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G-Protein-Coupled Estrogen Receptor (GPER) and Sex-Specific Metabolic Homeostasis

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Abstract

Obesity and metabolic syndrome display disparate prevalence and regulation between males and females. Human, as well as rodent, females with regular menstrual/estrous cycles exhibit protection from weight gain and associated chronic diseases. These beneficial effects are predominantly attributed to the female hormone estrogen, specifically l7β-estradiol(E2). E2 exerts its actions via multiple receptors, nuclear and extranuclear estrogen receptor (ER) α and ERβ, and the G-protein-coupled estrogen receptor (GPER, previously termed GPR30). The roles of GPER in metabolic homeostasis are beginning to emerge but are complex and remain unclear. The discovery of GPER-selective pharmacological agents (agonists and antagonists) and the availability of GPER knockout mice have significantly enhanced our understanding of the functions of GPER in normal physiology and disease. GPER action manifests pleiotropic effects in metabolically active tissues such as the pancreas, adipose, liver, and skeletal muscle. Cellular and animal studies have established that GPER is involved in the regulation of body weight, feeding behavior, inflammation, as well as glucose and lipid homeostasis. GPER deficiency leads to increased adiposity, insulin resistance, and metabolic dysfunction in mice. In contrast, pharmacologic stimulation of GPER in vivo limits weight gain and improves metabolic output, revealing a promising novel therapeutic potential for the treatment of obesity and diabetes.

Introduction: Obesity, Metabolism, and Sex Differences

Obesity represents a grave public health concern in the modern world. It is now recognized as a global epidemic with the number of obese individuals increasing drastically over the last two to three decades in both developed and more recently developing nations (Flegal et al. 2010; Guh et al. 2009). According to the latest demographic data from the Centre for Disease Control, it is estimated that in the United States alone, more than 65% of population

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is either overweight or obese (Center for Disease Control and Prevention: Adult Obesity Facts 2017). Obesity is not merely the presence of excessive body weight but represents a major risk factor for metabolic syndrome, a collection of conditions that includes high blood sugar, increased waist circumference, high blood pressure, and abnormal cholesterol (i.e., low high-density lipoprotein levels) or triglyceride levels (Han and Lean 2016; Keller and Lemberg 2003; Alberti et al. 2006; Mittendorfer 2011). Together, these conditions raise the risk of diabetes, heart disease, stroke, as well as certain forms of cancer (Eckel and Krauss 1998; Poirier et al. 2006; Kernan and Dearborn 2015; Basen-Engquist and Chang 2011). The socioeconomic burden of obesity is enormous, with current annual cost estimates in the United States alone ranging from $147 to 210 billion, and simultaneous reductions in the quality of life and life expectancy (Guh et al. 2009; Hammond and Levine 2010). Obesity can result from a multitude of factors ranging from genetic, behavioral to environmental (McAllister et al. 2009). However, the principal reasons for the recent obesity epidemic include increased consumption of calorie-dense foods, high in saturated fats and refined sugars, coupled with a sedentary lifestyle, resulting in a chronic energy imbalance. The precise mechanisms responsible for the development of obesity and metabolic dysfunction are complex and not yet fully understood. As a result, there is an urgent need to identify novel molecular targets and therapeutic agents capable of preventing or limiting the development of obesity and its resulting metabolic abnormalities.

Obesity, diabetes, and cardiovascular diseases exhibit a significant sexual dichotomy, with the incidence being lower in females in their premenopausal years compared to age-matched men or postmenopausal women (Kotani et al. 1994; Garaulet et al. 2002; Nakhjavani et al. 2014; Tandon et al. 2010; Regitz-Zagrosek et al. 2006). Furthermore, the quantity and site of fat distribution also varies between men and women, leading to differential health outcomes (Regitz-Zagrosek et al. 2006; Blaak 2001). Overall, men have less body fat than women; however, in men this fat is distributed in the upper body or abdominal area, reflecting an “android” pattern, whereas women display a “gynoid” pattern, with lower body or subcutaneous fat distribution. Properties of android and gynoid fat differ, with the former being more prone to lipolysis and secreting increased levels of pro-inflammatory cytokines and therefore associated with an increased risk of metabolic syndrome and cardiovascular disease (Kotani et al. 1994; Blaak 2001; Jensen 2008; Ritchie and Connell 2007; Monteiro et al. 2014; Shulman 2014). Typical sex-specific fat distributions in males and females suggest distinct regulatory mechanisms that control energy balance and adiposity. Obesity leads to altered secretory profiles of adipose-specific hormones and cytokines, increased levels of circulating glucose and lipids, and systemic inflammation, resulting in ectopic lipid deposition in peripheral metabolic tissues, such as the pancreas, liver, and skeletal muscle (Shulman 2014; Klop et al. 2013; Consitt et al. 2009; Bays et al. 2013; Shoelson et al. 2007; Tchernof and Despres 2013). The ensuing glucotoxicity, lipotoxicity, and inflammation result in dysfunction of these tissues, including defects in insulin production and secretion by the pancreas, inhibition of insulin-stimulated glucose uptake in skeletal muscle, and increases in glucose production by the liver (Consitt et al. 2009; Muoio and Neufer 2012; Mota et al. 2016; Brons and Vaag 2009; Poitout and Robertson 2008).

Differences also exist in insulin sensitivity between the sexes with premenopausal women being more insulin sensitive compared to age-matched men or postmenopausal women

(Walton et al. 1993; Geer and Shen 2009; Lindheim et al. 1994; Mauvais-Jarvis 2011). Postmenopausal women experience a decline in metabolic health due to increased android fat deposition, reduced energy expenditure, insulin resistance, impaired glucose/lipid metabolism, and inflammation (Mauvais-Jarvis 2011; Poehlman et al. 1995; Lobo 2008; Abu-Taha et al. 2009; Godsland 2005; Bruns and Kemnitz 2004; Zhang et al. 2002; Andersson et al. 1997; Mauvais-Jarvis et al. 2013). The systemic loss of estrogen after menopause is linked to an elevated risk of age-related metabolic disease and cardiovascular disease in women (Tandon et al. 2010). Conversely, hormone replacement therapy in postmenopausal women improves multiple aspects of metabolism (Bonds et al. 2006; Gurney et al. 2014; Kanaya et al. 2003; Margolis et al. 2004; Pereira et al. 2015). With increases in overall life expectancy, it is critical to develop therapeutic agents that will ameliorate weight gain and the effects of metabolic dysfunction in women and improve the quality of life after menopause. As an experimental model, mice also display similar sex differences in weight gain and metabolism (Hong et al. 2009; Della Vedova et al. 2016). Male mice fed a high-fat diet exhibit more pronounced effects on adiposity, hormonal imbalance, and impaired glucose metabolism compared to females (Yang et al. 2014; Pettersson et al. 2012; El Akoum et al. 2011). In both human and rodent females, these protective metabolic effects are largely attributed to the female hormone estrogen. Although recognized for its effects on reproduction and development, the complex mechanisms of estrogen action on metabolic tissues remain incompletely understood.

Estrogen Action in Metabolic Tissues

Estrogen (most importantly 17β-estradiol, E2) is a steroid hormone critical for the development and function of reproductive organs as well as the development of secondary sex characteristics. In addition, E2 action is involved in the nervous, immune, vascular, muscular, skeletal, and endocrine systems, all of which contribute to multiple aspects of metabolism (Deroo and Korach 2006; Prossnitz et al. 2008a). The regulation of metabolic functions by E2 and its receptors has been a topic of great interest owing to the sexual dimorphisms that exist in body weight, food intake, glucose/lipid homeostasis, and insulin sensitivity (Mauvais-Jarvis 2011, 2015; Barros and Gustafsson 2011; Barros et al. 2006; Meyer et al. 2011). The decline of circulating E2 due to either natural or surgical menopause induces rapid changes in whole body metabolism, fat distribution, inflammation, and insulin action both in animals and humans (Abu-Taha et al. 2009; Mauvais-Jarvis et al. 2013; Lee et al. 2009; Straub 2007). Loss of E2 or its function increases the risk of central weight gain, abnormal lipid profiles, diabetes, and cardiovascular disease (Hewitt et al. 2005; Louet et al. 2004). Similarly, E2 insufficiency in male and female mice lacking aromatase, a key enzyme in the biosynthesis of E2 from testosterone, results in increased adiposity, higher circulating lipid and insulin levels, as well as reduction in lean mass (Jones et al. 2000). In rodent models of obesity, such as high-fat (aka Western) diet, leptin deficiency, or ovariectomy, supplementation with E2 or its mimetics attenuates weight gain and alleviates metabolic abnormalities (Stubbins et al. 2012; Lundholm et al. 2008; Shen et al. 2014). In postmenopausal women, although hormone replacement therapy is a viable treatment option to alleviate the symptoms of menopause (Kaunitz and Manson 2015), it is associated with

oncogenic and cardiovascular risks (Hormone Replacement Therapy and Cancer 2001; Nabulsi et al. 1993).

One of the most widely used rodent models to study E2 action in vivo is ovariectomy, wherein surgical removal of the ovaries drastically reduces the levels of circulating endogenous E2 (Diaz Brinton 2012). The systemic loss of E2 in ovariectomized mice reveals that E2 is critical to several aspects of metabolism. E2 mediates certain metabolic effects via the central nervous system, as revealed by increased food intake, reduced energy expenditure, and weight gain in ovariectomized mice compared to ovary-intact littermates (Mauvais-Jarvis et al. 2013; Brown and Clegg 2010). In ovariectomized mice, the pancreas exhibits a loss of β-cell function and death (Louet et al. 2004; Le May et al. 2006). In contrast, supplementation with E2 promotes pancreatic β-cell survival in a proapoptotic environment (e.g., exposure to oxidative stress and/or pro-inflammatory cytokines), induces glucose-stimulated insulin secretion (GSIS), and suppresses lipogenesis, the latter by downregulating the expression of key transcription factors involved in lipid synthesis (Liu and Mauvais-Jarvis 2009; Liu et al. 2009; Balhuizen et al. 2010; Tiano et al. 2011; Tiano and Mauvais-Jarvis 2012). Furthermore, ovariectomy leads to aberrant glucose and lipid homeostasis resulting in increased fat mass, insulin resistance, glucose intolerance, dyslipidemia, and ectopic fat deposition (Vieira Potter et al. 2012; Yonezawa et al. 2012; Lin et al. 2013). Following ovariectomy, mice exhibit increases in visceral fat with larger adipocytes and increased expression of lipogenic and pro-inflammatory genes (Hong et al. 2009; Vieira Potter et al. 2012; Kamei et al. 2005; D'Eon et al. 2005). In addition, loss of E2 action increases susceptibility to oxidative stress and lowers fatty acid oxidation in multiple metabolic tissues (Muthusami et al. 2005; Paquette et al. 2009; Bokov et al. 2009). Rodent models of obesity and patients with type 2 diabetes also exhibit increased oxidative stress and inflammation (Furukawa et al. 2004; Fernandez-Sanchez et al. 2011; Wright et al. 2006; Folli et al. 2011; Domingueti et al. 2016). E2 supplementation in mice increases the expression of antioxidant enzymes and reduces inflammation (Monteiro et al. 2014; Strehlow et al. 2003; Borras et al. 2010). Finally, ovariectomized mice display increased susceptibility to the deleterious effects of HFD that can be reversed by supplementation with E2 at physiological concentrations (Stubbins et al. 2012; Litwak et al. 2014). Surprisingly, male mice fed a HFD also exhibit reduced body weight and improved glucose tolerance upon treatment with E2 or its mimetics (Huang et al. 2017; Vinayagam and Xu 2015; Kim et al. 2005).

Many natural or synthetic compounds with estrogenic activity can regulate endocrine signaling pathways, exerting either beneficial or adverse effects. Animal studies have revealed that prenatal exposure to endocrine disruptors, such as bisphenol A and diethylstilbestrol, causes abnormal programming of differentiating E2 target tissues that leads to the development of obesity later in life (Garcia-Arevalo et al. 2014; Liu et al. 2013; Newbold et al. 2007, 2009). On the other hand, dietary intake of genistein, an isoflavone that mimics certain actions of E2, exerts antidiabetic effects by improving hyperglycemia, glucose tolerance, and insulin levels in multiple mouse models of metabolic dysfunction (Lei et al. 2011; Liu et al. 2006). Taken together, these observations suggest that E2 exerts pleiotropic effects on multiple tissues involved in metabolism. Thus, a more complete

understanding of the mechanisms underlying E2 action on metabolic tissues requires a thorough investigation of the roles of individual estrogen receptors.

Estrogen Receptors, Signaling, and GPER Selectivity

The actions of E2, as an important physiological modulator of the complex events that regulate body weight and metabolism in multiple tissues, are mediated by its multiple receptors. The classical genomic actions through nuclear estrogen receptors (ERα and β) involve dimerization upon ligand activation and eventual binding to ER response elements in the promoters of target genes to facilitate regulation of gene expression (Barkhem et al. 2004; Dahlman-Wright et al. 2006; Jia et al. 2015). In addition to genomic responses, E2 also elicits rapid non-genomic signaling through extranuclear ERs and the G-protein-coupled estrogen receptor (GPER, previously known as GPR30) (Prossnitz et al. 2008a, b, c; Levin 2015). Long-term effects of GPER activity, however, also involve transcriptional regulation of target genes (Pandey et al. 2009; Prossnitz and Barton 2014; Vivacqua et al. 2015). GPER was initially discovered as an orphan receptor (Owman et al. 1996) but has since been demonstrated to bind E2 and activate multiple non-genomic, as well as genomic, pathways (Prossnitz et al. 2008a; Barton et al. 2017; Prossnitz and Arterburn 2015; Revankar et al. 2005, 2007; Filardo et al. 2000, 2002). GPER is expressed in diverse cell types and tissues, including the reproductive tissues, pancreatic islets, adipose, liver, skeletal muscle, CNS, heart, intestine, and inflammatory cells (Prossnitz and Barton 2011). It has been functionally implicated in metabolic regulation (Prossnitz and Barton 2014; Sharma et al. 2013; Sharma and Prossnitz 2016; Martensson et al. 2009; Barton and Prossnitz 2015), immune regulation (Blasko et al. 2009; Brunsing et al. 2013; Brunsing and Prossnitz 2011), cardiovascular physiology (Haas et al. 2009; Meyer et al. 2014, 2015, 2016; Fredette et al. 2017), reproduction (Thomas et al. 2010; Wang et al. 2008), the nervous system (Srivastava and Evans 2013; Xu et al. 2009; Hazell et al. 2009), and cancer (Prossnitz and Barton 2011; Arias-Pulido et al. 2010; Lappano et al. 2014; Marjon et al. 2014; Petrie et al. 2013; Smith et al. 2007, 2009). Stimulation of GPER activates a multitude of cellular signaling pathways including MAPK, PKC, PI3K, adenylyl cyclase, eNOS, and Ca^{2+} mobilization (Prossnitz and Barton 2014; Prossnitz and Arterburn 2015; Revankar et al. 2005; Filardo et al. 2000, 2002).

Whereas the roles of nuclear ERs in metabolism are more established (Barros and Gustafsson 2011; Jia et al. 2015), the physiological or pathological roles of GPER in metabolic signaling are still emerging (Sharma and Prossnitz 2016; Nilsson et al. 2011; Sharma et al. 2017) (Fig. 1). The effects of E2 and its multiple receptors on metabolism, as in other systems (Hadjimarkou and Vasudevan 2017), may be direct or indirect and furthermore may exhibit synergism or antagonism that may impact overall metabolic status. Thus, to assess the mechanisms involved in these complex interactions, the specific contribution of individual receptors must be assessed. Interestingly, mice lacking either ERα or GPER share similarities in metabolic phenotypes to varying degrees, such as increased adiposity, decreased insulin sensitivity, defective glucose/lipid homeostasis, and inflammation (Sharma et al. 2013; Martensson et al. 2009; Davis et al. 2014; Heine et al. 2000; Ribas et al. 2010; Prossnitz and Hathaway 2015). This suggests that both receptors might act cooperatively to mediate metabolic effects through similar or alternatively distinct

mechanistic pathways. It is important to note, however, that in the chronic absence of an individual receptor, compensatory effects may take place, masking the role of a given receptor in normal physiology or disease.

Since E2 binds to multiple receptors, pharmacological and genetic approaches can be employed to discriminate the contributions of individual receptors. Through the use of GPER-selective pharmacological agents, such as the agonist G-1 or antagonists G15 and G36, it is now possible to investigate the specific functions of GPER compared with those of ERs in complex systems expressing multiple estrogen receptor types (Prossnitz and Arterburn 2015; Revankar et al. 2007; Prossnitz 2017; Bologa et al. 2006; Dennis et al. 2009, 2011). However, experiments employing selective estrogen receptor modulators (SERMs) and selective estrogen receptor downregulators (SERDs) should be carefully interpreted as multiple SERMs, such as tamoxifen and raloxifene, and the SERD fulvestrant have been shown to lack ER specificity, acting as GPER agonists (Prossnitz and Arterburn 2015; Revankar et al. 2005; Filardo et al. 2000; Petrie et al. 2013). In addition, the availability of various genetic tools such as GPER knockout (GPER KO) mice (Prossnitz and Hathaway 2015) and siRNA or shRNA directed against GPER has significantly advanced our knowledge of GPER function. GPER KO mice with a global gene deletion of GPER (of which four independent genetically modified strains have been developed as reviewed in Prossnitz and Hathaway 2015) have been employed to evaluate metabolic phenotypes, generally revealing weight gain and metabolic dysfunction (Sharma et al. 2013; Martensson et al. 2009; Davis et al. 2014). Pharmacological and genetic approaches have often complemented each other, wherein treatment of mice with the GPER-selective agonist G-1 results in the opposite effect observed in GPER KO mice (Prossnitz and Hathaway 2015; Sharma and Prossnitz 2011). Furthermore, treatment of GPER KO mice with G-1 lacks the stimulatory effect of G-1 in WT mice, thereby confirming the selectivity of this compound for GPER via the absence of off-target effects (Prossnitz and Hathaway 2015; Sharma and Prossnitz 2011). In many systems, the lack of effects upon stimulation of GPER KO mice with E2 further validates the importance of GPER signaling in the actions of E2 (Prossnitz and Hathaway 2015; Sharma and Prossnitz 2011). Thus, recent studies utilizing GPER-selective approaches have provided strong evidence of the contributions of GPER signaling to metabolic homeostasis.

GPER, Body Weight, and Energy Homeostasis

Multiple studies have examined whether GPER regulates overall body weight, fat content, and energy balance (Tables 1 and 2). In the first such study, in 2009, Barton and colleagues reported that both male and female mice lacking GPER exhibited increases in body weight and visceral adiposity compared to their WT counterparts (Haas et al. 2009). At about the same time, but contrary to these results, Leeb-Lundberg and coworkers reported that female GPER KO mice exhibited slightly lower body weights than the corresponding WT mice, whereas GPER deficiency in males had no effects on body weight (Martensson et al. 2009). In 2013, we reported increased adiposity in male GPER KO mice throughout life, from 6 to 24 months of age, compared to WT mice (Sharma et al. 2013). MRI analysis revealed an overall increase in fat content of GPER KO mice with increased fat deposition in subcutaneous depots as well as visceral fat depots, such as the epididymal and perirenal fat

pads. The increase in adiposity of GPER KO mice occurred in the absence of altered food intake or locomotor activity. Furthermore, weight gain in male GPER KO mice was correlated with a significant increase in circulating levels of cholesterol, triglycerides, and LDL (Sharma et al. 2013), suggesting that GPER regulates key pathways involved in lipid homeostasis.

Consistent with these latter results, in a subsequent study in 2014, Clegg and colleagues observed that both male and female GPER KO mice demonstrated increased body weight and decreased energy expenditure in the absence of any changes in food intake (Davis et al. 2014). Furthermore, male and female GPER KO mice displayed a divergence in body weights compared to WT mice at different ages. Thus, while male GPER KO mice began to develop adiposity by 8 weeks of age, female GPER KO mice displayed detectable weight gain only at 14 weeks of age. Importantly, expression of two thermogenic genes, UCP1and β_3-adrenergic receptor, was reduced in brown adipose tissue of GPER KO mice. Interestingly, although expression of the β_3-adrenergic receptor was reduced in both male and female GPER KO mice, UCP1 expression was only decreased in male GPER KO mice. Furthermore, compared to WT mice, female GPER KO mice were less sensitive to the inhibitory effects of leptin on food intake and cholecystokinin (CCK) on satiety, whereas males did not reveal any differences. Interestingly, whereas E2 induced hypothalamic ERK activation in ovariectomized WT female mice, GPER KO mice failed to do so. Several studies have implicated hypothalamic ERK1/2 in the regulation of energy homeostasis (Rahmouni et al. 2009). Reduced E2-mediated ERK1/2 phosphorylation in hypothalamus may thus explain the diminished anorectic effects of leptin and CCK in female GPER KO mice. These findings strongly support the idea that the interaction of E2 with GPER is an important mediator of body weight regulation. Very recently and contrary to the multiple independent studies just outlined, Wang et al. reported that female GPER KO mice (the same as used in the previous studies by us and Clegg) were unexpectedly protected from diet-induced obesity, exhibiting lower body weight, decreased adipogenesis, and increased dark phase energy expenditure (Wang et al. 2016). The reasons behind these contradictory results are not clear. In general, factors such as chow, bedding, or environment can confound an observed phenotype. Furthermore, differences between studies could also arise as a result of the method used to generate GPER KO mice (e.g., homologous recombination of embryonic stem cells vs. cre/loxP, where chromosomal translocations are possible due to cryptic or pseudo-loxP sites) as recently reviewed (Prossnitz and Hathaway 2015).

Because a number of studies examining GPER KO mice reported that loss of GPER resulted in adiposity, we hypothesized that selective activation of GPER in a mouse model of obesity might attenuate weight gain and alleviate other chronic disease states arising from obesity. To this end, we tested the therapeutic potential of selective GPER agonism, employing G-1 in a mouse model of metabolic dysfunction, namely, ovariectomy. This model mimics menopause in women and results in adiposity and metabolic dysfunction due to loss of endogenous E2, as revealed by the reversal of metabolic dysfunction by E2 supplementation. In this model, treatment of ovariectomized mice with G-1 resulted in attenuation of overall weight gain and fat content as revealed by DEXA and MRI scans, without effects on bone mineral density, bone mineral content, or lean mass (Sharma and Prossnitz, unpublished data). In addition, a significant reduction in the mass of multiple fat depots was observed

upon G-1 treatment, as well as increased energy expenditure and higher expression of UCP1 in brown adipose tissue. Although G-1 exerted similar actions to those of E2 on the regulation of body weight and fat deposition, unlike E2, it did not increase uterine weight, reflecting a lack of the potent feminizing effects of E2 (Dennis et al. 2009). Thus, activation of GPER may represent a novel strategy to counteract weight gain and fat deposition.

GPER and Glucose Homeostasis

GPER is emerging as a key player in glucose homeostasis (Tables 1 and 2) (Sharma and Prossnitz 2016; Sharma et al. 2017). Evidence for an in vivo role of GPER in the regulation of glucose metabolism first emerged from studies of ERα/β double knockout mice exposed to streptozotocin (STZ). These mice did not exhibit a further increase in the incidence of diabetes when compared to either ERα KO or ERβ KO mice alone (Liu et al. 2009). In addition, ovariectomy increased the severity of insulin-deficient diabetes in ERα/β double KO mice following STZ treatment, an effect that was reversed by E2 supplementation, suggesting the presence of an additional distinct response mechanism to E2. Taken together, these studies suggested that even in the absence of ERα and ERβ, E2 continues to exert antidiabetic actions, consistent with the possible involvement of another estrogen receptor, such as GPER. Roles for GPER in glucose homeostasis have been established by a number of groups through the study of GPER KO mice. In 2009, Leeb-Lundberg and colleagues reported that deletion of GPER in mice resulted in impaired glucose tolerance and hyperglycemia with differential effects on male and female mice (Martensson et al. 2009). Whereas female GPER KO mice exhibited impaired glucose tolerance, increased plasma glucose, and defective GSIS compared to their WT counterparts, males did not reveal any differences between WT and GPER KO genotypes. In ovariectomized mice, GPER deficiency completely abolished E2-mediated increases in serum insulin. Furthermore, Mauvais-Jarvis and colleagues revealed that only female but not male GPER KO mice, upon exposure to STZ, exhibited a higher propensity toward insulin-deficient diabetes, displaying higher blood glucose levels, loss of β-cells, and a decrease in pancreatic insulin content (Liu et al. 2009). However, in contrast to the above reports, our observations revealed that male GPER KO mice exhibit age-dependent effects on glucose tolerance and insulin resistance (Sharma et al. 2013). At 6 months, although GPER KO mice were already insulin resistant as revealed by insulin tolerance tests (ITTs), they did not display any differences in glucose tolerance tests (GTTs), the latter consistent with the previous study by Leeb-Lundberg and colleagues (Martensson et al. 2009). However, at 12 months of age, GPER KO mice exhibited a trend toward impaired glucose tolerance, which was statistically significant by 18 months of age, with a concomitant exacerbation of insulin resistance (Sharma et al. 2013). The detection of higher fasting plasma insulin levels in GPER KO mice, with normal fasting glucose levels, further confirmed the presence of insulin resistance, in which elevated insulin levels are required to maintain normal glucose levels (Abdul-Ghani and DeFronzo 2009).

Weight gain, specifically visceral adiposity, is linked to a chronic inflammatory state and a decrease in the serum levels of adiponectin, an insulin-sensitizing adipokine that also exhibits anti-inflammatory properties (Bastard et al. 2006; Mangge et al. 2010). Consistent with these observations, we observed that adiposity in GPER KO male mice was

accompanied by increases in systemic markers of inflammation, such as TNFα, MCP1, IL-1β, and IL-6, along with a decrease in adiponectin levels (Sharma et al. 2013). The glucose intolerance present in aged GPER KO mice could have resulted from the cumulative effects of adiposity, insulin resistance, dyslipidemia, and inflammation. The existence of a pro-inflammatory state in GPER KO mice was subsequently confirmed by Davis et al., who demonstrated that both male and female GPER KO mice exhibit systemic increases in levels of the inflammatory marker SAA3 and a decrease in adiponectin levels compared to WT mice (Davis et al. 2014). Furthermore, treatment of ovariectomized GPER KO mice with E2 did not yield any improvements in glucose tolerance as in ovariectomized WT mice, indicating a definitive requirement for GPER in E2-mediated glucose metabolism. Taken together, these studies clearly indicate that both male and female GPER KO mice, in a sex-specific manner, exhibit regulation of glucose metabolism via GPER.

To determine whether selective activation of GPER could alleviate the symptoms of metabolic dysfunction with respect to insulin resistance and glucose tolerance, we treated ovariectomized mice with the GPER-selective agonist G-1 (Sharma and Prossnitz, unpublished data). Our results revealed that G-1 treatment led to a significant improvement in glucose tolerance in ovariectomized mice, with lower fasting glucose and insulin levels. In addition, G-1 treated mice exhibited improved insulin sensitivity and reduced the levels of circulating pro-inflammatory cytokines and hormones leptin and resistin. Lower fasting blood glucose and insulin levels in the ovariectomized mice suggest beneficial effects of G-1 on glucose homeostasis in both the liver and skeletal muscle, leading to speculation that G-1 may directly modulate glucose production in the liver and glucose uptake in skeletal muscle. However, as discussed above, since treatment with G-1 also prevents weight gain and visceral fat deposition in ovariectomized mice, an improvement in glucose homeostasis could be due to either direct or indirect effects of GPER-mediated signaling events in the tissues involved in metabolic regulation, which exhibit substantial cross talk with respect to glucose homeostasis (Samdani et al. 2015; Kim 2016).

GPER and Pancreatic Function

Pancreatic β-cells produce, store, and release insulin, the critical hormone in glucose homeostasis. GPER promotes the survival and function of multiple cell types in islets, particularly β-cells, the mechanisms of which have been examined in some detail and exhibit definite sex differences (Tables 1 and 2) (Liu et al. 2009; Mauvais-Jarvis 2016; Ropero et al. 2012). GPER expression was considerably higher in islets from females compared to males, both in mice and humans (Balhuizen et al. 2010; Kumar et al. 2011). Under basal conditions, islets isolated from male and female GPER KO mice exhibited reductions in insulin secretion compared to WT controls, as well as in the presence of glucose or tolbutamide, a potassium channel blocker that causes insulin secretion by blocking potassium channels in pancreatic β-cells (Martensson et al. 2009). Interestingly, with respect to their WT counterparts, islets isolated from female GPER KO mice exhibited a greater reduction in insulin secretion compared to islets from male GPER KO mice. Furthermore, islets from female GPER KO mice under basal conditions exhibited a decrease in pancreatic insulin content compared to islets from WT mice, which may have resulted from a defective E2 signaling in the absence of GPER. Similarly, islets isolated from both

male and female GPER KO mice completely lacked the E2-stimulated insulin secretion present in islets from WT mice, despite the use of supraphysiological concentrations of E2 (5 μM). Although E2 treatment of ovariectomized GPER KO mice failed to increase serum insulin levels, islets from treated mice exhibited higher pancreatic insulin content (presumably via ERα) compared to WT controls, suggesting that GPER may be important for insulin secretion from the pancreas. Finally, G-1 stimulation of both human and murine islets modulated hormone secretion and exerted antidiabetic effects in a dose-dependent manner similar to E2, with both agents increasing insulin secretion while inhibiting glucagon and somatostatin secretion (Balhuizen et al. 2010; Kumar et al. 2011).

The mechanisms leading to insulin secretion upon GPER activation by E2 or G-1 involve increased signaling through the cAMP/PKA and PLC/IP3 pathways, as stimulation with both agents increased the formation of cAMP and IP3 in a dose-dependent manner in islets from human female donors (Kumar et al. 2011). Interestingly, G-1 was more potent in IP3 production, whereas E2 exhibited higher potency in cAMP generation. In cultured mouse insulinoma MIN6 cells, E2 and G-1 both stimulated insulin secretion that could be inhibited by pharmacologic GPER-selective antagonism with G15 and depletion of GPER by siRNA (Sharma and Prossnitz 2011). Similarly, insulin secretion in WT islets was inhibited by G15 in response to GPER activation by either E2 or G-1, both of which failed to induce insulin secretion in islets from GPER KO mice (Sharma and Prossnitz 2011). In MIN6 cells, stimulation of GPER results in intracellular calcium release as well as activation of the ERK and PI3K pathways (Sharma and Prossnitz 2011). As previously reported in some cancer and other cell lines, GPER-mediated ERK activation occurred via transactivation of the EGFR (Filardo et al. 2000; Sharma and Prossnitz 2011). Interestingly, whereas ERK activity exhibited a positive effect on insulin secretion, PI3K activity inhibited insulin secretion, as previously observed (Hagiwara et al. 1995; Longuet et al. 2005). Thus, whereas inhibition of either EGFR or ERK prevented E2- or G-1-induced increases in insulin secretion, inhibition of PI3K signaling led to an increase in insulin secretion compared to E2 and G-1 alone. These results suggest that E2- and G-1-mediated activation of the ERK and PI3K pathways oppose each other and may serve to balance the secretion of insulin in response to multiple signaling inputs.

Obesity and insulin resistance lead to the process of compensation in islets, increasing the biosynthesis and secretion of insulin as well as the number of β-cells to maintain normal blood glucose levels (Cerf 2013). Persistent cellular stresses associated with obesity and insulin resistance, such as cytokine-induced inflammation, mitochondrial dysfunction, oxidative stress, ER stress, and glucolipotoxicity, eventually lead to β-cell death and hyperglycemia. E2 is known to promote β-cell survival under these conditions (Prentki and Nolan 2006). Experimentally, activation of GPER by either E2 or G-1 promoted cell survival and counteracted apoptosis induced by pro-inflammatory cytokines and oxidative stress in both murine and human islets, as well as in MIN6 cells (Liu et al. 2009; Balhuizen et al. 2010; Kumar et al. 2011). In islets subjected to inflammatory injury, E2 or G-1 promoted islet cell survival via phosphorylation of pro-survival genes such as CREB, Akt, and ERK1/2 with concomitant suppression in the activity of stress proteins, such as SAPK/JNK and p38 (Kumar et al. 2011). Pretreatment with ER antagonists, ICI 182,780 or EM-652, did not inhibit the protective effects of E2, suggesting that E2 may function via GPER,

consistent with the protective effects of G-1. In addition, islets from GPER KO mice exposed to oxidative stress lacked G-1-mediated protection, although survival mediated by E2 was maintained, suggesting a parallel effect of E2 via ERα (Liu et al. 2009). Interestingly, islets isolated from mice lacking both ERα and ERβ still exhibited protection against cell death when challenged with STZ, suggesting the involvement of GPER or another unknown ER in mediating this response (Liu et al. 2009). In addition to the mechanisms described above, studies on isolated islets and cultured cells have shown that GPER activation also inhibits lipid accumulation, by suppressing the expression of important transcription factors involved in lipogenesis, such as chSREBP and SREBP1 via STAT3, potentially leading to even greater antiapoptotic effects as a result of reduced lipotoxicity (Tiano et al. 2011; Tiano and Mauvais-Jarvis 2012).

An additional physiological stressor, pregnancy, also leads to the expansion of pancreatic β-cell mass in order to compensate for maternal insulin resistance (Rieck and Kaestner 2010; Ernst et al. 2011). Increases in β-cell mass during pregnancy result from an increase in proliferation and survival of β-cells through the downregulation of islet-specific microRNA mi-338-3p (Jacovetti et al. 2012). In cultured β-cells and dissociated islets, downregulation of miR-338-3p increased proliferation and protected cells against pro-inflammatory cytokine-induced apoptosis. GPER expression increased in rat islets during pregnancy, peaking at day 14, with E2 repressing mi-338-3p in rat islets via GPER through a cAMP-dependent pathway (Jacovetti et al. 2012). Activation of GPER in rat islets reduced the expression of mi-338-3p, an effect that was reversed by treatment with GPER-targeted siRNA. These results indicate that E2 signaling via GPER suppresses the expression of miR-338-3p, which may be critical for the increase in β-cell mass during pregnancy.

Effects of GPER in Peripheral Metabolic Tissues

Although roles for GPER in overall body weight regulation and glucose homeostasis have been observed (Sharma et al. 2013; Martensson et al. 2009; Davis et al. 2014), little is known regarding the effects of GPER in the individual peripheral tissues that are actively involved in metabolism, such as the adipose, liver, and skeletal muscle, all of which act in a coordinated manner to maintain metabolic homeostasis. Although GPER is widely expressed in multiple insulin-sensitive tissues such as the liver, adipose, and skeletal muscle, female mice exhibited higher GPER expression in white adipose tissue compared to males (Davis et al. 2014). GPER expression was localized predominantly to adipocytes with little expression in the stromal vascular fraction (Davis et al. 2014). GPER expression has also been reported in 3T3-L1 preadipocytes, where it was upregulated during differentiation of preadipocytes into adipocytes (Zhu et al. 2013). Treatment of 3T3-L1 preadipocytes with E2 or G-1 during differentiation inhibited lipid accumulation in adipocytes, an outcome that was reversed by GPER knockdown with siRNA (Zhu et al. 2013). During adipogenic differentiation, after initial mitotic clonal expansion, cells arrest at the G1 growth phase of the cell cycle and subsequently express adipogenic factors (Tang et al. 2003; Patel and Lane 2000). Treatment with G-1 in 3T3-L1 cells results in an aberrant differentiation process wherein most of the cells continue to divide even after 48 h of differentiation, whereas in the control group, the majority of cells arrested in the G_0/G_1 state by 24 h following the induction of differentiation (Zhu et al. 2013). Furthermore, GPER activation increased the

expression of cell cycle-regulating factors, such as CDK4, CDK6, and cyclin D. Thus, GPER inhibited lipid accumulation in adipocytes at least in part by preventing cell cycle arrest and subsequent differentiation (Zhu et al. 2013).

As described above for multiple studies, mice lacking GPER exhibit an increase in overall adiposity with increased fat deposition in subcutaneous, perigonadal, and perirenal fat depots compared to their WT counterparts (Sharma et al. 2013; Haas et al. 2009; Davis et al. 2014). In the gonadal fat pads of GPER KO mice, adipocytes were larger compared to WT mice, as a result of increased lipid storage (Davis et al. 2014). Interestingly, in GPER-lacZ mice (a mouse mutant that harbors a β-galactosidase (lacZ) reporter within the *Gper* locus, disrupting *Gper* expression), only female GPER KO mice exhibited increased lipid accumulation in liver along with a decrease in circulating HDL levels compared to WT mice, whereas male mice displayed no such differences (Meoli et al. 2014). Consistent with GPER KO mice, a study of a human cohort of the Northern European descent has revealed that individuals carrying a hypofunctional P16L genetic variant of GPER have increased plasma LDL cholesterol (Hussain et al. 2015). These observations were further extended using HepG2 liver cells in which activation of GPER with G-1 increased the expression of the LDL receptor (Hussain et al. 2015). This upregulation was blocked by either GPER antagonist G15 or knockdown of GPER expression by shRNA. These results imply that GPER plays a crucial role in modulating central pathways involved in lipid metabolism in multiple tissues, suggesting that selective GPER activation might be beneficial in lowering lipid levels. Thus, we examined the effects of GPER stimulation on lipid homeostasis in vivo in an ovariectomized mouse model (Sharma and Prossnitz, unpublished data). G-1 treatment lowered the levels of circulating lipids, reduced the expression of lipogenic and pro-inflammatory genes, and increased the expression of genes involved in lipid oxidation in the adipose, liver, and skeletal muscle. Thus, GPER exerts pleiotropic effects in metabolic tissues leading to reductions in both lipid accumulation and inflammation.

Conclusions

It has now become clear that GPER regulates not only body weight but also multiple aspects of metabolism in numerous tissues throughout the body, such as the pancreas, adipose, liver, and skeletal muscle. However, mechanisms of GPER-mediated effects remain poorly understood and merit further study. Global GPER KO mice have been used by a number of groups to investigate the functions of GPER in vivo (Prossnitz and Hathaway 2015), but due to cross talk between metabolic tissues and the possibility of compensatory effects during development, conclusions from such studies must be interpreted with caution. The use of pharmacological approaches to modulate GPER activity has largely supported the conclusions from GPER KO studies (Prossnitz and Arterburn 2015). With the epidemic prevalence of obesity and metabolic dysfunction, it is more critical than ever to identify new therapeutic approaches to mimic the salutary effects of E2 without the feminizing and other side effects of estrogenic substances, particularly for men. The therapeutic targeting of GPER may represent one such approach to simultaneously treat multiple aspects of metabolic syndrome.

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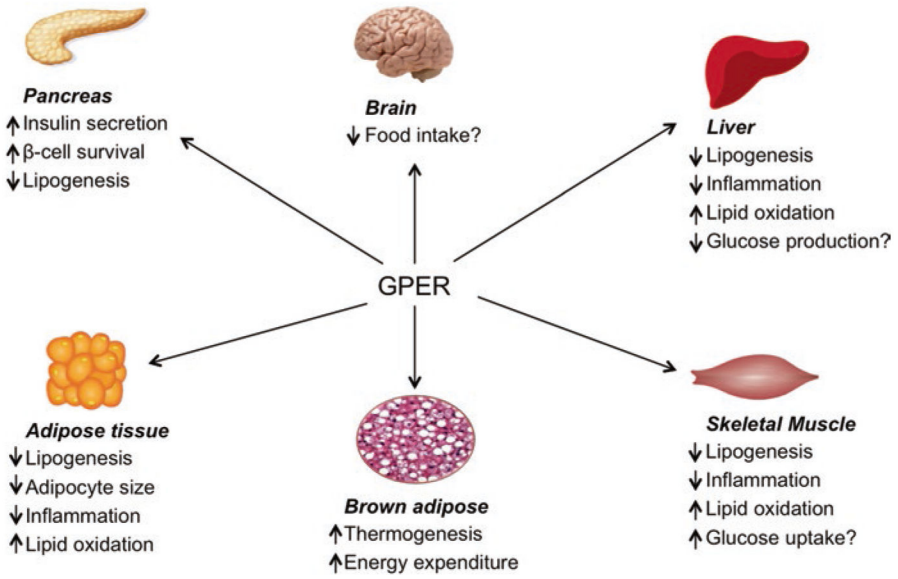

Fig. 1.

Schematic representation of the metabolic roles of GPER. GPER exerts pleiotropic effects on metabolically active tissues such as the pancreas, adipose, brown adipose, liver, and muscle. GPER controls body weight by regulating food intake and increasing energy expenditure as well as thermogenesis in brown adipose tissue. GPER activation in the pancreas promotes β-cell survival and insulin secretion. Stimulation of GPER in the pancreas, adipose, liver, and skeletal muscle reduces lipid deposition by inhibiting lipogenesis and promoting lipid oxidation. GPER activation also attenuates inflammation in multiple tissues. GPER-mediated prevention of lipotoxicity and inflammation in non-adipose tissues may improve glucose homeostasis by increasing insulin secretion, improving glucose uptake, and reducing hepatic glucose production in the pancreas, skeletal muscle, and liver, respectively. Effects not clearly known are indicated by. See text for details

Table 1

Effects of loss of GPER expression on metabolism. In vitro, ex vivo, and in vivo studies reveal that GPER regulates body weight, food intake, and energy expenditure. In addition, GPER modulates pancreatic cell survival and hormone secretion as well as glucose and lipid metabolism. Effects in GPER KO mice or islets are compared to WT control animals or islets. See the text for details

Cells/tissues/ mice	Treatment	Effect/(s)	Reference/year
GPER KO (F) mice	-	Slightly reduced body weight	Martensson et al. 2009
		Hyperglycemia and impaired glucose tolerance	
	OVX + E2	No increase in serum insulin levels	
GPER KO (F) islets	Basal	Reduced insulin and glucagon secretion	
	E2	No increase in insulin or decrease in glucagon secretion	
GPER KO (M) mice	-	No changes in body weight and normal glucose homeostasis	
GPER KO (M) islets	Basal	Reduced insulin secretion but glucagon secretion unchanged	
	E2	No changes in insulin or glucagon secretion	
GPER KO (F) mice	STZ	Increased incidence of diabetes (vs. WT mice + STZ)	Liu et al. 2009
		Lower insulin levels and pancreatic insulin content	
GPER KO (M) mice	STZ	Incidence of diabetes similar to WT mice	
GPER KO islets	STZ+E2	Increased pancreatic islet survival (possibly via ER)	
	STZ+G-1	No increase in pancreatic islet survival	
GPER KO (F) islets	E2 or G-1	Reduced insulin secretion under high glucose	Sharma and Prossnitz 2011
GPER KO islets	E2	No decrease in lipid accumulation	Tiano et al. 2011
GPER KO (M) mice	-	Increased body weight, fat content, and dyslipidemia	Sharma et al. 2013
		Impaired glucose tolerance and insulin resistance	
		Increased fasting insulin	
GPER KO (F) mice	-	Increased body weight	Davis et al. 2014
		Reduced energy expenditure and brown fat thermogenesis	
	OVX + E2	No decrease in body weight or improvement in glucose tolerance	
	Leptin or CCK	No decrease in food intake	
GPER KO (M) mice	-	Increased body weight	
		Reduced energy expenditure and brown fat thermogenesis	
	Leptin or CCK	Reduction in food intake	
GPER-lacZ (F) mice	HFD	No changes in body weight	Meoli et al. 2014
		Increased liver fat accumulation, decreased HDL	
GPER-lacZ (M) mice		No changes in body weight, liver fat accumulation, or HDL levels	

Table 2

GPER-mediated effects on metabolism. Selective pharmacological activation or genetic knockdown of GPER reveals that stimulation of GPER promotes human and murine pancreatic cell survival and insulin secretion, reduces lipid accumulation in adipocytes. In ovariectomized mice, GPER activation attenuates adiposity and improves glucose tolerance and lowers fasting glucose, insulin, and cholesterol

Cells/tissues/mice	Treatment	Effect/(s)	Reference/year
αβERKO (F) mice	OVX + STZ + E2	Lower incidence of diabetes (vs. OVX + STZ) (via non-ERs possibly GPER)	Liu et al. 2009
		Higher insulin levels and pancreatic insulin content	
Mice	G-1	Increased pancreatic islet cell survival	Liu et al. 2009,Balhuizen et al. 2010, and Kumar et al. 2011
Human islets			
MIN6 cells			
WT mice (F) islets	G-1	Increase in insulin secretion under high glucose	Balhuizen et al. 2010
		Decrease in glucagon secretion under low glucose	
		Decrease in somatostatin secretion under high glucose	
		Inhibition of pancreatic cell apoptosis	
Human (F) islets	G-1	Increase in insulin secretion under high glucose	Kumar et al. 2011
		Decrease in pancreatic cell apoptosis	
MIN6	E2 or G-1	Increased insulin secretion	Sharma and Prossnitz 2011
	(± G15 or ± siRNA)	Inhibition of E2 or G-1-mediated insulin secretion	
WT mice (F) islets	E2 or G-1	Increased insulin secretion	
	(± G15)	Inhibition of E2 or G-1-mediated insulin secretion	
ZDF (M) islets	G-1	Reduced lipid accumulation	Tiano et al. 2011 and Tiano and Mauvais-Jarvis 2012
INS-1 cells			
Human islets			
3T3-L1	E2 or G-1	Inhibition of Lipid accumulation	Zhu et al. 2013
	(± siRNA)	Reversal of GPER-mediated decrease in lipid deposition	
WT mice	OVX+G-1	Reduction in body weight and fat content	Unpublished data
		Improved glucose tolerance	
		Reduced insulin resistance	
		Lower fasting glucose, insulin, and cholesterol levels	
		Increased fatty acid oxidation in metabolic tissues	