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FSH increases the different LH- and hCG-dependent intracellular signalling and the downstream life/death signals *in vitro*

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Introduction

Luteinizing hormone (LH) and choriogonadotropin (hCG) are glycoprotein hormones regulating ovarian function and pregnancy. They were equivalently used in assisted reproduction techniques (ART) due to their binding to a common receptor (LHCGR). However, differences between LH and hCG were demonstrated at molecular and physiological level [1]. Our previous study revealed that LHCGR mediates hCG-dependent steroidogenesis-related signaling and LH-dependent proliferative and anti-apoptotic events in human granulosa cells [2].

Aim

The aim of this study is to evaluate how follicle-stimulating hormone (FSH) co-treatment, in the ART therapeutic dose-range, affects the different LH- and hCG-specific responses *in vitro*.

Study design

We evaluated phospho-CREB, -ERK1/2 and -AKT activation by Western blotting, gene expression by real-time PCR, cAMP, progesterone and estradiol production by ELISA, and cell viability by MTT assay in human granulosa-lutein cells (hGLC). LH and hCG dose-response experiments (0.1 pM-1.0 nM range) were performed, in the presence of 10 nM FSH.

Results

In the presence of FSH, hCG biopotency is higher than that of LH, and is about 5-fold increased, in terms of cAMP activation (fig.1), compared to previous data obtained in the absence of FSH [2]. Moreover, different LH and hCG dose-response curves were observed, in terms of 50% effective doses (EC50s), hill-slopes and maximal levels (Mann-Whitney's *U*-test; p<0.05; n=6), suggesting hormone-specific receptor cooperativity and biopotency. In the presence of FSH, the range of effective hCG doses increased, in terms of CREB phosphorylation (fig.2). FSH increased the LH-dependent ERK1/2 and AKT phosphorylation, the expression of the *X-linked inhibitor of apoptosis* (*XIAP*) gene (fig.3), and the cell viability (Mann-Whitney's *U*-test; p<0.05; n=4), resulting in anti-apoptotic effects (fig.4). Consistently with the effect on cAMP and pCREB activation, steroid production increased under hCG and FSH co-treatment (figs.5, 6).

Discussion

FSH potentiates the LH-dependent anti-apoptotic and the hCG steroidogenic (and pro-apoptotic) potential *in vitro*. The different modulatory activity of FSH on LH and hCG action *in vitro* reflects their different physiological functions, consisting in proliferative effects exerted by LH during the follicular phase and before trophoblast development, and the high steroidogenic potential of hCG requested to sustain pregnancy. These findings were recently published [3].

References

1. Grzesik et al. Front Endocrinol (Lausanne). 2015. 6:140; **2.** Casarini et al. PLoS One. 2012. 7(10); **3.** Casarini et al. Mol Cell Endocrinol. 2016. 422:103-14.;

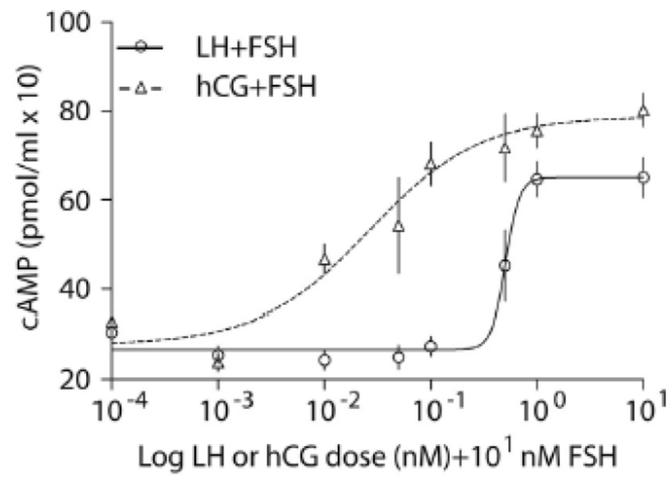

Figure 1. cAMP dose-response to LH and hCG, in the presence of 10 nM FSH treatment. hGLC were stimulated 2 h by 0-1x10^{1} nM LH or hCG together with the fixed FSH dose, in the presence of IBMX, then cAMP was measured by ELISA. cAMP levels were extrapolated by a standard curve and expressed as pmol/ml. All the results are represented as means±SD (n=6) in a logarithmic X-axis, then *non*-linear regressions were plotted. The EC50 (LH+FSH=440.9±271.4 vs hCG+FSH=20.3±1.2; p=0.0006), H-slope (LH+FSH=2.9±1.0 vs hCG+FSH=1.0±0.2; p=0.0023) and maximal cAMP values (LH+FSH=650.3±97.8 vs hCG+FSH=795.4±46.10; p=0.0175) were compared by Mann-Whitney's U-test.

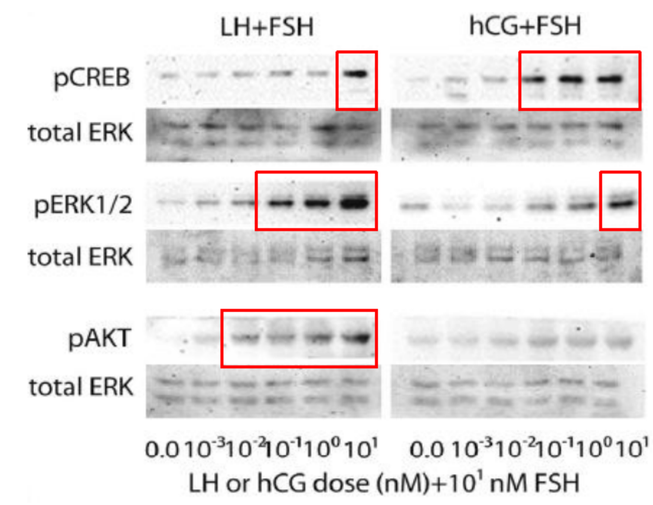

Figure 2. Phosphorylation of CREB, ERK1/2 and AKT induced by increasing LH or hCG concentrations, in the presence of a fixed FSH dose. hGLC were stimulated 15 min by 0-1x10^{1} nM LH or hCG together with 1x10^{1} nM FSH, then the signaling pathways activation was qualitatively evaluated by Western blotting using specific antibodies (image representative of 4 experiments). Total ERK served as normalizer. The signals detected by Western blotting were semiquantitatively evaluated to be represented as means±SD and used used for statistical analysis after background subtraction. Red squares indicates significant differences versus LH- or hCG-unstimulated (basals) (Mann-Whitney's U-test; p<0.05; n=4).

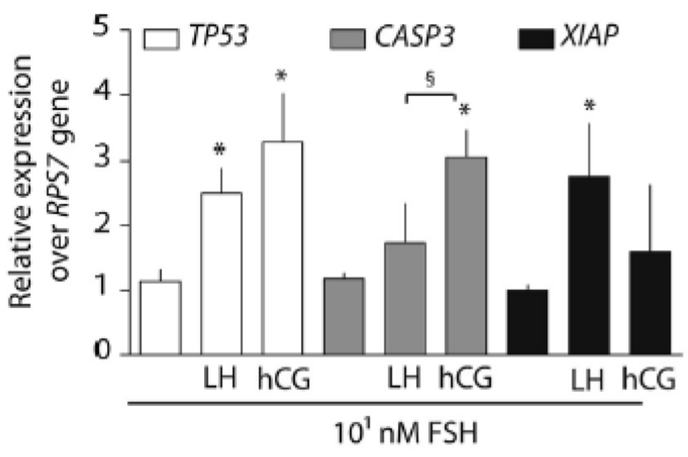

Figure 3. Gene expression analysis. The expression of target genes regulating pro- and anti-apoptotic events was evaluated in 2-h 1x10^{-1} nM LH- or hCG-stimulated hGLC, in the presence of 1x10^{1} nM FSH, after 12 h, by real time PCR. Controls were prepared by incubating the cells in media without LH or hCG. Each value was normalized by the *RPS7* control gene expression and graphically represented as fold increase over unstimulated controls in relative units scale (n=4; means±SD). Legend: *TP53* gene encoding the tumor protein 53 (P53); *CASP3* gene encoding the procaspase 3 protein; *XIAP* gene encoding the X-linked inhibitor of apoptosis factor protein. *=statistically significant difference versus FSH alone (basal); §=statistically significant difference between LH- and hCG-stimulated cells (ManneWhitney's U-test; p<0.05; n=4).

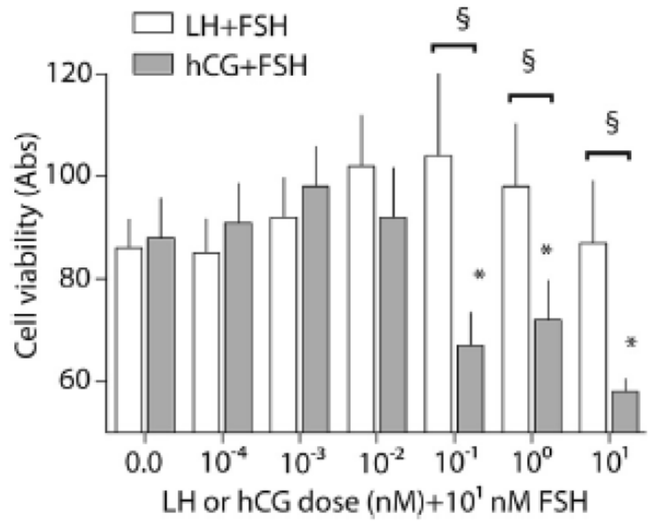

Figure 4. Cell viability assay in hGLC stimulated by different LH or hCG doses, in the presence of 1x10^{1} nM FSH. The cells were treated using the short-term stimulation protocol, then they were washed twice. The cell viability was assessed after 2 days by MTT assay and measured as absorbance using a spectrophotometer. The results were graphically represented as means±SD after background subtraction. *=statistically significant difference versus FSH (basal); §=statistically significant difference between LH+hCG- and LH+FSH-stimulated cells (Mann-Whitney's U-test; p<0.05; n=4).

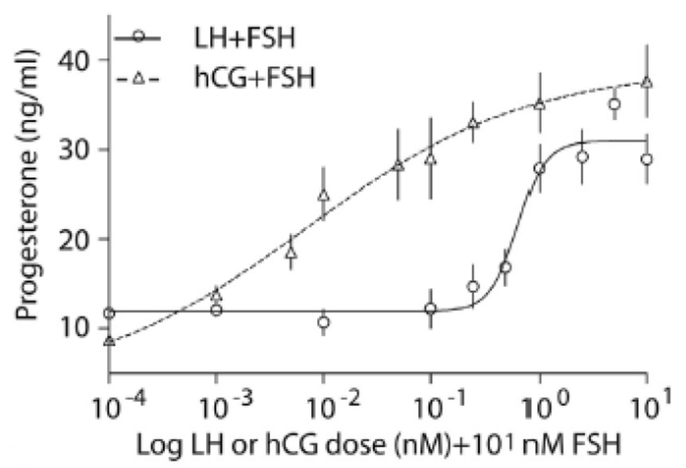

Figure 5. Progesterone dose-response in hGLC upon 2-h stimulation by increasing LH or hCG doses, together with 1x10^{1} nM FSH. Progesterone was measured by ELISA after 24 h then, hormone levels expressed as ng/ml in the graph using a logarithmic X-axis (means±SD; n=6). *Non*-linear regressions were calculate and the EC50 (LH+FSH=671.2±240.6 vs hCG+FSH=82.7±46.8; p=0.0095), H-slopes (LH+FSH=3.1±1.2 vs hCG+FSH=1.0±0.2; p=0.0087) and maximal progesterone levels (LH+FSH=35.0±2.11 vs hCG+FSH=39.0±3.4; p=0.3939) were extracted from the curves and compared by Mann-Whitney's U-test.

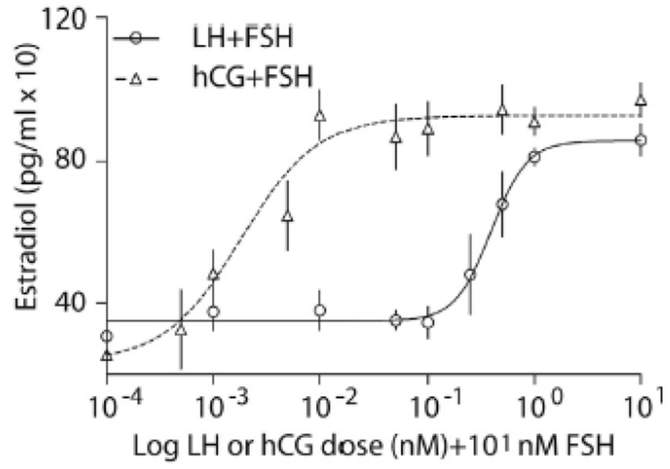

Figure 6. Evaluation of estradiol production. hGLC were stimulated 2 h by increasing LH or hCG doses, in the presence of 1x10^{1} nM FSH, then they were washed twice with PBS and the estradiol was measured by ELISA after 24 h 1 mM androstenedione was added to the cells media as substrate for the enzymatic conversion to estradiol. Estradiol levels were expressed as means±SD (pg/ml) and *non*-linear regression calculated (n=6). The EC50 (LH+FSH=225.1±80.2 vs hCG+FSH=1.3±0.8; p=0.0022), H-slopes (LH+FSH=2.0±1.1 vs hCG+FSH=1.2±0.8; p=0.4848) and maximal estradiol levels (LH+FSH=921.1±86.6 vs hCG+FSH=954.5±39.5; p=0.5887) were compared by Mann–Whitney's *U*-test.

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