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Conserved estrogen binding and signaling functions of the G protein-coupled estrogen receptor 1, GPER, in mammals and fish

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Abstract

Recent studies by several research groups have shown that G-protein estrogen receptor-1, GPER, formerly known as GPR30, mediates 17 β -estradiol (E2) activation of signal transduction pathways in a variety of human cancer cells and displays E2 binding typical of a membrane estrogen receptor. However, the importance of GPER as an estrogen receptor has been questioned by Otto and coworkers. Some of the pitfalls in investigating the functions of recombinant steroid membrane receptors that may explain the negative results of these investigators are discussed. The characteristics of GPER have also been investigated in a teleost fish, Atlantic croaker, where it has been shown to mediate E2 inhibition of oocyte maturation. Investigations on newly discovered homologous proteins from distantly-related vertebrate groups are valuable for determining their fundamental, evolutionarily-conserved functions. Therefore, the functions of croaker and human GPERs were compared. The comparisons show that croaker and human GPER have very similar estrogen binding characteristics, typical of estrogen membrane receptors, and activate the same estrogen signaling pathways via stimulatory G proteins (Gs) resulting in increased cAMP production. These results suggest that the estrogen binding and estrogen signaling functions of GPER arose early in vertebrate evolution, prior to the divergence of the teleosts from the tetrapods, more than 200 million years ago. The finding that estrogen membrane signaling through GPER has been conserved for such a long period in two distantly-related vertebrate groups, mammals and fish, suggests that this is a fundamental function of GPER in vertebrates, and likely its major physiological role.

Key terms

G protein-coupled receptor-1; GPER; GPR30; estrogen membrane receptor; oocyte maturation; fish

1. Introduction

It has been recognized for over 40 years that estrogens, in addition to their classic genomic actions mediated through activation of nuclear estrogen receptors (ERs), can also elicit rapid, cell surface-mediated responses that are often nongenomic [1,2]. However, the identities of the receptors on the plasma membranes of target cells mediating these nonclassical estrogen actions have been elusive and surrounded by controversy [3–5]. Some of these nonclassical estrogen actions have been attributed to ERs or truncated forms of ERs [6–9]. However, other

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receptors must also be involved because E2 actions have been described in cells lacking ERs [10–12]. For example, estrogens have been shown by Filardo and coworkers to cause rapid activation of second messengers in a human breast cancer cell line, SKBR3 cells, that lack ER α and ER β , but express the orphan GPCR-like protein, GPR30 (G protein coupled receptor 30) [13]. GPR30, recently renamed GPER, is widely distributed in both reproductive and non-reproductive tissues and has some sequence homology to chemokine receptors [14,15]; but extensive studies have shown that a wide variety of chemokines and angiotensins display no binding affinity for this orphan receptor [16,17]. On the basis of his findings with SKBR3 cells Filardo proposed that the effects of E2 are mediated by GPER [13], but direct evidence that estrogens interact with GPER was lacking.

In 2004 our laboratory and Prossnitz's research group independently showed that human GPER binds estrogens with high affinity and has the binding characteristics of a membrane estrogen receptor [18,19]. In addition, it was demonstrated that estrogen acts through human GPER to activate a stimulatory G protein (Gs) resulting in stimulation of adenylyl cyclase activity and increased cAMP production by plasma membranes of SKBR3 and GPER-transfected cells [18,20]. Estrogen has also been shown to act through GPER to release epidermal growth factor EGF-related ligands and transactivate the EGF receptor (EGFR) [13,21,22]. These findings have stimulated widespread research on GPER which has resulted in the publication of over 120 papers in the past 4 years on various aspects of GPER signaling, trafficking, regulation, tissue expression, and functions in health and disease. Studies on the functions of GPER are frequently complicated by its co-expression with ERs in estrogen target tissues. Therefore, the development of a selective GPER agonist that does not activate ERs, named G-1, has greatly facilitated research on GPER [23]. Experiments with cells and tissues in which GPER expression has been selectively knocked down by transfection with GPER siRNA have provided valuable clues of the physiological functions of the receptor, whereas studies with GPER knock-out mice have produced equivocal and conflicting results [24]. To date GPER has been implicated in the development or progression of breast, endometrial, and ovarian cancers [14,25–27], and in a broad range of physiological functions, including neurotransmitter and neuroendocrine regulation [28,29], protection against autoimmunity [30] and trauma-hemorrhage of the liver [31], lipid metabolism and cardiovascular tone [32], insulin secretion [33], primordial follicle formation [34], and regulation of oocyte meiotic arrest in teleost fish [35]. GPER is expressed in a wide variety of tissues and cell types so it is likely that many additional functions of GPER will be identified in the near future.

The role of GPER as an intermediary in estrogen activation of several signal transduction pathways has been independently confirmed by at least half a dozen research groups in a broad range of cellular models [13,18,19,30,31,36]. Moreover, the estrogen binding functions of GPER have been independently confirmed in several laboratories [18,19,37]. However, despite the existence of a large body of experimental data supporting a role for GPER as an intermediary in the rapid, nongenomic actions of estrogens, this proposed function of GPER as a membrane estrogen receptor has been challenged by two research groups on the basis of their negative results [5,38,39].

The criteria for designating a novel protein as a membrane steroid receptor have been discussed extensively for the novel membrane progesterin receptors, mPRs [40–42]. Many of these criteria are met by GPER. The predicted structure of the GPER protein with 7 transmembrane domains is plausible for a membrane receptor, and is characteristic of G protein coupled receptors (GPCRs). The receptor protein is localized to the plasma membranes of target cells and transfected cells [18,43]; although there are reports that GPER is expressed predominantly in the endoplasmic reticulum [19]. In addition, as discussed in the preceding paragraphs, recombinant mammalian GPERs have both the binding and signaling functions of membrane estrogen receptors. The criterion of biological relevance, for example, changes in receptor

abundance consistent with the proposed function of the receptor, has also been met for GPER in some animal and cell models, although additional examples are required to strengthen this criterion. Additional criteria that need to be met to fully establish that GPER is a membrane estrogen include determining the molecular structure of the ligand binding and G protein binding pockets by site-directed mutagenesis. In addition, a knowledge of the mechanisms by which GPER is transported to the cell membrane, receptor abundance is regulated, and the receptor is internalized upon ligand activation is required to understand how GPER functions in target cells. Finally, in view of the controversy over the estrogen binding and estrogen signaling functions of human GPER, it is necessary to confirm that GPERs from other vertebrate species also display the characteristics of steroid membrane receptors.

Comparisons of the characteristics of homologous proteins from distantly-related vertebrate species have proven valuable for identifying the fundamental, evolutionarily-conserved functions of newly discovered novel proteins [44,45]. Earlier evidence for the existence of a novel estrogen membrane receptor in croaker gonads (46) prompted us to clone croaker GPER [35]. Croaker GPER was transfected into HEK 293 cells, an ER negative cell line, in order to investigate the functional characteristics of the recombinant protein. Croaker and humans are representatives of two distantly-related vertebrate groups, teleost fish and mammals, which diverged from the vertebrate lineage about 200 million years ago. Therefore, a comparison of the functional characteristics of GPER in these two species should reveal whether estrogen binding and signal transduction are basic functions of these proteins in vertebrates.

2. Expression of recombinant GPER and characterization of E2 binding

The detection of specific steroid binding to the plasma membranes of cells transfected with steroid receptors is complicated by the presence of relatively high background steroid binding to these lipid-rich organelles. Therefore, in order to express sufficient amounts of novel recombinant steroid membrane receptors to properly investigate their ligand binding and signal transduction characteristics, it is necessary to stably transfect the receptor and select clones with the highest expression by limiting dilution or by other selection procedures such as cell sorting [41,43]. Typically for GPCRs only a small amount of the receptor protein in the cell is expressed on the plasma membrane and a large proportion remains in the endoplasmic reticulum [43,47]. The mechanisms regulating the trafficking of novel steroid receptors such as GPER and mPRs to the plasma membrane are unknown. We have observed that continued selection of mPR-transfected cells over 6 weeks is usually required to achieve strong expression of the receptor in the plasma membrane [41]. The protocol used to achieve plasma membrane expression of recombinant GPER is outlined in the following section. For the analysis of the steroid membrane receptors such as GPER which are very labile particular care is taken to limit their degradation during the homogenization, centrifugation and radioreceptor assay by performing all the procedures at 4°C and in the presence of protease inhibitors. Finally, the choice of receptor assay procedures that are inappropriate and have not been validated for the measurement of steroid membrane receptors is a likely cause of the failure of two groups of investigators to detect steroid binding to GPER and mPRs [38,41,48]. The membrane filtration assay is a standard method that is widely used to measure binding of a wide variety of ligands [49]. The protocol described below has proven to be a reliable procedure to measure estrogen, progesterin, androgen, and corticosteroid binding to both wild type and recombinant steroid membrane receptors [46,50–55].

3. Stable expression of recombinant human and croaker GPERs on the plasma membranes of HEK293 cells

Full length cDNAs encoding human and Atlantic croaker GPERs were cloned into mammalian expression vectors (pBK-CMV) and then stably transfected using Lipofectamine into human

HEK293 cells (ER α and ER β negative) as described in two publications [18,35]. G-418 was used for preliminary selection of GPER positive cells. Further selection of GPER positive cells was achieved by limited-dilution, in which single cells were selected and grown on separate plates to produce ten or more different clones. After several passages in the presence of G-418, each clone was evaluated for GPER protein expression and specific [3 H]E2 binding on the plasma membrane. Cell clones with both high GPER expression and membrane [3 H]E2 binding were selected as successful stably-transfected cell lines and used for subsequent experiments.

Significant deviations from this protocol resulted in inefficient expression of GPER on the plasma membranes of HEK293 cells and a lack of membrane [3 H]E2 binding. High amounts of GPER expression on the plasma membrane were observed after several passages (4–5) of stably-transfected cells whereas transient transfections were ineffective. Limited dilution and further selection proved to be an effective procedure for obtaining clones with high enough levels of GPER expression to clearly demonstrate specific [3 H]E2 binding characteristic of a membrane estrogen receptor and for investigating signal transduction pathways activated through GPER.

4. Preparation of plasma membranes and [3 H]E2 membrane filtration binding assay

Transfected cells were collected from the culture plates by scraping them into ice-cold HAED buffer (25 mM HEPES; 10 mM NaCl; 1 mM dithioerythritol; 1 mM EDTA, pH 7.6) containing 0.1% protease inhibitors. The cells were homogenized and the homogenate was centrifuged at 1000 $\times$ g for 7 min to remove the nuclear fraction prior centrifugation at 20,000 $\times$ g for 20 min to produce a crude plasma membrane pellet. The plasma membranes were further purified by centrifugation over a 1.2mM sucrose pad as described previously [18,35]. The final plasma membrane pellet was washed and re-suspended in binding assay buffer prior to the addition of [2,4,6,7- 3 H]E2 in the presence (non-specific) or absence (total binding) of nonradiolabeled E2. Sample mixtures were incubated at 4°C for 30 min and then filtered through GF/B glass fiber filters and washed 3 times. The [3 H]E2 binding to the plasma membrane fractions was determined by counting the radioactivity on the filters and the specific binding was calculated by subtracting the non-specific binding from total binding.

Procedures to minimize degradation of GPER during the entire cell harvesting, homogenization, centrifugation and radioreceptor assay steps are required to retain high [3 H]E2 binding to the plasma membrane fractions. Consequently, enzymatic removal of cells from the culture plates is avoided, all procedures are conducted at 4°C in the presence of a protease inhibitor cocktail, and the final filtration step of the binding assay is conducted in a cold room. The E2 binding activity of GPER appears to be labile since it is rapidly lost during storage of plasma membrane preparations at –80°C and is higher in unfrozen cell and tissue preparations. The extra centrifugation step using a sucrose pad to obtain a more purified plasma membrane preparation results in a decrease in non-specific binding in the radioreceptor assay.

5. Comparison of the estrogen membrane receptor binding characteristics of recombinant human and croaker GPERs

The membrane filtration procedure is a reliable and reproducible assay of the specific [3 H]-E2 binding to the plasma membranes cells and tissues expressing both wild type and recombinant GPERs, and represents 40–50% of the total binding. The E2 binding characteristics of human and croaker wild type and recombinant GPERs are very similar [18, 35] (Fig. 1). Saturation analyses and Scatchard plots for recombinant human and croaker GPERs (Fig. 1A,B) show that they have high affinity, limited capacity, specific, single binding sites for E2 characteristic of steroid receptors. The binding affinities of human GPER (K_d 3.3

nM) and croaker GPER (K_d 2.7 nM) are very similar, and are lower than the binding affinities of E2 for human ERs (K_d 0.13–0.6 nM) [56,57] and croaker ERs (K_d 0.4–0.6 nM) [58]. As shown in Figure 1C and D, [³H]-E2 binding to both human and croaker GPERs is readily displaceable with nonradiolabeled E2, thereby meeting an important criterion for designating these binding moieties as hormone receptors. Association and dissociation of radiolabeled E2 binding to wild type and recombinant human and croaker GPERs are very rapid, which is typical for steroid membrane receptors, and are completed with a few minutes. Steroid binding to the human and croaker recombinant GPERs is very similar and is specific for estrogens (Fig. 1E,F) [18,35]. Approximately 50% of [³H]-E2 binding to both receptors is displaced by 5×10^{-8} M E2, whereas an androgen, progestin and corticosteroid are ineffective competitors (Fig. 1E,F). The mammalian GPER-specific agonist, G-1, displays high affinity binding to croaker GPER and mimics the actions of E2 in the fish oocyte maturation bioassay, indicating that G-1 is a useful pharmacological tool for investigating GPER-specific estrogen actions across a broad range of vertebrate species. Similarly, the nuclear ER antagonists, tamoxifen and ICI182,780, act as estrogen agonists in both mammalian and fish bioassays, although their binding affinities for croaker GPER are lower than they are for human GPER. Recently the estrogen binding characteristics of GPER has been confirmed in another teleosts species, zebrafish, using the membrane filtration assay [37].

The finding that the estrogen binding functions of GPER have been retained in mammals and teleost fish, which diverged from the vertebrate lineage more than 200 million ago, and are remarkably similar in these two vertebrate groups, suggests that its physiological role as a membrane estrogen receptor is a fundamental, conserved function in vertebrates. Several lines of evidence indicate that the signaling functions of these receptors are also conserved in vertebrates. Both croaker and human GPERs are coupled to and activate stimulatory G-proteins (Gs), resulting in increased cAMP production [18,35]. It is concluded that GPERs have very similar functions at the biochemical and cellular levels in vertebrates, as membrane estrogen receptors mediating nonclassical estrogen actions through activation of stimulatory G proteins. Previous comparative endocrine studies with other hormone receptors have shown that their conserved functions do not always extend beyond the cellular level and that their functions may differ at the organismal level. Therefore, it is unclear at present whether the estrogen inhibition of oocyte maturation observed in fish is a mechanism restricted to species in which maturation of thousands of oocytes has to be synchronized for successful reproduction, or is of widespread importance among vertebrates.

6. Failure of a whole cell binding assay to detect specific [³H]-E2 binding to GPER

A recent study by Otto et al. reported that E2 does not bind to COS-7 cells transiently transfected with human GPER in a whole cell binding assay and that the receptor is not expressed on the plasma membranes of these cells but is exclusively located in the endoplasmic reticulum [38]. The reasons why these investigators failed to detect E2 binding to recombinant GPER, while other research groups have been successful [18,19,37], are unclear but are likely due to the use of transiently transfected cells and an inappropriate binding assay. As discussed previously, stable expression of novel membrane steroid receptors and further selection of clones is usually necessary in order to obtain efficient cell-surface expression of recombinant receptors and high amounts of specific steroid membrane binding. In contrast to the membrane filtration assay, there are no reports to our knowledge of the successful application of whole cell binding assays to detect specific binding of steroids to membrane receptors and characterize their binding sites. This is not surprising because whole cell binding assays appear to be inappropriate for measuring specific membrane binding of lipophilic ligands such as steroids. Radiolabeled steroids readily enter cells so that after relatively short incubation periods they will occupy multiple cellular compartments. Multiple brief washes of a few

seconds will remove the radiolabeled steroid ligand not bound to receptors on the cell surface of whole cells, as they do for isolated membranes in the membrane filtration assay, but will not remove unbound radiolabeled ligand from the intracellular compartment. We predicted that if the unbound intracellular component of the total steroid radioactivity is relatively high, it would obscure any differences in total and non-specific binding to steroid receptors on the cell surface, resulting in no detectable specific binding. To test this we compared specific [^3H]-E2 binding to recombinant human GPER stably produced in HEK293 cells in Otto and coworker's whole cell binding assay with that in our membrane filtration assay. The GPER-transfected cells were grown on culture plates in 5% charcoal-stripped serum for 48 hr prior to receptor assay. A non-enzymatic cell dissociation solution (CellStripper, Mediatech Inc) was used to remove the cells from the culture plates instead of the proteolytic enzyme procedure described in the original whole cell protocol in order to limit enzymatic degradation of the GPER protein on the cell surface. Approximately 150,000 live cells per measurement were incubated with increasing concentrations of [^3H]-E2 over the range of 0.5–8.0 nM in the absence and presence of 1000 fold excess cold E2 for 1 hr at room temperature in phenol-red free DMEM containing 0.5% BSA. Binding was terminated by rapid filtration (GF/B filters, Whatman), followed by rapid washing, and [^3H]-E2 was measured by scintillation counting. Both total and non-specific binding increased with increasing concentrations of radiolabeled E2 and no difference was detected between total and non-specific E2 binding at any ligand concentration (Fig.2A). Thus, using this method we could not detect measurable levels of specific [^3H]-E2 binding. In contrast, high amounts of specific [^3H]-E2 binding to plasma membranes prepared from cells were measured in a single point membrane filtration assay (Fig.2B). It is concluded from these experiments that the whole receptor assay cannot detect [^3H]-E2 binding to GPER, even when there is substantial cell-surface expression of the receptor and specific [^3H]-E2 binding that can be readily measured by the membrane filtration assay.

7. Investigations of the physiological functions of GPER

The clear demonstration of GPER-specific E2 receptor binding activity and physiological functions is a major challenge in most tissues and cell types because the majority of them also express abundant amounts of ER α and/or ER β . Although it remains unclear which of the pleiotropic actions of estrogens are mediated by GPER in many target cells, extensive recent research using various approaches has implicated GPER in a broad range of estrogen-dependent physiological processes and diseases. For example, selective cell-specific short-term knock down of GPER expression using siRNA techniques or microinjection of antisense oligo nucleotides has been used to demonstrate an association between GPER abundance and E2 membrane binding and its requirement for estrogen inhibition of fish oocyte maturation, respectively [18,35]. Two additional approaches to confirm the specificity of GPER mediation of an estrogen action, using fish oocyte maturation as an example, are briefly described below.

8. Identification of the major components of E2 binding in a target tissue

Ligand blot assays have proven to be useful for detecting the different binding proteins and receptors for hormones present in tissue extracts [59–61]. Ligand blot assays involve separation of tissue proteins by PAGE followed by transfer of the proteins onto membranes and detection of the protein bands that bind a tagged or radiolabeled ligand. For example, using peroxidase-conjugated progesterone as a probe Luconi and coworkers detected two protein bands of different molecular weights in whole human sperm lysates by ligand blot analysis that may represent two different membrane progesterone receptors [61]. A ligand blot procedure was developed to detect the major specific E2 binding components in croaker ovaries using radioactive E2 to detect the estrogen-binding protein bands. Previous studies using an estrogen-conjugate that cannot pass through the cell membrane had demonstrated that E2 acts on the surface of fish oocytes to inhibit oocyte maturation. Both GPER and ER α are present in fish

oocytes and therefore are candidates for the membrane estrogen receptor mediating this estrogen action.

Croaker ovarian plasma membranes were prepared from 2 g of fresh tissue as described previously described [35]. Ovarian tissue was homogenized in HAED buffer containing 0.1% protease inhibitors (Cell Signaling) on ice using a glass homogenizer. The nuclear fraction was removed by a centrifugation at $1000 \times g$ for 7 min at 4°C and the supernatant, containing cytosolic and membrane fractions, was centrifuged at $20,000 \times g$ for 20 min. The resulting crude plasma membrane pellet was resuspended in HAED buffer and layered over 5ml 1.2 M sucrose, followed by centrifugation at $9,600 \times g$ for 45 min. The plasma membrane fraction was collected and centrifuged at $20,000 \times g$ for 20 min to pellet the purified membranes. The enriched plasma membrane fraction was added to a non-reducing loading buffer and heated at 60°C for 5 min prior to loading (50µg/lane) onto a 10% SDS-PAGE gel. The proteins were separated by PAGE and then transferred to a nitrocellulose membrane. The resulting blot was washed for 4 hr in 0.01% TBST containing 0.1% BSA at room temperature. The membrane was then blocked in 0.1% TBST containing 1% BSA for 2 hr at room temperature and incubated overnight at 4°C with 30nM [³H]-E2 ± 1000 fold excess nonradiolabeled E2. The membrane was washed 5× for 5 min in 0.01% TBST containing 0.1% BSA. To determine the location of [³H]-E2 binding, the blot was scanned using a radioisotope thin layer analyzer (Raytest, Germany). To investigate whether the binding region contains GPER, the membrane was washed and subjected to Western blot analysis using croaker GPER antisera (1:2000).

A single peak of [³H]-E2 binding (total binding) corresponding to a molecular weight slightly less than 40kDa was detected on the membrane (Fig. 3). The amount of bound [³H]-E2 in the peak was decreased approximately 50% by co-incubation with 1000 fold excess nonradiolabeled E2 (non-specific binding), indicating high amounts of specific [³H]-E2 binding in this region. Subsequent Western blot analysis showed that the single peak of [³H]-E2 binding activity aligns with GPER which is present in this protein band (Fig.3). These results indicate that croaker ovarian plasma membranes contain a single membrane estrogen receptor with a molecular weight of around 40KDa that is most likely GPER. Whereas a contribution to the [³H]-E2 binding activity by proteins other than GPER in the ~ 40kDa peak cannot be excluded on the basis these results, no evidence was obtained for any E2 membrane binding through the full length ERα protein in croaker ovarian tissue. It was suggested in a recent editorial that GPER is not a membrane estrogen receptor but may act as a collaborator with the nuclear ERs, although no evidence was provided to support this claim [39]. The results of several previous studies clearly demonstrating that GPER displays all the functions of a membrane estrogen receptor in cells lacking ERα and ERβ such as SKBR3 cells are inconsistent with a collaborative role for GPER in mediating the actions of estrogens [13,18]. Similarly, the present results show that even when nuclear ERs are present in a tissue estrogen can bind to GPER without the cooperation of the nuclear ERs. The ligand blot shows that specific [³H]-E2 binding to croaker ovarian membranes is associated with a protein band less than 40kDa that coincides with GPER immunoreactivity, whereas full length ERα and ERβ and the truncated variants of the nuclear ERs have molecular weights around 60kDa and 50kDa, respectively [9].

9. Investigations of the specificity of estrogen receptor actions using selective estrogen receptor modulators

Previous experiments in which anti-sense oligo nucleotides to GPER microinjected into zebrafish oocytes blocked the inhibitory effects of E2 on oocyte maturation demonstrated an involvement of GPER in mediating this estrogen action [35]. However, it is possible that the nuclear estrogen receptors, ERα and ERβs, in either extra-nuclear or nuclear locations, also participate in estrogen regulation of oocyte maturation. A pharmacological approach with

selective estrogen receptor modulators can be used to confirm the results of receptor knock down experiments. Removal of the follicle layer surrounding fully grown fish oocytes by enzymatic digestion or manually, or inhibition of estrogen production with an aromatase inhibitor, results in spontaneous oocyte maturation within 3 hrs which can be partially blocked by E2 treatment [35,62]. Typical results obtained after enzymatically denuding zebrafish oocytes are shown in figure 4. Approximately 60% of the oocytes had completed oocyte maturation *in vitro* within 3 hrs after removal of the follicular layer. Incubation of the oocytes with the maturation-inducing steroid in this species, 17,20 β -dihydroxy-4-pregnen-3-one (DHP), a progestin which activates membrane progestin receptor alpha (mPR α), induced oocyte maturation of the remaining oocytes, whereas incubation with 10 nM E2 significantly reduced the number of oocytes that spontaneously matured (Fig.4). This inhibitory action of E2 was mimicked by the GPER-selective agonist, G-1, which also binds to fish GPERs at a concentration of 100 nM [35]. In contrast, 100 nM propyl-pyrazole-triol (PPT), an ER α -selective agonist, and diarylproprionitrile (DPN), an ER β -selective agonist showed no estrogenic activity in the *in vitro* oocyte maturation bioassay (Fig. 4). Both PPT and DPN have previously been shown to bind to fish estrogen receptors [63]. Moreover, diethylstilbestrol, which displays high binding affinity for fish ER α and ER β s but low affinity for fish GPER [56,36], also did not mimic the estrogenic effects of E2 in the bioassay (Fig.4). Finally, we have shown previously that the nuclear receptor antagonists, ICI182, 780 and tamoxifen, bind to fish GPER and act as estrogen agonists in the oocyte maturation assay [35]. Taken together, all these pharmacological studies are consistent with an involvement of GPER, but not ER α or ER β , in mediating the inhibitory effects of estrogens on oocyte maturation in fishes. Moreover, the results do not support a collaborative role for GPER with the nuclear ERs in mediating the inhibitory actions of estrogens in fish oocytes.

10. Conclusions and future studies

Significant progress has been made in elucidating the E2 binding and signaling functions of GPERs in the four years since it was first reported to have some of the characteristics of a novel membrane estrogen receptor. Comparative E2 binding studies with human and croaker GPER add further support to the suggestion that GPER functions as a membrane estrogen receptor in vertebrates. Moreover, the E2 binding and signaling functions of GPER have been confirmed by researchers in many laboratories in different cell models. In view of the preponderance of studies showing the estrogen receptor functions of GPER, it is puzzling why a few investigators continue to strongly dispute its function [38,39]. It is acknowledged that some additional criteria will need to be met before GPER's role as a receptor is fully established. In particular, it is important to understand at the molecular level what amino acids residues of GPER are required for binding E2 and the nature of the binding pocket. However, it is reasonable to conclude from all the available evidence to date that GPER most likely functions as a membrane estrogen receptor in those cell types where it is highly expressed.

Although considerable progress has also been made in determining the physiological roles of GPER, in many cases it remains unclear which of the pleiotropic actions of estrogens in target cells are specific to this receptor distinct from those mediated by the ERs. Multiple experimental approaches such as those employing silencing of both GPER and ERs, pharmacological tools [64], and characterization of the principal receptor proteins contributing to E2 binding on the plasma membranes of target cells, will be necessary to differentiate E2 actions mediated through GPER from those involving ER α and ER β . Using this approach clear evidence has been obtained for a specific role for GPER in estrogen inhibition of oocyte meiotic maturation in fish. The finding that expression of GPER in primary breast cancer is associated with clinicopathological indicators of tumor progression in patients [27] has important implications for the treatment of breast cancer, particularly in view of the previous demonstration that ER antagonists, used to treat breast cancer, tamoxifen and ICI182684, act

as estrogen agonists through GPER [18,20,24,36]. Therefore, it is important to test the new generation of ER selective modulators currently under development for their agonist activities through GPER before they are considered for use as therapeutic agents for the treatment of breast cancer.

Abbreviations

GPER	G protein coupled estrogen receptor-1
GPR30	G protein coupled receptor 30
ER α	estrogen receptor alpha
ER β	estrogen receptor beta
ERs	nuclear estrogen receptors
E2	17 β -estradiol

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hGPR30-HEK

croGPR30-HEK

Figure 1.

Comparison of estrogen binding characteristics of human (A, C, E) and croaker (B, D, F) recombinant GPER proteins expressed on the plasma membranes of HEK293 cells in the membrane filtration assay. A and B, representative saturation curves and Scatchard plots of specific [³H]-E2 binding to stably transfected HEK293 cell membranes. C and D, Time course of association and dissociation of specific [³H]-E2 binding to cell membranes. E and F, competition curves of steroid binding to transfected HEK293 cell membranes expressed as a percentage of maximal specific [³H]-E2 binding. E2, estradiol-17β; P4, progesterone; T, testosterone; Cort, cortisol. Reproduced with permission from Thomas et al., *Endocrinology*, 2005 [18] and Pang et al., *Endocrinology* 2008 [35].

Figure 2.

Comparison of [³H]-E₂ receptor binding to recombinant human GPER stably expressed in HEK293 cells in the whole cell binding assay of Otto et al. (2008)(A) and in the membrane filtration assay (B). A. Total binding and non-specific binding (in the presence of 1000-fold excess E₂) of [³H]-E₂ over the concentration range of 0.5 to 8.0 nM in the whole cell assay. Means ± SD are shown, n=3. B. Total binding and non-specific binding (in the presence of 500-fold excess E₂) of 4nM [³H]-E₂ in a single point membrane filtration assay. The whole cell binding assay was repeated several times and the same negative results were obtained on each occasion.

Figure 3.

Representative ligand blot of [3 H]E2 binding and detection of GPER by Western blot analysis of croaker ovarian membrane proteins separated by SDS-PAGE. Solubilized plasma membranes were separated on a SDS-PAGE gel, transferred to a nitrocellulose membrane, and incubated with 30nM [3 H]E2 in the presence (non-specific binding) or absence (total binding) of 1000-fold excess E2. The membranes were subsequently washed and Western blot analysis was performed using a specific human GPER antibody. The entire experiment was repeated 3 times and the same results were obtained each time.

Figure 4.

Effects of selective estrogen receptor modulators on inhibition of spontaneous maturation of denuded zebrafish oocytes *in vitro*. Fully grown zebrafish oocytes (mean diameter 550 μ m), capable of undergoing oocyte maturation, were separated from the surrounding follicle cells by enzymatic digestion, and incubated in 24-well plates in 1ml Dulbecco's media containing the various steroid treatments (approx. 20 oocytes/well, 4 wells/treatment) at 30°C for 3 hr in an *in vitro* oocyte maturation bioassay as described previously [35]. The steroids were added to the wells in ethanol (1 μ l). An equal volume of ethanol was added to the no treatment vehicle control (Veh) wells. At the end of the 3hr incubation period the oocytes were scored for completion of germinal vesicle breakdown (GVBD), an indication of oocyte maturation, by counting those that had a clear ooplasm lacking a nucleus under a binocular microscope, and the results presented as a percentage of the total oocytes that matured. DHP- 17,20 β -dihydroxy-4-pregen-3-one, the progestin that induces maturation in this species and a positive control to confirm the oocytes are capable of undergoing maturation. PPT- a selective ER agonist ; DPPN-a selective ER agonist. G-1: name, a selective GPER agonist. Asterisks denote means significantly different from vehicle controls ($P < 0.05$, one way ANOVA and nonparametric Bonferroni test). The entire experiment was repeated 3 times and similar results were obtained on each occasion.