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Research Article

Involvement of Ovarian Estradiol Biosynthesis and Pituitary FSH Expression in the Mechanism of Human Chorionic Gonadotropin Stimulation of Uterine Growth in Immature Female Rats

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In a previous work, we showed that human Chorionic Gonadotropin (hCG) alone is able to stimulate uterine ponderal growth in immature female rats. In the present paper, we provide new information indicating that a single 100 ng hCG injection increased ovarian androgen availability and aromatase activity. These changes are consistent with an increase in ovarian estradiol secretion. Ovarian activin βA and activin βB subunit RNAs expression were also increased following hCG injection. Most interestingly, this treatment also led to an increase in FSHβ subunit mRNA expression in the pituitary, which might be due to hCG-stimulated ovarian activin secretion. We have not been able so far to follow the kinetics of the plasma concentrations of activin and FSH in hCG-treated animals compared to control animals. This has to be explored in the future to provide a more complete assessment of our model.

1. Introduction

In a previous paper [1], we showed that the uterus weight response in immature rats is observed at much lower gonadotropin concentration than ovarian weight response (threshold of 0.8 IU versus 12.5 IU), with a slightly greater dynamic (×4.2 versus ×3.5) and a steeper dose-dependent slope.

It has been known for a long time that FSHs from various species trigger uterus weight gain in immature rats but only in the presence of a low dose of hCG [2, 3]. In addition, we found that hCG alone was also able to stimulate uterus weight gain although it exhibits only LH activity in contrast to equine CG (eCG) that exhibits both LH and FSH activities in rat [4–6].

In the present work, we aimed at understanding how hCG is able to stimulate uterus weight gain even in the absence of exogenous FSH. Our data demonstrate the central role of estradiol where synthesis is increased after enhanced mRNA expression of pituitary FSHβ mRNA as well as ovarian activin and aromatase mRNAs but not of ovarian FSHR mRNA.

2. Materials and Methods

The animals were Wistar rats (RjHan:WI) from the colony reproduced and bred at INRA-UEPAO facility (Nouzilly, France). The founders and renewal animals of this colony were from Janvier (Le Genest-Saint-Isle, France). Temperature of experimental room was kept at 21 ± 1°C and artificial lighting at 12L/12D. The animals were fed *ad libitum* with scientific animal food (SAFE, Augy, France) and drinking water was provided *ad libitum*. All procedures were approved by the Ethical Committee of Centre-Val de Loire region (CNRS, INRA, Universities of Tours and Orléans (France)).

Human Chorionic Gonadotropin (hCG) with a bioactivity of 8149 IU/mg was obtained from Hépartex (Saint-Cloud, France) and bovine FSH (bFSH) was from Aspen (Castle Rock, CO). The estrogen receptor antagonist ICI 182,780 was purchased from Santa Cruz Biotechnology (Dallas, TX) and testosterone from Sigma-Aldrich (Saint-Louis, MO).

Each control and treatment group comprised 6 immature 23-day-old female rats. The animals were injected

Table 1

Gene	Abbreviation	Aaccession nr	5′-3′ sequence	Size
luteinizing hormone beta	Lhb	NM_012858	Fw: CTGAGCCCAAGTGTGGTGT Rv: GTAGGTGCACACTGGCTGAG	201 pb
Follicle-stimulating hormone, beta polypeptide	Fshb	NM_001007597.1	Fw: AAGTCGATCCAGCTTTGCAT Rv: CAGCCAGGCAATCTTATGGT	248 pb
Follicle-stimulating hormone receptor	FSHR	NM_199237.1	Fw: GAGTCATCCCGAAAGGATCA Rv: TGAGACTGGGGAGATTCTGG	192 pb
Cytochrome P450, family 19, subfamily a, polypeptide 1	Cyp19a1	NM_017085.2	Fw: CTCCTCCTGATTCGGAATTG Rv: ATGCTCGAGGACTTGCTGAT	206 pb
Inhibin alpha	INHα	NM_012590.2	Fw: ATGCACAGGACCTCTGAACC Rv: GGGGCCTAGAGCTATTGGAG	247 pb
Inhibin beta-A	INHβA	NM_017128.2	Fw: GGAAAACGGGTATGTGGAGA Rv: TGAAACAGACGGATGGTGAC	243 pb
Inhibin beta-B	INHβB	NM_080771.1	Fw: GAGCGCGTCTCTGAGATCAT Rv: CTGCCCTTCTCCAGGACATA	167 pb
Actin, beta	actb	NM_031144.3	Fw: GGCATCCTGACCCTGAAGTA Rv: AACACAGCCTGGATGGCTAC	233 pb
Ribosomal protein L19	Rpl19	NM_031103.1	Fw: CATGGAGCACATCCACAAAC Rv: CCATAGCCTGGCCACTATGT	216 pb

subcutaneously with 100 μL of either sterile 0.9% NaCl (Braun, Melsungen, Germany) or olive oil (Lesieur, Asnières, France) containing the various molecules of interest at the concentrations and times indicated below for each experiment.

Reverse Transcriptase-qPolymerase Chain Reaction (RT-qPCR) analyses were performed essentially as previously described [7]. In brief, pituitary or ovarian tissues were extracted using Nucleospin RNAII kit (Macherey-Nagel, Düren, Germany) by mechanical homogenization with Fast-Prep-24 Instrument (MP Biochemical, Santa Ana, CA) and lysates were prepared in the presence of β-mercaptoethanol. After DNAse treatment, RNAs were washed extensively and stored at −80°C. Their concentration was estimated through their optical density at 260 nm using a Nanodrop (Thermo Scientific, Waltham, MA) photometer.

Reverse transcriptions were performed with MMLV-RT at 42°C for 60 min using 0.2 μg pituitary RNA or 1.5 μg ovary RNA with the GoScript™ Reverse Transcriptase System (Promega, Madison, WI) using 0.5 μg oligo$(dT)_{15}$, 0.2 μL RNAsin (40 U/μL), and 0.5 mM dNTPs in a final volume of 20 μL. The reaction was stopped by heating at 70°C for 15 min. The cDNAs were stored at −20°C until use in qPCR.

The primers were designed using the Primer 3 Plus software (Andreas Untergasser, Michelstadt, Germany) and were synthesized by Eurogentec (Seraing, Belgium). These primers are presented in Table 1. The qPCR reactions were carried out in duplicate in 20 μL total volume containing 10 μL IQ-Sybr Green Supermix (Biorad, Hercules, CA), 25 ng ovary cDNA or 10 ng pituitary cDNA, and 0.25 μL of each 10 μM primer. Amplifications were carried out in a Biorad iCycler (denaturation 94°C-30 sec/hybridization 60°C-30 sec/elongation 72°C-30 sec). The cycle threshold (Ct) was determined and relative expressions were calculated relative to the housekeeping genes βactin and Rp119. The data were expressed as mean ± standard deviation (SD) and analyzed by One Way ANOVA in the StatView Package.

3. Results

3.1. Mediation of Estradiol in hCG-Stimulated Uterus Weight Gain. In order to determine whether hCG stimulation of uterus weight gain is mediated by estrogen, the estrogen receptor antagonist ICI 182,780 (1–100 μg/animal in 100 μL olive oil) was injected subcutaneously at different times between 8 h before and 30 h after hCG (100 ng/animal in 100 μL saline). The uteri were removed and weighted 48 h after hCG injection.

Figure 1 shows that ICI 182,780 exerts a dose-dependent inhibition of hCG stimulation when injected simultaneously with the gonadotropin (Figure 1(a)). It was also found that ICI 182,780 at 100 μg/animal inhibited uterus weight gain over (48 h) even when injected up to 24 h after hCG (Figure 1(b)).

The data indicate that estrogens mediate hCG stimulation of uterus weight gain mainly between 24 h and 48 h after hCG stimulation since the estrogen receptor antagonist was able to inhibit uterus weight gain even when injected 24 h after hCG.

3.2. Involvement of Testosterone in FSH-Stimulated Uterus Weight Gain. Figure 2 shows that testosterone alone at a concentration of 1 μg/100 μL olive oil/rat has no stimulating effect on uterus weight over 48 h after injection. A dose of 1.5 μg bFSH alone injected in 100 μL saline exhibited a significant effect over the same period of time. However, when these doses of testosterone and bFSH were injected simultaneously (less than 2 min apart), a clear synergic effect was observed

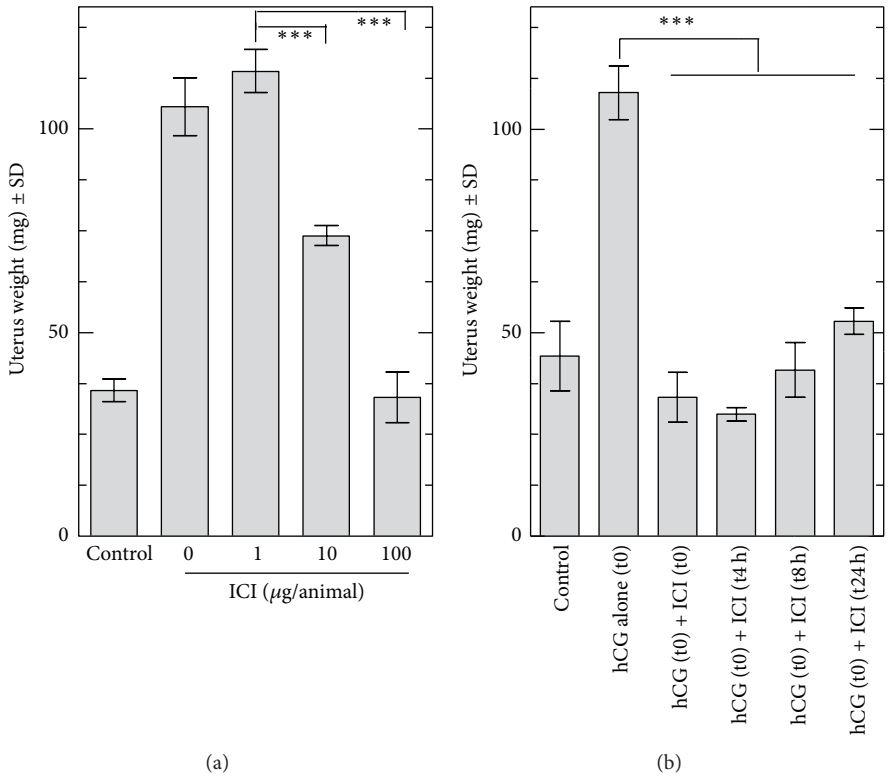

Figure 1: (a) Dose-dependent inhibition of hCG-stimulated uterus weight gain by ICI 182,780 in 23-day-old immature female rats ($n = 6$ per group). Human CG was injected at a dose of 0.1 μg/100 μL/rat quasi simultaneously with 0 to 100 μg ICI/100 μL/rat and uterus weights were determined 48 h later ($^{***}P < 0.01$). (b) Inhibition of hCG-stimulated uterus weight gain by ICI 182,780 given at different times after hCG injection ($n = 6$ per group). Human CG was injected at a dose of 0.1 μg/100 μL/rat and then 100 μg ICI/100 μL/rat either simultaneously (as in (a)) or 4 h, 8 h, or 24 h later. Uterus weights were determined 48 h after hCG injection ($^{***}P < 0.01$).

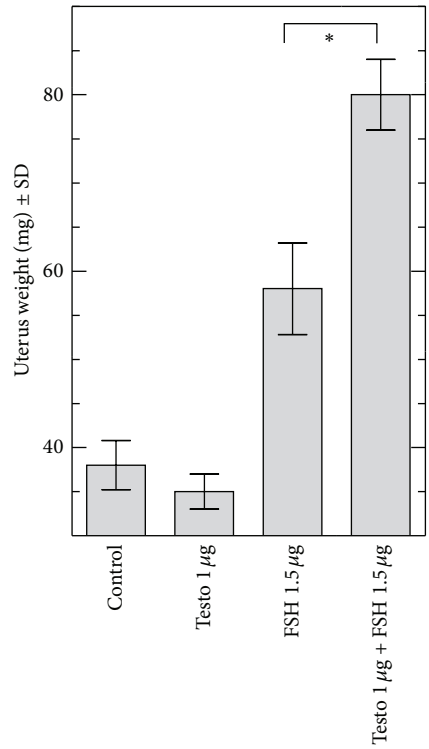

Figure 2: Effect on uterus weight response (means ± SD) of testosterone alone (1 μg/100 μL olive oil/rat) or bFSH alone (1.5 μg/100 μL saline/rat) compared to the simultaneous injection of both hormones in 23-day-old immature female rats. Uterus were weighted 48 h after injection(s).

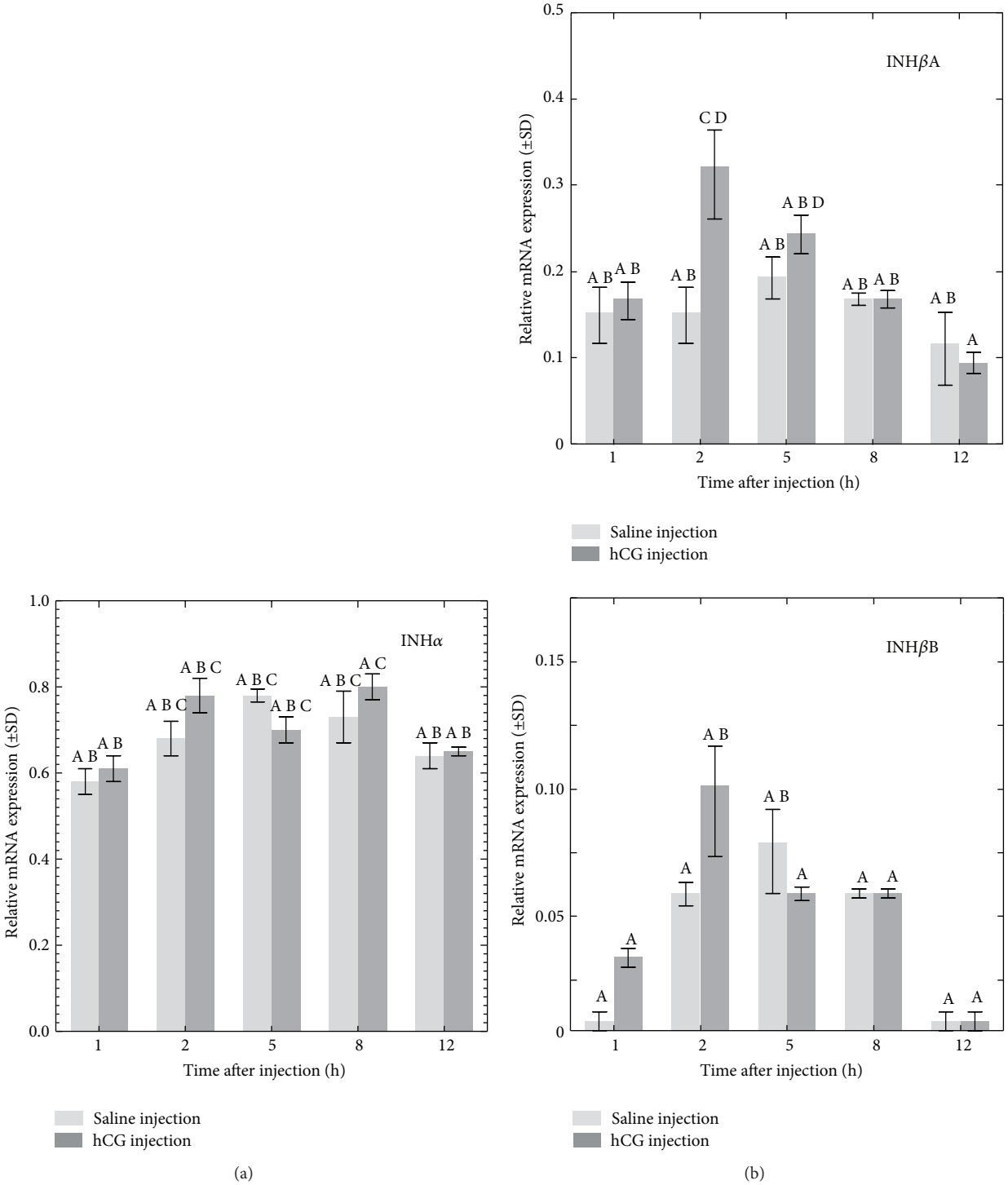

Figure 3: Kinetics of ovarian INHα (a) and INHβA and INHβA (b) mRNAs expression (means ± SD) after saline or hCG injection (0.1 μg/rat; $n = 4$) in immature 23-day-old female rats. Uppercase letters indicate statistically significant differences ($P < 0.05$).

in uterus weight gain. This result is in agreement with the view that FSH stimulates the aromatization of androgens into estradiol which in turn stimulates uterus growth.

3.3. Ovarian Inhibin/Activin Subunits and Aromatase mRNAs after hCG Injection. Figure 3 shows that inhibin βA and inhibin βB subunit mRNAs in the ovaries are slightly augmented 2 hours after hCG injection in contrast to the inhibin α subunit. This is in agreement with a possible increase of activin ($\beta\beta$) expression but not of inhibin ($\alpha\beta$) expression.

Figure 4 shows that hCG promotes a strong increase in ovarian aromatase mRNA within the 5 hours following

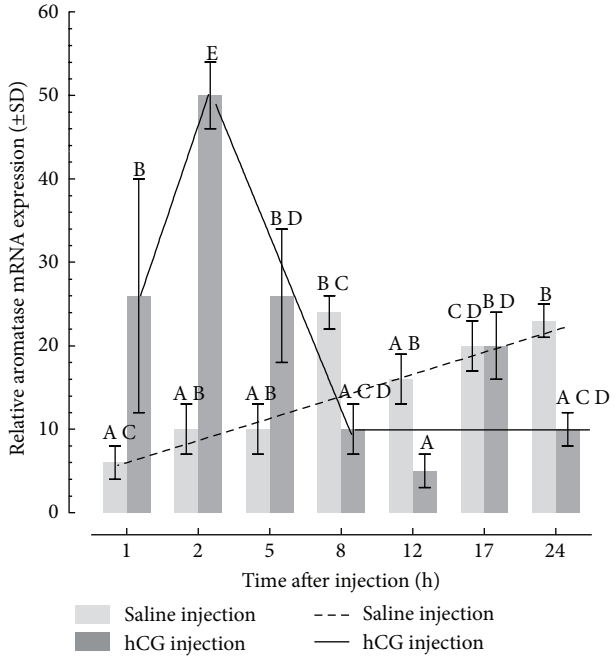

Figure 4: Kinetics of ovarian aromatase mRNA expression (means ± SD) after saline or hCG injection (0.1 μg/rat; $n = 4$) in 23-day-old immature rats. Uppercase letters indicate statistically significant differences between groups ($P < 0.05$).

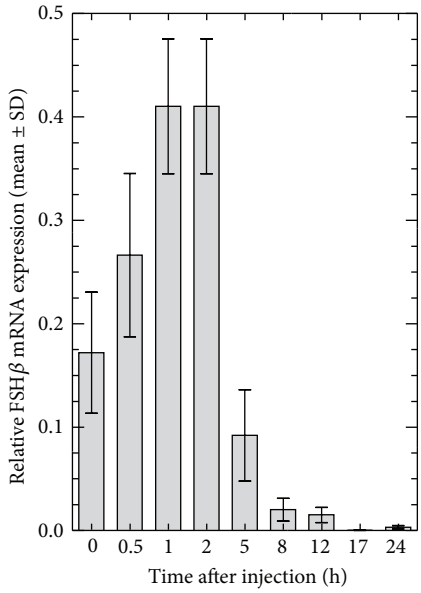

Figure 5: Kinetics of pituitary FSHβ mRNA expression (means ± SD) after single hCG injection (0.1 μg/rat; $n = 4$) in 23-day-old immature female rat.

injection. By contrast no significant modification was observed for FSH receptor mRNA and inhibin α, inhibin βA, inhibin and βB subunits mRNAs from the ovary.

3.4. Expression of Pituitary FSHβ and LHβ Genes after hCG Injection. Figure 5 shows that hCG exhibits a stimulatory effect on FSHβ expression during the first two hours after injection and promotes its total inhibition from 5 h on. In contrast, no modification was observed neither in the pituitary expression of LHβ gene nor of inhibin α, inhibin βA, and inhibin βB subunits genes (not shown).

4. Discussion

The LH activity of hCG has been known for decades to be required for the detection of FSH activity in gonadotropin preparations [2]. In a previous work concerning the mechanism of eCG and hCG stimulation of uterus weight gain in immature rats [1], we pointed out that hCG also exerted a strong uterotrophic effect even when injected alone, although it is devoid of any FSH activity (in contrast to eCG). We proposed a hypothesis in which hCG could exert this apparent *in vivo* FSH activity by (1) increasing FSHR expression in the ovary and (2) by indirectly stimulating FSH secretion by the pituitary. The data in the present study give some support to this hypothesis but mainly demonstrate that hCG activity on uterus growth is also mediated by the stimulation of ovarian aromatase gene transcription thus increasing estradiol production from hCG-stimulated testosterone.

The mechanism of action of hCG on uterus weight gain in immature rats is not easy to decipher as LH/CG receptors are present not only in the gonads as expected but also in the uterus itself [8, 9]. An additional difficulty comes from the fact that the uterus also expresses receptors for numerous ovarian mediators, particularly steroids [10].

The uterus growth stimulating activity of hCG alone was expected to be mediated through either its ovarian receptors and/or its uterine receptors. Our data clearly show that the estradiol antagonist ICI 182,780 totally inhibits hCG stimulation. This indicates that estradiol is indispensable in hCG-stimulated uterus growth. It is tempting to conclude that estradiol is produced by the ovaries after hCG injection since we showed in the present work that hCG injection led to enhanced expression of aromatase mRNA in the ovary (Figure 4). This effect of hCG on the ovary might be direct or indirect through stimulation of FSH secretion by the pituitary.

Follicle-stimulating hormone alone does not stimulate uterus growth but it exhibits a dose-dependent stimulatory effect in the presence of a low concentration of hCG. Since receptors for FSH have never been described in the uterus, FSH site of action must be its receptors in the ovary but the permissive effect of hCG could be through LH/CG receptors either in the ovary or in the uterus.

Our hypothesis [1] was that hCG could stimulate, directly or indirectly, the expression of ovarian FSH receptors. However, we observe no significant increase in the expression of the ovarian FSHR mRNA after hCG injection but, in contrast, we found that hCG significantly stimulated ovarian aromatase mRNA expression.

It has been previously reported that hCG stimulates androgens production by the rat theca [11–15] and androgens have been described to potentiate FSH action in the ovary [12, 16]. It is thus reasonable to make the assumption that hCG synergizes FSH-stimulated estradiol secretion by increasing

both aromatase activity as well as availability of its androgen substrate.

Aromatase activity might be enhanced either directly or through increase of its expression. Our data show that hCG injection to immature rats leads to an increase in ovarian aromatase mRNA expression (Figure 4).

A previous work [17] has shown that 40 IU FSH (~10 μg pure hFSH) exerted no stimulation of 17α-hydroxylase activity in immature hypophysectomized female rats whereas a dose-dependent increase of this activity was observed after injection of 0.1 to 10 IU hCG (~10 ng–1 μg). Therefore, it is clear that hCG but not FSH does stimulate ovarian 17α-hydroxylase activity which is present in theca cells but not in granulosa cells [15]. This is in agreement with the two cells-two gonadotropins hypothesis [12]; nevertheless, it does not explain the ovarian estradiol secretion under hCG stimulation alone.

The central point in our hypothesis [1] is that hCG injection would promote the secretion of an ovarian factor able to stimulate endogenous pituitary FSH secretion. Indeed we observed in the present work a transient stimulation of FSHβ mRNA expression (Figure 4) which is consistent with the hypothesis. Moreover, we observed a weak induction of ovarian INHβA and INHβB mRNA expression over the same period of time and therefore the stimulatory factor of FSHβ expression could possibly be activin secreted by the ovary.

There is a peak of serum FSH in untreated rat between postnatal days 10 and 20 and then a drop back to baseline values [18, 19]. At day 25, FSHR mRNA is localized exclusively in granulosa cells whereas LHR mRNA is only present in theca and interstitium and both mRNA expressions were found to be increased after eCG priming [20]. In the present work, we could not detect any change in FSHR mRNA in the ovary during the 48 h after hCG injection (not shown). It is thus the stimulation of aromatase expression by FSH plus the increase stimulated by hCG in its androgenic substrates from theca cells that leads to increased estradiol secretion.

FSH binding sites are already present in the granulosa cells of some antral follicles of rats at day 21 [21], whereas hCG binding sites (LHR) are mostly found in theca and interstitium cells and to a much smaller proportion in granulosa cells as well as in the uterus and pituitary [22]. It is thus most likely that the uterotrophic activity of hCG is primarily initiated through stimulation of theca cells but additional direct effects at the levels of uterus itself, granulosa cells, and pituitary cannot be excluded.

The gonadotropin-primed immature rat has become the most common model for the study of ovarian follicular development [18, 23, 24]. This method makes use of an initial injection of eCG to 23-day-old female rats followed by an injection of hCG 48 h later. It is generally accepted that eCG acts through its FSH activity allowing follicular development and that hCG can then stimulate ovulation. However, the observation that hCG that only expresses LH activity exhibits similar uterus stimulating activity does not fit with this view. It must also be kept in mind that eCG exhibits a 5-fold higher *in vitro* LH activity compared to its FSH activity in rats as well as in other nonequine species [25–27]. It thus could be that eCG acts in a similar way as hCG, that is, essentially through its LH activity.

In a previous report [1], we showed that hCG but not oLH or pLH could stimulate uterus weight growth in immature rats. We attributed the difference between all these purely LH-specific hormones to the much longer half-life of hCG compared to the pituitary hormones. But it has come to our attention that LHR can partially be alternately spliced and miss exon 10 [28–33]. The LHR missing the sequence encoded by exon 10 appears to be responsive to hCG but no more to LH [29]. One could raise the hypothesis that this truncated LHR is the major form expressed in immature female rats.

5. Conclusions

In brief, the present work reinforces our hypothesis that hCG alone stimulates uterus ponderal growth in premature rats by stimulating ovarian estradiol synthesis mainly through increased aromatase expression and testosterone availability. It also stimulates ovarian activin subunits mRNA expression and pituitary FSHβ mRNA expression but it remains to be established (1) that ovarian activin is indeed secreted and responsible for the observed increase in pituitary FSHβ mRNA expression and (2) that FSH is indeed secreted and responsible for uterus weight gain.

Disclosure

The present address for Sandrine Rafert is CHU Poitiers (France).

Conflict of Interests

The authors declare no conflict of interests regarding the publication of this paper.

Acknowledgments

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