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The ins and outs of GPR30: a transmembrane estrogen receptor

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Abstract

Estrogen is an important hormone in human physiology. It acts both via transcriptional regulation as well as via modulation of intracellular signaling through second messengers. Although estrogen's transcriptional effects occur through classical nuclear steroid receptors (ERs), recent studies reveal the existence of a novel 7-transmembrane G protein-coupled receptor, GPR30, which responds to estrogen and tamoxifen stimulation with rapid cellular signaling including ERK activation, PI3K activation, calcium mobilization and cAMP production. To distinguish between ER- and GPR30-mediated signaling, we have identified a novel GPR30 agonist that exhibits high specificity for GPR30. In this review, we will describe recent work to further our understanding of the role of GPR30 in estrogen biology.

Keywords

GPR30; estrogen receptor; estrogen; signaling

Introduction

Estrogen (E2) is a critical hormone in the human body, regulating functionally dissimilar processes in numerous tissues. Estrogen is one member of the family of steroid hormones, which also includes progesterone, testosterone, cortisol/glucocorticoids and aldosterone/mineralocorticoids that control many aspects of mammalian physiology. Steroid hormones are synthesized in tissues throughout the body, including the ovaries (estrogen, progesterone), testes (androgens/testosterone) and adrenal glands (cortisol, androgens and aldosterone). Additional estrogen-based steroids, estrone and estriol are also known to mediate biological functions. Among estrogen's diverse physiological effects are the regulation of growth, development and homeostasis of numerous tissues. The best understood of these are

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mammalian female reproduction and breast development [1]. In addition, estrogen regulates skeletal physiology [2], (cardio)vascular function [3] and the central nervous system [4] as well as the immune system [5]. Estrogen modulates processes ranging from adhesion and migration to survival and proliferation, cardiovascular and neuro-protection, organogenesis, angiogenesis and cancer. In addition, neurological processes such as stress responses, feeding patterns, sleep cycles and temperature regulation have been shown to be modulated by estrogen [6,7]. In the clinical arena, estrogen is perhaps most appreciated for its role in stimulating the proliferation of approximately two thirds of breast cancers [8,9].

Estrogen-like activity can also be found in a large variety of sources, both natural and man-made. These include phytoestrogens/isoflavonoids, from plants and fungi [10], as well as xenoestrogens, which include a variety of pesticides, polychlorinated biphenyls and plasticizers [11,12]. Diethylstilbestrol (DES), for example, was used from 1938–1971 as a treatment for pregnant women who experienced miscarriages or premature deliveries [13]. *In utero*, exposure to DES has been shown to have carcinogenic, teratogenic and reproductive effects on both the original patient as well as the children of treated individuals [14]. The majority of such compounds are thought to exert their effects through the inappropriate activation of estrogen receptor(s) [15].

The physiological effects of estrogens are traditionally mediated by nuclear hormone estrogen receptors (originally termed ER but later ER α), first characterized in 1973 [16]. A second estrogen receptor, ER β , discovered in 1996 [17], complicated our understanding of estrogen action. ER β is highly homologous to ER α and the two receptors are clearly evolutionarily related. The recent cloning of a mollusk estrogen receptor homolog that fails to bind estrogen suggests that the steroid receptors may be much more ancient than previously thought [18, 19]. Steroid receptors, including ER α and ER β , display a modular organization consisting of a ligand-binding domain, a DNA-binding domain and two transcriptional activation function domains. Binding of estrogen to ERs results in the release of the receptor from an inhibitory complex with heat shock proteins, allowing the receptor monomer to dimerize, translocate to the nucleus and associate with co-activating transcriptional factors. The DNA- and ligand-binding domains of ER α and ER β are 97% and 60% homologous, respectively. However, the amino terminus, which contains one of the transcriptional activation domains is only 18% homologous between the two ER subtypes [20]. Thus, both receptors bind estrogen and estrogen analogs with similar, though not identical, affinities/specificities and recognize identical DNA sequences. Distinct patterns of tissue distribution and the characterization of ER α and ER β knockout mice, however, reveal many differences in function [21].

Transmembrane G protein-coupled receptors for estrogen

The existence of G protein-mediated signaling by estrogen [22] and localization of estrogen binding sites to membranes [6] suggested the possibility of a 7-transmembrane G protein-coupled receptor family member being involved in certain aspects of estrogen function. The cloning of an orphan GPCR from estrogen-responsive MCF7 cells provided the impetus to test whether this receptor could mediate any of the effects of estrogen in cells lacking classical estrogen receptors [23–26]. In 2000, Filardo *et al.* demonstrated MAP kinase (Erk1/2) activation by estrogen in breast cancer cell lines expressing GPR30 but not in cell lines lacking the receptor [27]. ER antagonists, ICI 182,780 (considered a “pure” antiestrogens [28]) and 4-hydroxy-tamoxifen, at high concentrations, were also capable of mediating Erk activation in GPR30-expressing cells. This response proceeded through a pertussis toxin-sensitive pathway (indicating the involvement of Gi/o heterotrimeric G proteins) that involved the transactivation of EGFRs through the release of cell-surface heparin-bound EGF. Subsequent studies revealed a second phase of GPR30-dependent signaling via adenylyl cyclase that resulted in the eventual attenuation of Erk activation [29].

Subsequent studies by other groups reported that GPR30 was upregulated by progestin in MCF-7 cells [30] and that this expression is essential for progestin-mediated growth inhibition [31], [32]. GPR30 has been suggested to promote estrogen-mediated inhibition of oxidative stress-induced apoptosis by promoting Bcl-2 expression in keratinocytes [33] and furthermore, to promote cell growth by upregulation of cyclin D expression [34]. Stimulation of nerve growth factor production in macrophages [35] and upregulation of c-fos by estrogen in breast cancer cells [36] have also been shown. All in all, these reports suggest that estrogen may mediate, at least in part through the activation of GPR30, the regulation of cellular functions, including growth, proliferation and apoptosis.

Subcellular localization of GPR30

Alternative roles of GPR30 in estrogen signaling could be envisioned. For example, GPR30 could serve as a critical transactivated GPCR intermediate, much as EGFR appears to be in some GPCR-mediated signaling. Alternatively, GPR30 could act as a scaffold recruiting kinases and other signaling molecules, or its expression (and activation by an unknown endogenous ligand) could regulate the expression of conventional or novel estrogen receptors. In order to try to answer these and other questions regarding GPR30, we began by expressing GPR30 in cells that exhibit no endogenous responses to estrogen stimulation, such as COS7 cells [37]. To monitor expression of the receptor, we utilized a GFP chimeric construct fusing GFP to the carboxy-terminus of GPR30. Surprisingly, GPR30 appeared to be expressed in an intracellular tubuloreticular network. Using subcellular markers, we identified this compartment as the endoplasmic reticulum. In addition, we were unable to detect GPR30 on the plasma membrane, as defined by staining of the actin cytoskeleton to delineate the plasma membrane from the cell interior. This has been confirmed for endogenously expressed GPR30 as well. Such localization is at odds with the conventional paradigms of GPCR function, which place functional GPCRs in the plasma membrane. In fact Filardo *et al.* originally described GPR30 as a plasma membrane receptor, a logical assumption, despite the lack of experiments to examine receptor localization.

To complicate this issue further, Thomas *et al.* published an article providing evidence that GPR30 was in fact found in the plasma membrane [38]. Their argument was based on two observations: 1) an annular staining pattern of endogenously expressed GPR30 in SKBr3 breast cancer cells and 2) the presence of tritiated estrogen-binding sites in isolated cell membranes. More recently, Funakoshi *et al.* also reported expression of GPR30 in the plasma membrane [39]. Based on these apparently contradictory results, we suspect that the subcellular localization of GPR30 may be regulated, depending perhaps on cell/tissue type or culture conditions. Although the majority of GPCRs are expressed in the plasma membrane, it is becoming more evident that some GPCRs may be functionally expressed at intracellular sites [40]. This is particularly true of GPCRs with lipophilic ligands, and given that GPR30's ligand E2 is membrane permeable, an intracellular localization of the receptor is consistent with its function.

Estrogen-binding properties of GPR30

A role for GPR30 in cellular estrogen responsiveness was until recently suggested based on the fact that estrogen-responsive could be engendered through expression of the protein. To examine this directly, we created a fluorescent ligand derived from ethynyl estradiol. Using this fluorescent estrogen, we demonstrated that the bound fluorescent estrogen derivative colocalizes with either ER in the nucleus or GPR30 in the endoplasmic reticulum [37]. This latter result suggests that the GPR30 present in the endoplasmic reticulum is competent to bind ligand. The binding of this reporter to either classical ER and to GPR30 can be displaced by 17 β estradiol but not 17 α estradiol, demonstrating the need for the appropriate stereochemistry

of the ligand. The affinity of 17 β estradiol for GPR30 is approximately 6 nM, a value is similar to that reported by Thomas *et al.* for the binding of tritiated 17 β estradiol to membrane preparations [38]. Furthermore, the lack of detectable binding of the charged fluorescent estrogen to non-permeabilized cells is consistent with the intracellular binding we observe for GPR30. Finally, multiple cell lines that endogenously express GPR30 (but not ER) show fluorescent estrogen staining of the endoplasmic reticulum, which can be reduced by GPR30 antisense constructs.

Another important question relating to estrogen binding by GPR30 is the stoichiometry of binding. If, for example, GPR30 expression merely *enables* estrogen signaling, one would not expect a high correlation between the levels of GPR30 expression and estrogen binding. To address this issue, we used our fluorescent estrogen in conjunction with cells expressing GFP chimerae of ER α , ER β and GPR30 [37]. Receptor-GFP expressing cells were stained with fluorescent estrogen and analyzed by flow cytometry. By gating on populations of cells expressing different levels of GFP, we demonstrated a linear relationship between the number of receptors (either ER or GPR30) expressed in a cell population and the level of fluorescent estrogen binding. In addition, for a given level of receptor-GFP expression, we found the GPR30 binds almost as much fluorescent estrogen as ER α . In other words, since ER α binds 1 mol estrogen per mol receptor, GPR30 binds approximately 0.8 mol estrogen per mol receptor. These results, combined with the subcellular colocalization of fluorescent estrogen with GPR30, provide the strongest evidence to date that GPR30 directly binds estrogen. Ultimately, complete purification of detergent-solubilized receptor will be required to demonstrate direct physical binding of estrogen.

Identification of a GPR30-specific ligand

A major hindrance in the identification of the physiological significant functions of GPR30 in normal and disease states has been the lack of reagents that can specifically target (activate or inhibit) the receptor. This is exemplified by the fact that E2 and 4-OH-tamoxifen both bind to GPR30, as well as classical estrogen receptors, thus showing no specificity towards either receptor type. To identify novel compounds capable of specifically binding to GPR30, we undertook a combination of virtual and biomolecular screening [41]. Ligand-based virtual screening, which uses no input from the presumed receptor target, was used since steroid receptors as a class are particularly approachable resulting from the fact that their endogenous ligands (e.g., estradiol, testosterone, progesterone, cortisol) are very rigid in structure. This leaves no room for speculation regarding flexibility of the bioactive ligand conformation [42]. Using this approach, we selected the top 100 compounds (out of a library of almost 10,000) that resembled 17 β -estradiol in shape, chemical structure similarity and pharmacophoric pattern. Biomolecular screening was carried out using flow cytometry based on the competitive binding of the fluorescent estrogen by 17 β -estradiol in GPR30-transfected cells [41]. The results of this screening regime yielded a single compound, referred to as G-1 (GPR30-specific compound 1, a substituted dihydroquinoline) that consistently competed for binding of the fluorescent estrogen reporter.

Using the assay described above to further characterize G-1, additional competition binding assays were carried out. Whereas 17 β -estradiol yielded K_i values of 0.3 and 0.4 nM for ER α and ER β , respectively, GPR30 yielded a K_i of approximately 6 nM [37,41]. G-1 had a K_i of 11 nM for GPR30, which was about two-fold lower than that observed for 17 β -estradiol. Furthermore, G-1 displayed no significant binding to either ER α or ER β even at concentrations of 10 μ M, showing high selectivity for GPR30. Functional assays, including calcium mobilization and PI3K activation, support the selectivity of G-1 for GPR30 over the classical estrogen receptors. Additional studies are currently underway to further characterize the specificity and physiological properties of this compound.

Future prospects

GPR30 is becoming recognized as an estrogen receptor, perhaps complementary to the classical estrogen receptors, and responsible for the non-genomic effects induced by estradiol. This however raises more questions than it answers. These include the physiologic function of GPR30 in normal tissues as well as disease states, the overlapping and distinct functions of GPR30 with respect to ER α and ER β , and the development of probes/drugs that selectively target GPR30 vs. ER α and ER β and vice versa. With the description of the first GPR30-selective agent, the door has been opened to uncover the biology surrounding GPR30.

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