Primary Leydig cells naturally expressing mouse LHR do not discriminate between LH- and hCG-mediated signaling *in vitro*

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Abstract

Human luteinizing hormone (LH) and chorionic gonadotropin (hCG) are glycoprotein hormones fundamental for development and reproduction. These hormones were considered biologically equivalent for decades due to structural similarities and binding to the same receptor (LHCGR), although they mediate different physiological roles. Previous reports demonstrated LH- and hCG-specific intracellular signaling mediated by LHCGR in human primary granulosa cells, but few studies using rodent receptor (Lhr) are available. We investigated the Lhr-mediated activation of the cAMP/PKA-pathway, ERK1/2 and CREB phosphorylation, gene expression and steroidogenesis, in murine Leydig cells treated with LH and hCG. We found that hCG is more potent than LH in inducing cAMP production, as well as downstream the pERK1/2 activation. However, similar levels of CREB phosphorylation, *Stard1* gene expression and testosterone production occurred upon LH and hCG treatment *in vitro*. These findings revealed that rodent Lhr mediates quantitatively, but not qualitatively, different LH- and hCG-dependent signaling, which results in similar testosterone synthesis. These data suggest that *in vivo* bioassay using a model expressing rodent receptor, which rely on the evaluation of testosterone-dependent endpoints, may be not suitable to quantify gonadotropins activity for clinical purpose.

KEY WORDS:LH, hCG, Leydig, gonadotropins, LHCGR.

Introduction

Human luteinizing hormone (LH) and chorionic gonadotropin (hCG) are heterodimeric glycoprotein hormones which play crucial roles in human reproduction. LH is produced by the pituitary in a pulsatile fashion, supporting the folliculogenesis and inducing ovulation and *corpus luteum* maintenance in fertile females. In adult males, LH stimulates the production of testosterone by Leydig cells, regulating spermatogenesis. hCG is secreted by trophoblast cells to support pregnancy through progesterone production (1). LH and hCG bind the common G protein-coupled receptor (LHCGR) expressed in gonads. LHCGR displays an extracellular domain which serves for hormone binding and is connected to a seven-transmembrane domain by a hinge region, and an intracellular C-terminal tail. Interestingly, both hormones retain

binding capability of *non*-human LH receptors (Lhr), even if choriogonadotropin is primate-specific and is linked to different physiological functions than LH (2) (Table 1).

Upon LH or hCG binding, the receptor mediates the activation of multiple, ligand-specific signaling pathways (3). Activation of the Gas protein/cAMP/protein kinase A (PKA)-pathway is a requisite for steroid production and it is linked to extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation and cAMP-response element-binding protein (CREB) activation. CREB is a transcription factor mediating the expression of target genes, such as the steroidogenic acute regulatory protein (*STARD1*), and steroidogenic events (2). pERK1/2 activation may occur *via* intracellular molecules alternative to G proteins, such as β-arrestins, associated with receptor internalization and proliferative signals, in steroidogenic cells (4). Moreover, the action of gonadotropins involves several other signals, such as the anti-apoptotic phosphatidyl-inositol 3-kinase (PI3K)/protein kinase B (AKT)-pathway, resulting in a complex network of intracellular signaling pathways.

Several studies found LH- and hCG-specific interaction at the receptor levels (5-8), as well as signal transduction *in vitro* (3, 9-12). Especially, the human receptor is able to discriminate the hormone binding by primate-specific amino acids located within the hinge region, triggering qualitatively and quantitatively different intracellular signaling. Especially, hCG is more potent in inducing cAMP-mediated steroidogenic response than LH, which instead preferentially modulates proliferative and anti-apoptotic events *via* ERK1/2- and AKT-pathway, at least in primary granulosa cells *in vitro*. Therefore, LH/hCG binding to the human receptor modulates a number of intracellular pathways mediating physiological effects, which may be summarized as the net result of a balance between life and death signals.

Gonadotropins are used for infertility treatment in assisted reproduction techniques (ART) and they are marketed as several drug formulations differing in source, purification methods and biochemical composition (13). Gonadotropin bioactivity is established by *in vivo* bioassays and an example is provided by the so-called "Van Hell bioassay". In this procedure, a fixed dose of LH/hCG is injected into old immature male rats for five days to evaluate the seminal vesicles weight gain (SVW) (14). Then, the preparation is calibrated against an International Standard and expressed in international units (IU), instead of molarity. Therefore, hormone dosage to be used in ART depends on gonadotropin bioactivity in animal models expressing *non*-human receptors.

Activation of steroidogenic signals in Leydig cells

Intracellular cAMP increase is the first signal to rise steroid synthesis. Recruitment and accumulation of the second messenger occurred in murine primary Leydig cells upon stimulation by increasing doses of LH and hCG (pM-μM range), in the presence of a inhibitor of phosphodiesterase enzymes (11). These experiments revealed that hCG treatment features significantly lower maximal effective concentrations (EC_{50}) than LH, and that hCG is about 10-fold more potent than LH in triggering cAMP production (LH EC_{50}=192±53.96 pM; hCG EC_{50}=18.64±10.14 pM; means±SEM). This

Table 1 - Main differences between luteinizing hormone and chorionic gonadotropin.

Luteinizing hormone	Chorionic gonadotropin
Exists in all the vertebrates	Exists only in primates
β-subunit is encoded by a unique *LHB* gene	β-subunit is encoded by six *CGB* genes, in human
Produced by pituitary in a pulsatile fashion	Produced by trophoblast cells in a constant, increasing manner
Regulates gametogenesis, ovulation and steroid synthesis during the fertile age	Support pregnancy through progesterone production
Low number of glycoforms	High number of glycoforms
Short half-life (~90 minutes)	Long half-life (several hours)

finding was confirmed in the mouse Leydig tumor cell line mLTC-1 and in human LHCGR-expressing HEK293 cells, transiently trasfected with a BRET-based cAMP sensor (CAMYEL) (12). All together, these data corroborate what previously demonstrated in human primary granulosa cells, confirming that hCG retains higher steroidogenic potential than LH.

cAMP increasing at the intracellular level is linked to ERK1/2 phosphorylation occurring within 15 minutes, which, consistently with its cAMP/PKA dependence, results highly activated upon hCG rather than LH treatment in primary Leydig cells (11). This result differs from what observed in human primary granulosa cells, where LHCGR mediates higher LH- than hCG-mediated pERK1/2 activation, not reflecting cAMP data (3) (Figure 1). Thus, the impact of hCG treatment on the activation of intracellular signaling pathways is higher than that of LH. In fact, BRET experiments revealed that hCG was more potent than LH in inducing β-arrestin 2 recruitment, with hCG EC_{50} 12-times lower than that of LH (12). This intracellular event occurred upon treatment by gonadotropin in the nM concentration range, suggesting that a higher level of receptor occupancy is required to trigger β-arrestin 2 recruitment, compared to cAMP accumulation. Interestingly, *in vitro* experiments evaluating the kinetics of β-arrestin 2 recruitment revealed that LH acts as a partial agonist, in terms of β-arrestin 2 recruitment, likely impacting on LH-dependent pERK1/2 activation. Indeed, β-arrestin 2-mediated ERK1/2 phosphorylation has been demonstrated for several G protein-coupled receptors (15-17). Taken together, these data revealed that hCG induces relatively high levels of early intracellular events activation downstream Lhr, suggesting that choriogonadotropin exerts

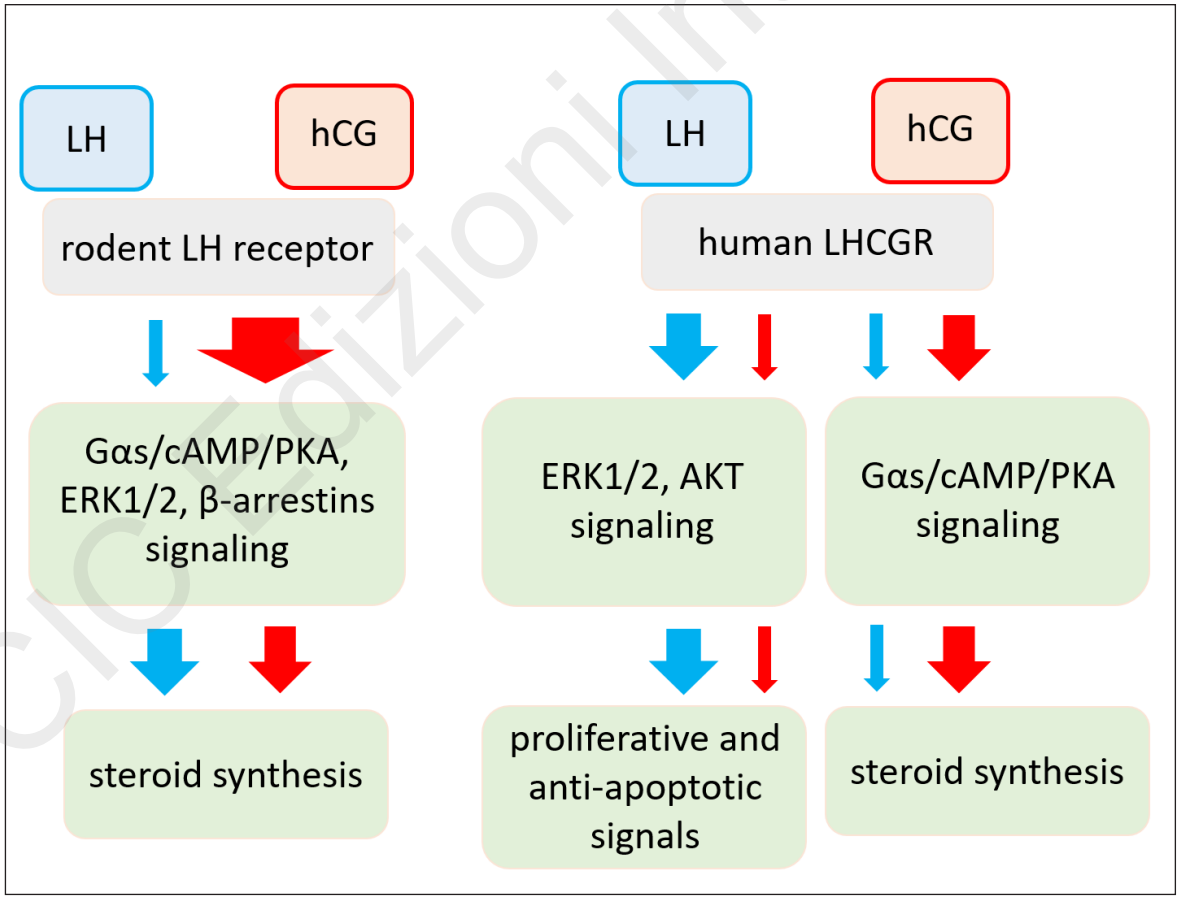

Figure 1 - Different LHR- and LHCGR-mediated signaling and downstream events. Upon human LH and hCG binding, LHR triggers quantitatively different, gonadotropin-specific intracellular signaling, however resulting in similar testosterone synthesis. LHCGR is able to differentiate quantitatively and qualitatively the LH- and hCG-induced signaling, resulting in preferential proliferative/anti-apoptotic or steroidogenic events, respectively.

higher agonistic activity than LH at the rodent receptor level (18). However, no different hCG- *versus* LH-induced pCREB activation, as well as *Stard1* gene expression was found in murine primary Leydig cells (11). This is surprising since CREB phosphorylation is a cAMP-dependent event (19). Most probably, LH and hCG mediate quantitatively different cAMP and pERK1/2 levels, which converge in similar pCREB activation, as an effect of a balance between pro- and anti-steroidogenic intracellular events simultaneously activated through Lhr.

The pattern of LH- and hCG-mediated pCREB activation and *Stard1* gene expression are reflected by testosterone production, which is similarly induced upon treatment by gonadotropins (11). Since testosterone production was abolished by the specific inhibitors H-89 and U0126, both the cAMP/PKA- and ERK1/2-pathways are required for steroid synthesis. Interestingly, hCG treatment resulted in higher testosterone levels than those obtained by LH treatment, in mLTC1 cells (12), suggesting the existence of specific, intracellular mechanisms featuring the response to gonadotropins in tumor cells. In this cell model, the depletion of β-arrestins by specific small-interfering RNA probes (siRNA) is linked to decreased progesterone and testosterone production, demonstrating that these molecules may be involved in the modulation of steroidogenic signals, at least in tumor cells (12).

Testosterone dependence of spermatogenesis

These data demonstrate that rodent Lhr expressed in mouse primary Leydig cells does not modulate qualitatively LH- and hCG-specific intracellular signaling and testosterone production. *In silico* studies support the concept that LH- and hCG-specific signaling depends on specific amino acid residues encoded by the exon 10 of the *LHCGR* gene, corresponding to a portion of the hinge region of the protein receptor (1, 7). In fact, the deletion of exon 10 found in a 18-year-old boy affected by hypogonadotropic hypogonadism was linked to impairment of LH action (20, 21). This boy had very low testosterone levels, retarded pubertal development and small testicles, but, surprisingly, clinical treatment by exogenous hCG restored testosterone synthesis, revealing the importance of exon 10 for discriminating the actions of LH and hCG. Moreover, exon 10 is naturally lacking in the LH receptor mRNA of the new world monkey *Callithrix jacchus*, where transcripts presumably corresponding to chorionic gonadotropin, rather than LH sequence were found in the pituitary (22). On the other hand, testosterone is crucial for the maturation of gametes and fertility maintenance in both primates and rodents (23, 24). Testosterone administration recovered fertility in mLhr knock-out mice (25), while, in gonadotropin-suppressed men, spermatogenesis was restored by exogenous hCG-induced testosterone production (26). Interestingly, only about 80% identity is shared by the sequences encoded by the mouse and human exon 10 of the LH receptor, suggesting that amino acids fundamental for discriminating LH- and hCG-specific signaling may be lacking in rodents. In fact, chorionic gonadotropin is not existing in rodents, where progesterone production is sustained by LH during pregnancy, thus providing the evolutionary basis to understand why it is not required a discrimination between LH- and hCG-induced signaling by Lhr. This is a relevant issue for clinicians using gonadotropins quantified by mouse or rat bioassay, where testosterone-dependent endpoints serve to infer hormone bioactivity. Whether Lhr is not able to differentiate the effect of human ligands, *in vivo* bioassays should be optimized by using different strategies. Therefore, mouse primary Leydig cells may be a limited system to quantify gonadotropins to be used in human, especially in ART. Indeed, folliculogenesis is a complex process involving both life and death signals, which are not sufficiently evaluable using models expressing orthologous receptors, i.e. mice.

The action of LH and hCG at the intracellular level reflects the physiological role of these gonadotropins (12). In human, hCG prompts relatively massive progesterone production to sustain pregnancy, while LH mediates gametogenesis, when proliferative and anti-apoptotic signals occur. Especially, LH triggers testosterone synthesis in the male, where spermatogenesis may be sustained by low testosterone levels (27). Moreover, steroid synthesis is exerted through different pathways, the Δ5-pathway is mainly dedicated to 17-OH-pregnenolone pro-

duction rather than the Δ4-pathway, which instead leads to progesterone accumulation (28). Different steroidogenesis between primary Leydig cells, mLTC-1 and human primary granulosa cells may be due to cell type-dependent variations of the steroidogenic pathway preferentially activated upon LH and hCG binding to the receptor.

Implications for ART

Current data provide the concept that gonadotropins are not identical and mediate molecule-specific effects. The deep characterization of these effects is a matter of interest for assisted reproduction, where the administration of exogenous gonadotropins should aim to replicates a complex cross-talk between intracellular signals occurring during folliculogenesis. In fact, the differentiation between LH and hCG action provided by *in vitro* studies is supported by *in vivo* data. A recent meta-analysis demonstrated that specific combinations of FSH and LH or hCG, or FSH alone, may impact on different ART outcomes (29). For instance, oocyte number may be optimized by using FSH alone, while hCG supplementation positively impacts on mature oocytes, embryos, and implantation rate, and LH addition is linked to higher pregnancy rate. These are important evidences support the LH- and hCG-specific intracellular signaling observed *in vitro*. Moreover, these data suggest that gonadotropins may be selectively used in order to optimize specific ART outcome, as a possible strategy for future personalized protocols. In this view, the development of bioassays able to properly quantify gonadotropins to be used in human will be a crucial issue.

Conclusions

In vitro data provide the molecular basis to understand the differences between gonadotropins used as equivalent in clinical practice. Especially, they allow to elucidate the specific regulation of steroidogenic signals in mouse and human primary Leydig and granulosa cells, as well as tumor cells. These studies provide new concepts and knowledge to optimize ART protocols and the comprehension of steroidogenic tumor cells features.

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