated calcium mobilization has been demonstrated in micro-dissected renal tubules and cortical collecting ducts, effects that were absent in GPER knockout mice (Hofmeister et al., 2012). These findings corroborate a novel and previously unknown role for GPER in non-genomic effects of estrogens on intracellular calcium.

Recent reports have extended our knowledge about GPER functions in vascular smooth muscle and regulation of arterial tone. In mesenteric resistance arteries, which partly dilate via endothelium-dependent hyperpolarization, Lindsey and colleagues have recently identified vascular smooth muscle adenylyl cyclase/cAMP signaling as contributing to

relaxation (Lindsey et al., 2013b). A role for GPER/G-1-mediated EGFR transactivation, as observed in many cancer cells (Filardo et al., 2000), in vascular relaxation is supported by a recent study showing that two different EGFR antagonists abolished the relaxant effects of G-1 (Jang et al., 2013).

Several factors appear to affect vascular GPER function. Aging in normotensive and hypertensive rats results in a downregulation of GPER in both males and females, and is associated with a reduced relaxation to G-1 or E2 (Lindsey et al., 2013a). As has been shown for other agonists, estrogen receptor-selective agonists display a marked anatomic heterogeneity in arteries of female rodents, with some vessels responding negligibly while others show rapid relaxation in response to activation of ERs or GPER (Reslan et al., 2013). A similar heterogeneity has recently been reported to be present in human uterine and placental arteries (Corcoran et al., 2013). The implications of such heterogeneity for vascular protection and susceptibility to disease development have been recently discussed (Barton et al., 2013). Activation of GPER is also protective to the heart, as it improves diastolic function and left ventricular remodeling in hypertensive rats (Wang et al., 2012) and ameliorates cardiac function in a model of isoproterenol-induced heart failure (Kang et al., 2012). Preliminary reports have also demonstrated that GPER activity, but not that of ER α or ER β , is critical in the protective effects of estrogen on myocardial reperfusion injury (Bopassa et al., 2011; Bopassa et al., 2012a; Bopassa et al., 2012b) with the identification of multiple differentially up- and down-regulated mitochondrial proteins in GPER KO mice (Bopassa et al., 2013). Finally, responses to treatment with G-1 may show a sexual dimorphism, as recently reported by Broughton *et al.* in a stroke model (Broughton et al., 2014) and by Caron's group using a genetic model of cardiac hypertrophy and fibrosis (Lenhart et al., 2013).

5. GPER in Immunity and Inflammation

Estrogen and, as more recently demonstrated, drugs commonly used as “anti-estrogens”, exert multiple effects upon the development and function of the immune system (Ray and Ficek, 2012; Bonds and Midoro-Horiuti, 2013; Sakiani et al., 2013), as exemplified by the developmental effects of estrogen in estrogen-promoted atrophy of the thymus during pregnancy (Pernis, 2007). Expression of GPER in multiple immune cells, including B and T cells, monocytes/macrophages and neutrophils, suggested that some estrogenic effects in the immune system could be mediated by GPER (Wang et al., 2008a; Blasko et al., 2009; Rettew et al., 2010; Cabas et al., 2013). A role for GPER in estrogen-mediated thymic atrophy was first suggested employing ER α , ER β , and GPER knockout mice (Wang et al., 2008a). Whereas ER α was shown to mediate exclusively the early developmental blockage of thymocyte development, GPER was required for thymocyte apoptosis that preferentially occurs in T cell receptor double-positive thymocytes. Furthermore, G-1 induced thymic atrophy and thymocyte apoptosis, but not a developmental blockage. Although supported by an increased apoptosis rate for naïve T cells in GPER knockout mice (Isensee et al., 2009) distinct from those used in the original study (Wang et al., 2008a), others, however, have not observed similar effects with G-1 (Otto et al., 2008) or in ovariectomized GPER knockout mice (Martensson et al., 2009) distinct from the other two GPER knockout mice employed, suggesting further clarification is necessary.

Estrogens have also more recently received increased attention as potential anti-inflammatory/anti-apoptotic therapeutic agents for stroke, reperfusion injury associated with myocardial infarction, and autoimmune diseases, particularly multiple sclerosis (Niino et al., 2009). Employing a widely used murine model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), estrogen-mediated protection was significantly decreased in GPER knockout mice (Wang et al., 2009), whereas in studies that employed G-1 treatment with the EAE model, protection against clinical and histological manifestations of EAE was similar to that of estrogen (Blasko et al., 2009; Wang et al., 2009). Importantly, the effects of G-1 were completely absent in GPER knockout mice, demonstrating a requirement for GPER, and as the protective effects of estrogen were partially lost, this suggests that estrogen acts through independent yet overlapping mechanisms via both ER α and GPER, that can be completely mimicked through G-1 activation of GPER in the absence of ER α -mediated effects (Wang et al., 2009). Whereas G-1 was demonstrated in one study to enhance the suppressive activity of CD4⁺Foxp3⁺ T regulatory cells through upregulation of programmed death 1 (Wang et al., 2009), in another study G-1 was observed to inhibit inflammatory cytokine production of macrophages (Blasko et al., 2009). These data are consistent with a more recent study that has also demonstrated the estrogen-induced inhibition of LPS-induced TNF α production in bone marrow-derived macrophages is mediated by GPER through calcium signaling but not ER α activation (Liu et al., 2013). In addition, the therapeutic effect of ethinyl estradiol in established disease was demonstrated to require expression of GPER but not ER α , and was associated with the production of the anti-inflammatory cytokine IL-10 (Yates et al., 2010). Demonstrating this effect explicitly, G-1 was shown to elicit *de novo* IL-10 production in pro-inflammatory Th17-polarized cells *in vitro* as well as *in vivo* following G-1 administration (Brunsing and Prossnitz, 2011). Furthermore, using *ex vivo* cultures of purified CD4⁺ T cells, G-1 elicited Foxp3 expression (a marker of natural and induced regulatory T cells) under Th17-polarizing conditions, which exist in most autoimmune diseases (Brunsing et al., 2013). Lastly, GPER has recently been shown to modulate vertebrate (fish) granulocyte (neutrophil) functions, including reducing the respiratory burst and pro- and anti-inflammatory gene expression through a cAMP/protein kinase A/CREB-mediated pathway (Cabas et al., 2013). Together, these reports suggest that endogenous GPER conveys immunoprotective effects, which, like the direct generally anti-inflammatory effects of G-1 on the immune system, are multifaceted and complex.

As many cardiovascular diseases involve strong inflammatory and immune components (e.g. atherosclerosis (Moore et al., 2013) and reperfusion injury (Frangogiannis, 2006)), the functions of GPER could have additional important roles, beyond the direct effects on the endothelium and smooth muscle discussed above, in the development and progression of these conditions. Estrogen has long been recognized for its anti-atherogenic effects, reflecting prevention of atherosclerosis through inhibition of inflammation and endothelial/vascular dysfunction (Resanovic et al., 2013), and in the early stages of atherosclerosis, where estrogen-dependent protective effects are independent of ER α (Villablanca et al., 2009). One recently reported additional direct immunomodulatory effect of GPER in the endothelium is the attenuation of TNF α -induced upregulation of pro-inflammatory adhesion proteins such as ICAM-1 and VCAM-1 (Chakrabarti and Davidge, 2012), likely to result in

decreased leukocyte adherence, important in atherogenesis and other vascular diseases (Golias et al., 2007). GPER deficiency has also been shown to result in insulin resistance, dyslipidemia, obesity, and increased circulating pro-inflammatory cytokines (Haas et al., 2009; Sharma et al., 2013), all likely to contribute to vascular dysfunction and disease (Meyer and Barton, 2009; Meyer et al., 2011b), suggesting that GPER contributes in important ways to a healthy metabolism and inflammatory state. Immune effects via GPER have also been shown to be involved in the reduction of infarct size and improvement of peripheral immunosuppression following stroke with estrogen deficiency in female mice (Zhang et al., 2010). Similar effects following myocardial infarction are possible and thus, in addition to the direct cardioprotective effects of G-1 observed in myocardial ischemia-reperfusion models involving activation of PI3K/Akt and ERK1/2 (Deschamps and Murphy, 2009; Bopassa et al., 2010; Patel et al., 2010; Weil et al., 2010), GPER may play additional important roles in the immune system that might have profound effects on the course of diseases with a strong underlying inflammatory component, such as atherosclerotic vascular disease (Meyer and Barton, 2009).

6. Outlook and Potential Clinical Applications

It has been almost 20 years since the first report identifying and cloning GPR30 (Owman et al., 1996), yet only over the past few years, with the publication rate of scientific reports on GPER now exceeding 10 per month on average, has our understanding of GPER's cellular functions in physiology and disease truly begun to advance. GPR30 was officially designated G protein-coupled estrogen receptor (GPER) by the International Union of Basic and Clinical Pharmacology in 2007 based on the preponderance of results demonstrating estrogen action through GPER (Alexander et al., 2011). However, despite academic laboratories providing molecular and pharmacological tools (Huryn et al., 2013) as well as GPER knockout mice (Wang et al., 2008a; Martensson et al., 2009), deciphering the unique roles of GPER as an estrogen receptor in health and disease remains challenging due to the complexity of physiological responses to estrogen and the multiple receptors and interacting partners involved. These issues have been further complicated at times by seemingly controversial research findings, leading to premature conclusions (Tiano et al., 2011; Barton, 2012), such as statements that GPER is "a receptor for aldosterone, but not estrogen" (Funder, 2011). The field of non-genomic ("non-classical") effects mediated by ER α and its many splice variants with varying cellular localization, as well as the multitude of ligands with widely varying effects depending on cellular context, has experienced similar difficulties (Heldring et al., 2007; Kim et al., 2011). Further complications arise from sex differences in GPER and sex steroid receptor expression and function (Barton, 2012; Miller, 2014), only now beginning to be investigated (Lenhart et al., 2013; Broughton et al., 2014).

In the future, the measurement of GPER expression levels, its cellular and tissue distribution, or the use GPER agonists or antagonists may provide new approaches in diagnostic, prognostic, or theranostic evaluation (Smith et al., 2007; Smith et al., 2009), drug therapy, or molecular imaging in disease (Fowler, 2014). With regard to clinical effects mediated by GPER, it is now clear that previous views must be reassessed regarding SERMs, such as raloxifene, and SERDs, such as ICI182,780 (Fulvestrant or Faslodex®). Instead of acting solely as ER-modulating agents (Barton, 2012), these drugs, known and

clinically used as cancer therapeutics, also have the potential to act as agonists for GPER *in vivo*. The fact that GPER activation causes vasodilation is consistent with the hypotensive side effects observed in some patients receiving Faslodex® (Vergote and Abram, 2006). The understanding of fulvestrant action has however been further complicated by the recent finding that this compound may also act as an ER α agonist when the activation function-2 (AF-2) of ER α is mutated (Borjesson et al., 2011; Moverare-Skrtic et al., 2014). Finally, the question as to whether polymorphisms of the GPER gene, which maps to chromosome 7p22, a locus also associated with resistant arterial hypertension in genetic linkage studies in humans (Lafferty et al., 2000; Haas et al., 2009), may be associated with changes in cardiovascular disease risk, is beginning to be addressed in arterial hypertension (Feldman et al., 2013) as is the relationship of GPER polymorphisms to human seminoma risk (Chevalier et al., 2014). Finally, information related to GPER (e.g. responses to certain treatments, expression in tumors (van't Veer and Bernards, 2008; Venkatakrishnan et al., 2013)) may also be used for theranostic purposes that could provide optimization of current treatments and allow personalized medicine for many diseases (Shastry, 2006), including cancer and cardiovascular diseases (Ozdemir et al., 2006; Offit, 2011).

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Table of Abbreviations

eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular signal-related kinases-1/2
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
GPER	G protein-coupled estrogen receptor 1
GPR30	G protein-coupled receptor 30
MAPK	Mitogen-activated protein kinase
mER	Membrane-associated estrogen receptor (α or β)
NO	Nitric oxide
PI3K	Phosphatidylinositol-3-kinase

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Figure 1.

Ligands of GPER and ERs. Agonists (green arrows), partial agonists (SERMS, orange arrow) and antagonists (red arrows) of GPER and the classical estrogen receptors ER and ER. SERMS and SERDs that function as ER antagonists in many tissues act as agonists of GPER. GPER-selective ligands (G-1, G15 and G36) are highly selective for GPER over ERs. Modified from (Meyer et al., 2011b), with permission of Elsevier Publishers.

Figure 2.

Signaling pathways initiated by GPER and ERs. Endogenous estrogens, including 17 - estradiol (E2), represent nonselective activators of the three known ERs, ER, ER and GPER. Estrogen activates nuclear ERs, inducing dimerization of the receptors and binding of receptor dimers to target gene promoters. Alternatively, activated ERs modulate the function of other classes of transcription factors (TFs) through protein-protein interactions. Subpopulations of ERs at the plasma membrane following activation by estrogen interact with adaptor proteins (adaptor) and signaling molecules such as *c-src*, which mediates rapid signaling via PI3K/Akt and MAPK pathways. GPER, which is predominantly localized intracellularly, can be activated by estrogen, or selective GPER agonists (such as G-1), but also by selective estrogen receptor downregulators (such as fulvestrant), or selective estrogen receptor modulators (such as tamoxifen and raloxifene). GPER activation stimulates cAMP production, calcium mobilization and *c-src*, which activates MMPs. These MMPs cleave pro-HB-EGF, releasing free HB-EGF that transactivates EGFR, which in turn activates MAPK and PI3K/Akt pathways that can induce additional rapid (nongenomic) effects (X), or genomic effects regulating gene transcription. Estrogen-mediated transcriptional regulation may involve phosphorylation (P) of ER or other TFs that may directly interact with ER, or bind independently of ER within the promoters of target genes. Abbreviations: E2, 17 - estradiol; EGFR, epidermal growth factor receptor; ER, estrogen receptor; GPER, G-protein-coupled ER; MMP, matrix metalloproteinase; pro-HB-EGF, pro-heparin-binding epidermal growth factor; TF, transcription factor. Figure reproduced from (Prossnitz and Barton, 2011).

Figure 3.

Current experimental and clinical evidence implicates a physiological role (shown in black) for GPER in neuroendocrine and cerebral functions, immunity and immune cell function, metabolic and endocrine regulation, vascular, myocardial, and kidney function, as well as development and reproductive functions. In addition, data obtained in experimental models of disease and/or tissue from patients with disease suggest roles for GPER in pathological conditions (shown in red), such as diabetes mellitus, arterial hypertension, proteinuric renal disease, osteoporosis, arthritis, immune diseases, such as multiple sclerosis, and cancer. Targeting GPER activity with highly selective ligands in humans may represent a novel approach for the treatment of these conditions, for molecular imaging, as well as for theranostic approaches and personalized medicine. Figure reproduced from (Prossnitz and Barton, 2011).