Published in final edited form as:

Mol Cell Endocrinol. 2007 January 2; 260-262: 244–248.

Potential Leydig cell mitogenic signals generated by the wild-type and constitutively active mutants of the lutropin/choriogonadotropin receptor (LHR)

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Introduction

Although chronic activation of the LHR by injection of hCG was shown to cause Leydig cell hyperplasia over 20 years ago (Christensen et al., 1980), the ability of the LHR to stimulate the proliferation of Leydig cells and to behave as a potential Leydig cell oncogene was not clearly recognized until recently, when the phenotypes individuals harboring naturally occurring activating or inactivating mutations of the hLHR were described (reviewed by Latronico et al., 1999; Themmen et al., 2000; Ascoli et al., 2002). 46XY individuals harboring germ line activating mutations of the hLHR are associated with Leydig cell hyperplasia whereas those harboring germ line inactivating mutations of the hLHR are associated with Leydig cell hypoplasia. Moreover, the finding of a somatic activating mutation of the hLHR in Leydig cell adenomas of several unrelated boys with precocious puberty (Liu et al., 1999; Canto et al., 2002; Richter-Unruh et al., 2002) suggests that the LHR may even be involved in the transformation of Leydig cells. The mitogenic and oncogenic potential of LH, CG and the LHR are also supported by several observations made in different mouse models. For example, targeted deletion of the LHR results in Leydig cell hypoplasia (Lei et al., 2001; Zhang et al., 2001) and LH induces the development of Leydig cell tumors in inhibin-deficient mice (Kumar et al., 1996) or in mice expressing an SV40 T-antigen transgene under the control of the inhibin α -promoter (Kananen et al., 1997). The LHR is also ectopically expressed in the adrenal cortex of these two transgenic models and these mice develop gonadotropin-dependent adrenocortical hyperplasia or adrenocortical tumors (Rilianawati et al., 1998; Kero et al., 2000). In addition, transgenic mice overexpressing a modified form of LH β with a long circulatory half-life are characterized by an increased incidence of gonadal tumors (Risma et al., 1995) as do mice with high levels of LH induced by administration of 5 α -reductase inhibitors (Pralhada et al., 1994).

In this paper I (a) briefly review studies describing the mechanisms by which the LHR regulates a classical mitogenic pathway, the ERK1/2 cascade and (b) highlight the similarities in the signaling transduction pathways activated by the agonist-engaged LHR-wt and its constitutively active mutants.

Molecular basis of the LHR-induced activation of the ERK1/2 cascade

The ERK1/2 cascade is a prominent mitogenic pathway that has been studied in much detail (reviewed by Marinissen et al., 2001; Pearson et al., 2001; Pierce et al., 2001a; Stork et al., 2002; Lefkowitz et al., 2005). The phosphorylation of ERK1/2 proceeds by the sequential

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*This work was supported by a grant from the National Institutes of Health (CA-40629 to MA)

activation of three kinases, Raf1 which phosphorylates MEK1 and MEK1 which phosphorylates ERK1/2 (Marinissen et al., 2001; Pearson et al., 2001; Pierce et al., 2001a; Stork et al., 2002; Lefkowitz et al., 2005).

Raf1, the first kinase of this cascade, is activated when it associates with the active (GTP-loaded) forms of Ras and/or Rap1. These two are members of the family of small GTPases which toggle between an active (GTP-bound) or inactive (GDP-bound) state. The transition between these two states is facilitated by guanine nucleotide exchange proteins which promote the exchange of GDP and GTP and by GTPases which aid in the hydrolysis of the bound GTP (Cullen et al., 2002; Stork et al., 2002; Hancock, 2003). Thus, extracellular signals that activate the ERK1/2 cascade usually do so by indirectly modulating the activation of small GTPases such as Ras and/or Rap1. A particularly well characterized mode of Ras activation is the formation of multi-protein complexes involving activated growth factor receptors, adaptor proteins and a Ras guanine nucleotide exchange factor called SOS (Hackel et al., 1999; Yarden et al., 2001).

An early report showed that addition of hCG to heterologous cells expressing the recombinant LHR resulted in the phosphorylation of ERK1/2 (Faure et al., 1994). This was subsequently shown to be the case in porcine (Cameron et al., 1996) or rat granulosa cells (Salvador et al., 2002) expressing the endogenous LHR and in an immortalized rat granulosa cell line expressing the recombinant LHR (Seger et al., 2001). When expressed in a heterologous cell type (COS-7 cells) the LHR-mediated activation of the ERK1/2 cascade was reportedly mediated by G β / γ (Faure et al., 1994), but subsequent studies done in granulosa cells expressing the endogenous LHR (Cameron et al., 1996; Salvador et al., 2002) or immortalized rat granulosa cells expressing the recombinant LHR (Seger et al., 2001) indicated that this effect was a cAMP/PKA-dependent process.

Recently we investigated the mechanisms by which the LHR may mediate the activation of the ERK1/2 cascade in MA-10 Leydig tumor cells (Hirakawa et al., 2003b). Although the low density of endogenous LHR expressed in MA-10 cells can mediate an increase in the phosphorylation of ERK1/2 when MA-10 cells are exposed to hCG, the magnitude of this response can be enhanced several fold by transient expression of the hLHR-wt (Hirakawa et al., 2002). Therefore most of our experiments were done using MA-10 cells transiently transfected with the hLHR-wt to help us delineate the pathways by which the activation of this receptor leads to an increase in the phosphorylation of ERK1/2.

The G protein-coupled receptor (GPCR)-mediated phosphorylation of ERK1/2 can occur by G protein-dependent and/or -independent pathways (reviewed by Murga et al., 1999; Marinissen et al., 2001; Pierce et al., 2001a; Luttrell et al., 2002; Perry et al., 2002; Pierce et al., 2002; Stork et al., 2002; Lefkowitz et al., 2005). The most prominent G protein-independent pathway involves the formation of multi-protein complexes containing the receptor, a β -arrestin and one or more components of the ERK1/2 cascade (Pierce et al., 2001a; Luttrell et al., 2002; Perry et al., 2002; Pierce et al., 2002; Stork et al., 2002; Lefkowitz et al., 2005). The participation of this pathway seems unlikely, however, because overexpression of the β -arrestins or their dominant-negative counterparts have little effect on the hCG-induced phosphorylation of ERK1/2 in MA-10 cells (Hirakawa et al., 2003b).

The G protein-dependent phosphorylation of ERK1/2 can occur by multiple pathways that are mediated by the G α or G β / γ subunits liberated from the GPCR-promoted activation of different families of G proteins (Murga et al., 1999; Marinissen et al., 2001; Pierce et al., 2001a). The G β / γ -mediated pathway is utilized mostly by GPCRs that couple to the G $_{i/o}$ family of G proteins and it likely involves the G β / γ -mediated trans-activation of the EGF receptor (Carpenter, 1999; Prenzel et al., 1999; Pierce et al., 2001b). Although the hLHR activates the G $_{i/o}$ family

of G proteins (Herrlich et al., 1996; Rajagopalan-Gupta et al., 1998; Hirakawa et al., 2003a) the activation of this G protein family appears to play a minor role (if any) on the LHR-provoked phosphorylation of ERK1/2. This conclusion is based on the findings that the use of pertussis toxin and sequestrants of $G\beta/\gamma$ induced only a 20–30% inhibition of the hCG-induced stimulation of ERK1/2 phosphorylation (Hirakawa et al., 2003b). In addition, we have not been able to detect enhanced phosphorylation of the EGF receptor in MA-10 cells treated with hCG (unpublished data).

G_{α_s} and $G_{\alpha_{q/11}}$, which are liberated as a consequence of the activation of the LHR (Herrlich et al., 1996; Rajagopalan-Gupta et al., 1998; Hirakawa et al., 2003a), activate second messenger cascades that can also lead to the phosphorylation of ERK1/2 (Marinissen et al., 2001). The involvement of $G_{\alpha_{q/11}}$ -activated pathways in the LHR-induced phosphorylation of ERK1/2 in MA-10 cells can be readily excluded by three independent lines of experimentation (Hirakawa et al., 2003b). First, activation of another $G_{q/11}$ -coupled GPCR endogenous to MA-10 cells (the V1 arginine vasopressin receptor, see Ascoli et al., 1989) does not result in the phosphorylation of ERK1/2. Second, selective inhibitors of protein kinase C have little or no effect on the hCG-induced phosphorylation of ERK1/2. Third, when engaged by hCG, the low levels of endogenous LHR present in MA-10 cells do not mediate an increase in inositol phosphate accumulation but they do mediate an increase in cAMP accumulation and ERK1/2 activation (Ascoli et al., 1989; Hirakawa et al., 2002). In contrast, the involvement of the G_{α_s} /adenylyl cyclase/cAMP signaling cascade in the LHR-induced phosphorylation of ERK1/2 in MA-10 cells can be readily documented by a number of different experimental approaches. The most direct demonstration for a role of cAMP is the finding that co-transfection of MA-10 cells with a cAMP phosphodiesterase (which blunts the hCG-induced increase in cAMP accumulation) also blunts the LHR-induced phosphorylation of ERK1/2 (Hirakawa et al., 2003b).

The effects of cAMP on the ERK1/2 cascade are complex and often they are cell-type specific (reviewed by Stork et al., 2002). The actions of cAMP are mediated by protein kinase A (PKA, see Edelman et al., 1987) or by cAMP-dependent guanine nucleotide exchange factors (cAMP-GEFs, see ref. de Rooij et al., 1998; Kawasaki et al., 1998) and both of these cAMP targets have been reported to serve as intermediates in the cAMP-induced phosphorylation of ERK1/2 (Figure 1 and Enserink et al., 2002; Stork et al., 2002). In MA-10 cells PKA seems to be an important mediator of the cAMP-induced phosphorylation of ERK1/2, however as judged by the effects of two structurally unrelated inhibitors of PKA and a cAMP analog that selectively activates cAMP-GEFs. Thus, co-transfection of MA-10 cells with a vector coding for the heat stable protein kinase inhibitor (a specific inhibitor of PKA, see Day et al., 1989) or treatment of the cells with H89 (a selective PKA inhibitor, see Davies et al., 2000) inhibit the LHR-mediated increase in ERK1/2 phosphorylation (Hirakawa et al., 2003b). Conversely, addition of 8CPT-2Me-cAMP, a cAMP analog that is selective for cAMP-GEFs (Enserink et al., 2002) has little or no effect on ERK1/2 phosphorylation (Hirakawa et al., 2003b).

Small GTPases such as Ras, Rap1 and Rap2 have also been implicated as mediators of the actions of cAMP on ERK1/2 phosphorylation. These are located downstream of PKA or the cAMP-GEFs (see Figure 1) and they participate in ERK1/2 phosphorylation by virtue of their ability to bind to and activate Raf, the first kinase in the ERK1/2 cascade (Stork et al., 2002). Our results indicate that the actions of hCG and cAMP on ERK1/2 phosphorylation in MA-10 cells are mediated by PKA and Ras rather than cAMP-GEFs and Rap (Figure 1). These conclusions are supported by two key findings. First, when added to MA-10 cells hCG and cAMP cannot activate Rap1 but they both activate Ras and they do so in an H89 sensitive fashion (Hirakawa et al., 2003b). Second, co-transfection of MA-10 cells with a dominant negative mutant of Ras inhibits the effects of hCG on ERK1/2 phosphorylation but co-transfection with a dominant negative mutant of Rap1 does not (Hirakawa et al., 2003b).

Importantly, many of the details of the pathway described above can also be documented in primary cultures of Leydig cells isolated from 21-day old rats. These cells were chosen as the Leydig cell model to study because they are believed to be good representatives of the precursors of the adult population of mammalian Leydig cells (Hardy et al., 1990; Shan et al., 1992; Ge et al., 1996; Pelliniemi et al., 1996; Lee et al., 1999). In these primary cultures 8Br-cAMP, but not 8CPT-2Me-cAMP, induce the activation of Ras and the phosphorylation of ERK1/2 and both of these effects are effectively inhibited by H89 (Hirakawa et al., 2003b). The ability of hCG to stimulate ERK1/2 phosphorylation in primary cultures of immature rat Leydig cells was also documented subsequently by other investigators (Martinelle et al., 2004). They concluded that this effect was mediated by PKA and PKC, however.

Collectively our data delineate a pathway in which the LHR-provoked activation of Gs resulting in the stimulation of adenylyl cyclase leading to an increase in cAMP which then activates Ras in a PKA-dependent manner. The activated Ras in turn switches on the classical Raf/MEK kinase cascade that ultimately results in ERK1/2 phosphorylation (Figure 1). The PKA-dependency of the activation of Ras is surprising because in most cases this event has been reported to be PKA-independent (see Busca et al., 2000; Tsygankova et al., 2000; Schmitt et al., 2002). The potential involvement of a PKA-dependent activation of Ras as a mediator of the effects of cAMP on the ERK1/2 cascade was only recently recognized during the development of cAMP analogs that activate cAMP-dependent guanine nucleotide exchange factors but do not activate PKA (Enserink et al., 2002) and in studies on the mechanisms by which another Gs-coupled GPCRs (i.e., the serotonin receptors) activate the ERK1/2 cascade in heterologous cell types (Norum et al., 2002). Lastly, it is important to note that the involvement of other isoforms of Rap (such as Rap2) have not been yet been examined and a potential role for Rap2 in the hCG-induced phosphorylation of ERK1/2 cannot be formally excluded.

The PKA-mediated activation of Ras can occur by a PKA-mediated activation of a guanine nucleotide exchange factor for Ras (a Ras-GEF) or by a PKA-mediated inactivation of a Ras GTPase (a Ras GAP). There are many Ras-GEFs and GAPs that have been described (Cullen et al., 2002; Hancock, 2003), including RasGRF1, a Ras-GEF that is activated by PKA-mediated phosphorylation (Yang et al., 2003). We do not yet know which Ras-GEFs and GAPs are present in Leydig cells and if they are targets for PKA (Cullen et al., 2002; Hancock, 2003).

Another important issue has not yet been investigated is the potential involvement of the LHR-activated ERK1/2 cascade on Leydig cell proliferation. In this respect it should be noted that ERK1/2 cascade does not have to be involved only on Leydig cell proliferation. In fact the LHR-induced, cAMP-mediated phosphorylation of ERK1/2 has been recently implicated as a negative regulator of steroidogenesis in granulosa cells (Seger et al., 2001) and as a positive regulator of steroidogenesis in Leydig cells (Martinelle et al., 2004).

Signaling pathways activated by the agonist-engaged wild-type LHR and its constitutively active mutants

As already mentioned above a rather large number of naturally occurring activating mutations of the hLHR gene associated with precocious puberty in boys have been reported in the last decade (reviewed by Latronico et al., 1999; Themmen et al., 2000; Ascoli et al., 2002). The overwhelming majority of these are germ line mutations found in boys with familial or sporadic precocious puberty and Leydig cell hyperplasia (Latronico et al., 1999; Themmen et al., 2000; Ascoli et al., 2002) but there is one somatic mutation (D578H in transmembrane helix 6) that has been found in Leydig cell tumors of several unrelated boys with precocious puberty (Liu et al., 1999; Canto et al., 2002; Richter-Unruh et al., 2002).

When expressed in a heterologous cell type the D578H mutant of the hLHR was initially reported to display constitutive activity when assayed for cAMP and inositol phosphate accumulation, whereas a similar germ line mutation associated with Leydig cell hyperplasia and male precocious puberty (D578Y, see refs. Laue et al., 1995; Yano et al., 1996; Muller et al., 1998) displayed robust constitutive activity when assayed for cAMP accumulation and little or no constitutive activity when assayed for inositol phosphate accumulation (Kosugi et al., 1996; Kosugi et al., 1998; Liu et al., 1999). These findings lead to the proposal that the unique, dual constitutive activation of the cAMP and the inositol phosphate signaling pathways by the D578H mutant contributed to the neoplastic transformation of Leydig cells (Liu et al., 1999).

Subsequent experiments performed by us (Min et al., 2000; Hirakawa et al., 2002; Min et al., 2002; Hirakawa et al., 2003a) generated an extensive comparison of the functional properties of two germ line hLHR CAMs associated with Leydig cell hyperplasia (hLHR-L457R and hLHR-D578Y, see Laue et al., 1995; Yano et al., 1996; Latronico et al., 1998; Muller et al., 1998) with the functional properties of the single somatic hLHR CAM (hLHR-D578H) associated with Leydig cell adenomas. Our experiments, which were done by expressing the recombinant hLHR-wt or mutants thereof in MA-10 cells allowed us to conclude that the G proteins and signaling pathways activated by constitutively active mutants of the hLHR associated with Leydig cell hyperplasia or tumors are identical and are the same as those activated by the agonist engaged hLHR-wt.

The hLHR-wt and the three mutants activate the G_s , $G_{i/o}$ and $G_{q/11}$, but not the $G_{12/13}$, families of G proteins. The activation of these G proteins by the hLHR-wt occurs only when engaged by agonist, but their activation by the L457R, D578Y and D578H mutants occurs independently of agonist stimulation (Hirakawa et al., 2003a). In addition, the L457R, D578Y and D578H mutants were found to be constitutively active towards the steroidogenic pathway, towards more general and proximal signaling cascades such as cAMP and inositol phosphates and towards ERK1/2 phosphorylation, which as discussed above, is a classical mitogenic pathway (Hirakawa et al., 2002). In fact, our data show that the constitutive activity of these three mutants is pleiotropic in nature.

We concluded that the G proteins and signaling pathways activated by constitutively active mutants of the hLHR associated with Leydig cell hyperplasia or tumors are identical and are the same as those activated by the agonist-engaged hLHR-wt. If there was preferential activation of some G protein families by the somatic D578H mutation found in Leydig cell tumors as opposed to the germ line mutations found in Leydig cell hyperplasia then one could envision mechanisms by which the D578H mutant would be oncogenic. Our data suggest that such mechanisms do not need to be considered. On the other hand, although our data exclude this possibility they provide no clues about the reasons why the D578H mutation is uniquely associated with Leydig cell adenomas.

Summary and Perspective

Although it seems clear that LH/CG is mitogenic to Leydig cells there is a paucity of information regarding the mitogenic signaling pathways activated by the LHR and their potential involvement in Leydig cell proliferation and transformation. Clearly, however, other hormones and growth factors such as Mullerian inhibiting substance (Behringer et al., 1990; Behringer et al., 1994), ghrelin (Barreiro et al., 2004), IGF-1 (Wang et al., 2004), the Kit ligand (Kissel et al., 2000) and PDGF (Gnessi et al., 2000) can also influence the proliferation and/or transformation of Leydig cells. Perhaps a comparison of the signaling pathways activated by these receptors and by the LHR as well as studies using established cell culture systems, primary cultures of Leydig cells and animal models will help us address these important issues.

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Figure 1. Pathways involved in the LHR-provoked phosphorylation of ERK1/2

A pathway (shown by the transparent shapes) involving the activation of the Gs/adenylyl cyclase/cAMP signaling cascade seems to be prominently involved in the LHR-provoked phosphorylation of ERK1/2 in MA-10 cells. Cyclic AMP seems to stimulate ERK1/2 phosphorylation by activating protein kinase A (PKA), which in turn activates Ras by a pathway that has not yet been characterized (indicated by the broken arrows and question marks). Additional LHR-mediated pathways for Ras activation are also possible as indicated by the curved arrow and question marks. The Ras-induced activation of the Raf1-MEK1-ERK1/2 cascade is presumed to occur by the classical pathway that has been so well described

in many other cell types. A role for MEK has been documented by the inhibition of ERK1/2 phosphorylation induced by the addition of UO126, a selective MEK inhibitor.

A role for cAMP can be readily documented by the inhibition of ERK1/2 phosphorylation induced by overexpression of a cAMP phosphodiesterase (PDE) and by the ability of 8Br-cAMP (a cAMP analog that activates PKA or cAMP-GEFs) to stimulate ERK1/2 phosphorylation. A role for PKA can be documented by the inhibition of ERK1/2 phosphorylation induced by overexpression of the heat stable PKA inhibitor (PKI) or by addition of H89, a selective PKA inhibitor. A role for Ras can be documented by ability of hCG to enhance the levels of Ras-GTP and by the inhibition of ERK1/2 phosphorylation induced by overexpression of a dominant-negative mutant (Ras-S17N).

The involvement of cAMP-GEFs, and Rap1 can be excluded (shown by the gray shapes) by the inability of 8CPT-2Me-cAMP (a cAMP analog that selectively activates cAMP-GEFs) to stimulate ERK1/2 phosphorylation, by the inability of hCG to activate Rap1 and by the inability of a dominant negative mutant of Rap1 (Rap1b-S17N) to inhibit the hCG-induced increase in ERK1/2 phosphorylation.