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GPR30: a G protein-coupled receptor for estrogen

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Abstract

Estrogen is a critical steroid in human physiology exerting its effect both at the transcriptional level as well as at the level of rapid intracellular signaling through second messengers. Many of estrogen's transcriptional effects have long been known to be mediated through classical nuclear steroid receptors but recent studies also demonstrate the existence of a 7-transmembrane G protein-coupled receptor, GPR30 that responds to estrogen with rapid cellular signaling. There is currently controversy over the ability of classical estrogen receptors to recapitulate GPR30-mediated signaling mechanisms and *vice versa*. This article will summarize recent literature and address the relationship between GPR30 and conventional estrogen receptor signaling.

Keywords

GPR30; estrogen receptor; estrogen; signaling

1. Introduction

The effects of all hormones, including steroids such as estrogen, are mediated by specific receptors that recognize and bind the hormone transmitting this information to downstream effectors. The first described estrogen receptor (termed ER and later ER α) was characterized in 1973, based on specific binding activity in rat uterus/vagina extracts (Jensen and DeSombre, 1973). The first DNA sequence of the estrogen receptor was determined in 1986 (Greene et al., 1986) and the first crystal structure of an ER ligand-binding domain was described in 1997 (Brzozowski et al., 1997). The discovery of a second estrogen receptor, ER β , in 1996 (Kuiper et al., 1996), complicated the understanding of estrogen action, resulting in initial skepticism over its physiological significance (Gustafsson, 2003). Though highly homologous overall, the relative lack of homology between ER β and ER α in the transcriptional activation domain and differences in the tissue distribution of the two receptors (Dechering et al., 2000; Kuiper et al., 1997) suggested functional differences, a conclusion supported by characterization of ER α and ER β knockout mice (Hewitt and Korach, 2003). As the functional characterization

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of the two classical estrogen receptors continued, it became largely accepted that the diverse physiological functions of estrogen could be entirely ascribed to the combined effects of ER α and ER β (Koehler et al., 2005). However, over the same time period, reports also described estrogen binding properties and cellular/physiological effects of estrogen that were not easily explained by transcriptional activation mediated by soluble estrogen receptors (Ho and Liao, 2002).

2. Membrane-associated estrogen receptors

Classical steroid receptors, localized in the cytosol and/or nucleus, traditionally mediate their primary effects at the genomic level. In recent years, a large number of reports have described membrane-associated estrogen receptors, either similar to or distinct from the classical nuclear estrogen receptors (Acconcia et al., 2004; Li et al., 2003; Razandi et al., 2003; Toran-Allerand et al., 2002). These receptors have been postulated to mediate aspects of cellular estrogen function, including traditional genomic (transcriptional) signaling as well as novel non-genomic (rapid) signaling (Govind and Thampan, 2003). These non-genomic signaling events include pathways that are traditionally thought of as arising from transmembrane growth factor receptors and G protein-coupled receptors. Whereas some reports described estrogen binding sites on intracellular membranes (Evans and Muldoon, 1991), other reports suggest that palmitoylation (Acconcia et al., 2004; Li et al., 2003) or phosphorylation (Balasenthil et al., 2004; Wang et al., 2002) may target classical ERs to the cytoplasmic face of the plasma membrane. Additional reports have suggested the involvement of adaptor proteins, such as Shc (Evinger and Levin, 2005) and MNAR (Boonyaratanakornkit and Edwards, 2004), in the recruitment of ER α to the plasma membrane and caveolae in particular. Further reports describe estrogen-mediated cell activation from the cell exterior using impermeant estrogen-conjugated BSA (Benten et al., 2001), which is reportedly prone to artifact(s) due to free and dissociating estrogen and must therefore be used with great care (Taguchi et al., 2004). Interestingly, reports of membrane binding sites for estrogen are not a recent phenomenon as they were first reported in 1977 (Pietras and Szego, 1977).

3. Estrogen-mediated non-genomic signaling

In addition to transcriptional regulation, which occurs on a time scale of hours, estrogen also mediates cellular effects with response times from seconds to minutes. These rapid non-genomic estrogen signaling events include the generation of the second messengers Ca²⁺, cAMP, and NO, as well as activation of receptor tyrosine kinases, such as EGFR and IGF-1R, and protein/lipid kinases (e.g. PI 3-kinase, Akt, MAPK family members, Src family members, and PKA/PKC) (Hall et al., 2001; Ho and Liao, 2002; Kelly and Levin, 2001; Levin, 2001; Levin, 2002; Razandi et al., 2003). In many reports, the estrogen-responsive receptor is proposed to be ER itself (either α or β), or a modified form of the protein (Acconcia et al., 2004; Li et al., 2003). Complexes between the classical ERs and G proteins (Navarro et al., 2003) as well as with PI3 kinase (Simoncini et al., 2003) have been described. Recently, ER associations with plasma membrane Gi proteins have been reported to mediate NO production (Wyckoff et al., 2001) and cAMP inhibition (Navarro et al., 2003). From these examples, it is clear that estrogen can mediate a multitude of complex rapid cellular activation events. However, not all such responses can be attributed to the classical ERs. The diversity and number of reported estrogen receptors and estrogen-stimulated signaling pathways described require careful analysis and interpretation of experimental data in order to understand the mechanisms involved in estrogen signaling and biology.

4. G protein-coupled seven transmembrane estrogen receptors

The description of G protein-dependent estrogen signaling and membrane localization of estrogen binding sites with varying affinities led to speculation of a transmembrane estrogen

receptor, possibly of the 7-transmembrane G protein-coupled receptor family. In the late 1990's, a putative GPCR was cloned by 4 different groups using highly disparate approaches (Carmeci et al., 1997; O'Dowd et al., 1998; Owman et al., 1996; Takada et al., 1997). The GPCR identified in these studies displayed little homology to other GPCRs. GPR30 mRNA was shown to be expressed in numerous tissues throughout the body (e.g. placenta, lung liver, prostate, ovary, placenta) although substantial contradictions between the tissue expression patterns were reported (Carmeci et al., 1997; Owman et al., 1996; Takada et al., 1997). Since no ligand was known for this receptor, it was labeled an orphan GPCR. It was not until 2000 that a possible function for this GPCR was identified from experiments demonstrating MAP kinase (Erk1/2) activation by estrogen, as well as ER antagonists, ICI 182,780 and tamoxifen. Responses were demonstrated in breast cancer cell lines expressing GPR30 but not in cell lines lacking GPR30 (Filardo et al., 2000). Signaling in response to estrogen could be restored in the latter cell lines by expressing GPR30. Estrogen-dependent signaling 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. A second report described a second phase of GPR30-dependent signaling via adenylyl cyclase that led to attenuation of Erk activation over time (Filardo et al., 2002).

Soon after, it was also reported that in keratinocytes, GPR30 promotes estrogen-mediated inhibition of oxidative stress-induced apoptosis by promoting Bcl-2 expression (Kanda and Watanabe, 2003) as well as cell growth by stimulation of cyclin D expression (Kanda and Watanabe, 2004). Furthermore, GPR30-mediated upregulation of nerve growth factor production in macrophages by induction of c-fos expression has also been demonstrated (Kanda and Watanabe, 2003). The upregulation of c-fos by estrogen and phytoestrogens has also been shown in breast cancer cells (Maggiolini et al., 2004). Recently, GPR30 has been demonstrated to mediate the proliferative effects of both estrogen and tamoxifen in endometrial cancer cells (Vivacqua et al., 2005) and thyroid cancer cells (Vivacqua et al., 2006). Taken together, these observations suggest that GPR30 may play a role in the regulation of cellular growth, including proliferation and apoptosis. However, despite the implications that GPR30 may represent a novel estrogen-binding receptor, a description of its ligand binding properties was absent from the literature.

5. GPR30 and its subcellular localization

Although the majority of GPCRs are expressed in the plasma membrane, it is becoming accepted that some GPCRs may be functionally expressed at intracellular sites (Gobeil et al., 2006). This is particularly true of GPCRs with lipophilic ligands. Our results revealed that GPR30 appeared to be expressed in an intracellular tubuloreticular network. Using subcellular markers, we identified this compartment as the endoplasmic reticulum. Staining of the nuclear envelope, which is contiguous with the endoplasmic reticulum, was also observed. In addition, we were unable to detect transfected or endogenously expressed GPR30 on the plasma membrane, as defined by staining of the actin cytoskeleton to delineate the plasma membrane from the cell interior for example. At the same time however, Thomas *et al.* showed expression of GPR30 in the plasma membrane, though no staining of subcellular markers was provided (Thomas et al., 2005). Recently, Funakoshi *et al.* also reported expression of GPR30 in the plasma membrane (Funakoshi et al., 2006). In conclusion, there remains controversy over the site of GPR30 expression. Interestingly, it has been shown that G protein $\beta\gamma$ subunits are initially targeted to the endoplasmic reticulum, where they subsequently associate with G protein α subunits, providing the requisite machinery for GPR30 to initiate signaling. From that point, soluble mediators such as Src could mediate EGFR transactivation at the cell surface. It is certainly possible that under the appropriate conditions, intracellular GPR30 could exist or translocate to the cell surface or *vice versa*. However, given the fact that GPR30's ligand

estrogen is membrane permeable, an intracellular localization of the receptor is certainly consistent with its function.

6. GPR30 and its ligands

As discussed above, the proposed role of GPR30 in cellular estrogen responsiveness was, until recently, based on the correlation of receptor expression with estrogen-mediated signaling (Filardo et al., 2000; Kanda and Watanabe, 2003; Kanda and Watanabe, 2003; Kanda and Watanabe, 2004; Ylikomi et al., 2004). To test whether GPR30 binds estrogen, we synthesized a fluorescent derivative of ethinyl estradiol (a high affinity ER agonist). Such derivatives offer several advantages over tritiated estrogen, including high-resolution subcellular localization of estrogen binding sites by confocal fluorescence microscopy and quantitative flow cytometric measurements. The fluorescent estrogen displays subcellular colocalization with either ER in the nucleus or GPR30 in the endoplasmic reticulum, consistent with anti-GPR30 staining patterns (Revankar et al., 2005). Binding is also highly specific as demonstrated by competition with 17 β estradiol but not 17 α estradiol for both ER and GPR30, the latter displaying a K_i for 17 β estradiol of approximately 6 nM. This value is similar to the 3 nM value reported by Thomas *et al.* for the binding of tritiated 17 β estradiol to membrane preparations (Thomas et al., 2005). Furthermore, other steroids such as estrone and estriol exhibited very low affinities whereas progesterone, testosterone and cortisol displayed no binding (Thomas et al., 2005). Interestingly, ER antagonists/SERMs such as tamoxifen and ICI182,780 were also shown to bind GPR30 (Thomas et al., 2005) consistent with previous functional studies showing that these same compounds were agonists for GPR30 (Filardo et al., 2000). With the recent report of a highly selective, non-steroidal GPR30 agonist, future studies of GPR30 function should be greatly facilitated by this novel reagent (Bologa et al., 2006).

7. GPR30-mediated signaling

As discussed above, estrogen initiates multiple intracellular signaling cascades. Although classical estrogen receptors have been demonstrated to be capable of mediating many of these responses, the signaling capabilities of GPR30 in response to estrogen have just begun to be described. Estrogen-mediated GPR30-dependent activation of the MAP kinase Erk1/2 via EGFR transactivation was first described in 2000 (Filardo et al., 2000). Subsequently, adenylyl cyclase activation by GPR30 has also been demonstrated (Filardo et al., 2002; Thomas et al., 2005). However, following these studies, Levin and colleagues questioned these original conclusions in a paper where they reported similar EGFR transactivation and Erk activation mediated by estrogen through ER α (Razandi et al., 2003). To directly compare the signaling capabilities of ER α and GPR30, we expressed each receptor in COS7 cells and examined the signaling pathways contributing to estrogen-mediated mobilization of intracellular calcium and the activation of PI3K (Revankar et al., 2005). Our results demonstrated that only in the case of GPR30 was EGFR transactivation required, consistent with Filardo's original observations (Filardo et al., 2000). Furthermore, that GPR30 is the sole receptor responsible for mediating PI3K activation in response to estrogen via EGFR transactivation in ER-negative breast cancer cells such as SKBr3 is demonstrated by the loss of estrogen-responsiveness following GPR30 antisense transfection. Here antisense transfection reduces both GPR30 protein expression and fluorescent estrogen binding commensurate with the loss in functional responsiveness. Our results also demonstrate that tamoxifen activates PI3K through GPR30 but not ER α , suggesting a possible involvement in tamoxifen-resistant breast cancers and/or the increased incidence and severity of endometrial cancers in women treated with tamoxifen (Rieck et al., 2005; Senkus-Konefka et al., 2004). The circumstances under which ER α and/or GPR30 activation lead to EGFR transactivation remain to be fully determined.

PI3K is traditionally activated at the plasma membrane in response to growth factor receptor or G protein-coupled receptor stimulation, resulting in PIP3 accumulation at the plasma membrane. However, we observed PIP3 accumulation in the nucleus in response to estrogen stimulation of both ER α and GPR30. That this was the result of *de novo* synthesis of PIP3 was confirmed by pharmacological inhibition of PI3K activity. Although our understanding of the role of nuclear phosphoinositides is limited (Lian and Di Cristofano, 2005; Neri et al., 2002), recent studies confirm the presence of PI3K, PDK1, Akt and Pten in the nucleus (Lian and Di Cristofano, 2005). Additional work has revealed that the activity of steroidogenic factor-1 and liver receptor homolog 1, two members of the nuclear receptor superfamily of transcription factors thought to exhibit ligand-independent activity, is regulated by binding phosphatidylinositol-3,4,5 trisphosphosphate (Krylova et al., 2005). Thus, accumulation of nuclear PIP3 in response to estrogen-mediated activation of ER α and/or GPR30 may regulate gene expression through this family of nuclear receptors. Additional functions of PIP3 in the nucleus also remain to be explored.

8. Unresolved questions

With the diverse functions of estrogen in development, adult physiology and disease in almost every tissue of the body and with GPR30 now defined as a *bona fide* estrogen receptor (both in terms of binding and signaling), many significant questions remain: What is the physiologic function of GPR30? Does GPR30 serve redundant or overlapping functions to ER α and ER β , or does it initiate mostly independent responses? Is GPR30 expressed in the same or different cells and tissues compared to ER α and ER β ? Will drugs that selectively target GPR30 vs. ER α and ER β and *vice versa* be superior to drugs currently available for the treatment of cancer, cardiovascular, neurological and immune disorders? As these questions are addressed and the physiological role(s) of GPR30 are elucidated, our understanding of the complex physiological responses to estrogen will be further advanced.

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