Failure of hCG/LH receptors to stimulate the transmembrane effector adenylyl cyclase in human endometrium

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ABSTRACT

The functional significance of the endometrial hCG/LH receptors has been related to a rapid release of prostaglandins. However, as compared to gonads and myometrium, in-endometrium mechanisms of transmembrane signalling of the hCG/LH receptors are probably not conventional and remain unclear. Here we investigated, in vivo, the potential of hCG to interact with, and stimulate the membrane effector enzyme, adenylyl cyclase (AC), in human endometrium. Hormonal and nonhormonal activation of AC was tested in membrane fractions prepared from endometrial biopsies obtained from patients undergoing evaluation cycles for hormone replacement therapy (HRT) and controlled ovarian hyperstimulation (COH). AC activity was determined by the direct conversion of the substrate ATP into cAMP under unstimulated conditions and in the presence of the non-hormonal activators guanyl nucleotide and forskolin. Also AC activity was tested in the presence of hCG under conditions allowing maximal enzyme stimulation. Isoproterenol and prostaglandin E2 (PGE2) were included for comparison. Immunoblot analyses demonstrated the presence of hCG/LH receptors and Gsα protein and other members of the G protein family in the membrane fractions. Endometrial membranes also exhibited high levels of AC activity compared to luteal membranes used as control. Stimulation by GMP-P(NH)P alone was 196 ± 63 ($n = 8$) (pmol/mg/min $\pm$ SD). Neither hCG nor isoproterenol showed stimulation of endometrial AC (210 ± 65, and 197 ± 53, respectively; $n = 66$ assays). But PGE2 stimulated the enzyme system significantly (264 ± 63, $p < 0.05$; $n = 66$ assays). These data show that membrane fractions from human endometrium express all the AC system components, namely, hCG/LH receptors, Gsα protein and AC; however, hCG does not stimulate the endometrial AC system. Our data indicate that, in great contrast to gonadal receptors, endometrial hCG/LH receptors are not coupled to the transmembrane AC effector. The well known release of eicosanoids in response to hCG suggests that these receptors are functional in human endometrium but throughout a signalling system different from AC. This enzyme is certainly coupled to and directly activated by eicosanoids and other embryonic signals.

Keywords: hCG/LH Receptors; Human Endometrium; Membrane Signal Transduction; Adenylyl Cyclase

1. INTRODUCTION

Following our original studies on hCG/LH receptors, G proteins and adenyl cyclase enzyme characterization in human endometrium [1,2], a number of clinical and molecular biology studies have increasingly been published on this topic. Detection of non-gonadal hCG/LH receptors has been reported in many reproductive and non-reproductive human tissues including uterus, fallopian tubes, placenta, lymphocytes of pregnant women, adrenal, mammary, prostate glands, and even skin and brain [3-7]. Current information is now available to candidate the hCG binding hormone and its cognate receptor to be, at least in primates and humans, the main biochemical markers and key regulators of the entire embryo-maternal dialogue from the start until the end of the pregnancy [8-10]. Accordingly, there is general evidence that hCG regulates the endometrial blood flow through vasoactive molecules (PGE2 and VEGF) and controls in combina-

tion with other embryonic signals (interleukin 1) the decidual transformation of the stroma and the invasion process [11-13]. Despite this, the functions of these extragonadal hCG/LH receptors, under normal and physiological conditions have yet to be established. In particular, how these receptors generate transmembrane signalling and whether these mechanisms are similar to those described for gonadal hCG/LH receptors are controversial. In gonadal tissues, it is well known that the actions of hCG and LH are initiated by a specific binding to the membrane-bound hCG/LH receptors, followed by coupling of the receptors to GTP-binding stimulatory (Gsα) protein and activation of the adenylyl cyclase (AC), the transmembrane effector enzyme responsible for the intracellular synthesis of cyclic AMP (cAMP) [14]. In the uterus the presence of hCG/LH receptors has been shown in all parts including myometrium, large, small blood vessels and endometrium. During pregnancy they are active and favour vasodilatation and uterine tone relaxation by coupling to probably different signal transduction pathways associated with prostaglandins and cAMP production, respectively [15,16]. Immunohystochemical studies show that endothelial cells contain more receptors than vascular smooth muscle and both cell types in vessels of smaller diameter contain more hCG/LH receptors than larger vessels [6,15]. Whether these receptors could behave differently according to the type of specific cellular tissue where they are expressed it is, at present, unknown. Similar considerations hold true for endometrium: where and to which exact signal transduction the hCG/LH receptors are coupled, are the subject of controversy. Available information basically relies on *in vitro* studies of human endometrial cells or *in vivo* studies on the baboon uterus [17-20]. This notwithstanding, all the investigators agree that whenever the endometrium is exposed to hCG a consistent and constant activation of the COX2 enzyme is derived along with an increased eicosanoids biosynthesis [17,21-23]. Up to date, there are no *in vivo* data in human endometrium to support that the hCG/LH receptors are or not coupled to the adenylyl cyclase effector system. We report here never published data from a study designed to gain new insights on the physiological mechanisms involving hCG/LH receptors in human endometrial tissue. Specifically we investigated the transmembrane receptor-adenylyl cyclase system in membrane fractions obtained from human endometrium and evaluated the capacity of hCG/LH receptors to interact with and directly stimulate the enzyme system. We will comment in the discussion why we believe these data are of interest and capable to explain missing points of the complex transduction signals initiated by the embryonic hCG at the time of implantation. These data also allow a reasonable interpretation of the premature stromal maturation of endometrium apparently associated with reduced implantation rates

during cycles of controlled ovarian stimulation when an exogenous injection of hCG is administered.

2. MATERIAL AND METHODS

2.1. Animals

Luteal cells membrane fractions were harvested from ovaries of adult female rats maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Collection of rat corpora lutea was performed after inducing superovulation [24]. The protocol allows to achieving a rather homogenous population of corpora lutea within the ovary.

2.2. Ethics of Human Tissues Collection

Endometrial specimens (n = 22) were performed by means of biopsy from patients (n = 6) undergoing evaluation cycles for hormone replacement (HR) therapy (mean patients age ± SD: 41.4 ± 1.6) and from women (n = 4) undergoing controlled ovarian hyperstymulation (COH) cycles for oocyte-donation (mean patients age ± SD: 26.6 ± 0.5). From each patient, 2 biopsies were obtained in subsequent dates of the same cycle. For hormone replacement therapy cycles this occurred on the last day of oestrogen administration (day 0) and on day plus 3 (n = 2) or 6 (n = 2) or 9 (n = 2) of progesterone (P) supplementation. For controlled ovarian hyperstymulation cycles this occurred on the day of transvaginal oocytes aspiration (day 2) and 3 (n = 1) or 5 (n = 1) or 8 (n = 2) days later (days 5, 7 and 10 respectively). A part of each tissue sample obtained was reserved for histological examination and dated according to Noyes *et al*. (1950) [25]. Hormonal replacement therapy was achieved by sequential and combined administration of oestradiol and progesterone. 17-β oestradiol was given per os at increasing dosage (2 - 6 mg) for a period of 14.3 ± 0.5 days. At this time, concomitant administration of natural progesterone (100 mg i.m., daily) was started. During evaluation cycles, oestradiol and progesterone serum levels were measured by immunoassay on cycle day 20 (day plus 6 of progesterone supplementation) and resulted 367 ± 124 pg/ml and 22 ± 5.0 ng/ml, respectively. LH serum levels were measured by an immunoradiometric assay in patients undergoing evaluation cycles of hormone replacement therapy. These patients were expected to present different levels of circulating LH (3 patients were down-regulated with GnRH to suppress the interference of any gonadal function while other 3 were premature ovarian failure patients). Controlled ovarian hyperstimulation was achieved by a combination protocol of LHRH-analogue plus FSH-HMG as previously published [26].

In experiments concerning measurement of adenylyl cyclase activity, corpora lutea obtained from ovaries of 4 women undergoing exploratory laparotomies for benign

gynaecological conditions were used as controls.

2.3. Membrane Preparation and Detection of hCG/LH Receptors and G Proteins

Preparation of membrane fractions for determination of receptor-adenylyl cyclase system was carried out according to procedures previously published in our laboratory [27]. Briefly, endometrial tissue was flushed with phosphate-buffered saline (PBS) and weighed. The tissue was minced and homogenised in a small Dounce homogenizer and homogenate centrifuged at $10,000 \times g$ for 45 min, at 4˚C. Resulting pellet was then resuspended in 5 parts of 27% (vol/wt) sucrose in 1 mM EDTA and 10 mM Tris-HCL, pH 7.5.

hCG/LH-receptors and GTP-binding proteins [(Gsα, Gi1, 2α, Gi3α and common β sub-unit)] were determinated by immunoblot analysis as reported previously [1]. Briefly, protein content in the homogenates was determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL). Proteins were precipitated by adding 2.5 volumes of acetone, incubated for 1 hour at −80˚C in a freezer, and then centrifuged at $70,000 \times g$ for 20 minutes at 4˚C. The supernatant was then removed, and the pellet resuspended in sample buffer. Same procedure was used to prepare control tissue (rat corpus luteum). After extracting proteins in presence of proteinase inhibitors, the proteins were separated on discontinuous sodium dodecyl sulphate-polyacrilamide gel electrophoresis (12%) under reducing conditions. Same experiments were performed under no reducing conditions (7.5% gel). Different types of molecular weight markers were used depending on the experiment (this is specified in the figure legend). The migrated proteins were then transferred to Immobilon-P transfer membranes (Millipore Corp., Bedford, MA) using a transfer unit. After blocking the membranes for 1 hour with 2% non-fat dry milk Tris-buffered saline, we incubated the blots overnight at 4˚C with different dilutions of polyclonal first antibodies. Receptors were detected by 1:10,000 dilution of polyclonal antibodies to synthetic fragments of rat luteal hCG/LH receptors [anti-PCR II-(1-11) and anti-LHR-(15-38)], generously provided by Dr. P. Roche (Mayo Clinic, Rochester, MN). The G proteins were detected by 1:5000 dilution of polyclonal antibodies to fragments of rat Gsα, Gi1/2α, Gi3α and common β (DuPont, Boston, MA). The blots were washed and treated with anti-rabbit immunoglobulin G horseradish peroxidase conjugate. Rat and human corpus luteum membranes were used as positive controls. The molecular sizes of proteins were determined by running standard marker proteins in an adjacent lane. An enhanced chemiluminescence detection system (ECL kit; Amersham, Arlington Heights, IL) was used to detect bands. Autoradiographs were then scanned with a gel digitiser (Ultraviolet Products, Cambridge, England) to obtain a semiquantitative evaluation of proteins levels.

2.4. Adenylyl Cyclase Assay

Optimal experimental conditions to determine expression of membrane-bound adenylyl cyclase and coupling of the enzyme system to hormonal receptors have been described elsewhere [28,29]. Quantitative determination of adenylyl cyclase activity was performed by the direct conversion of the substrate ATP into the reaction product, cAMP. Briefly, aliquots (10 μl) of membrane particle preparation (10 μg protein) were assayed in duplicate for adenylyl cyclase activity. The final volume of 50 μl contained: [α-32]ATP (150 cpm/pmol), 0.5 mM ATP, 5.0 mM MgCl$_2$, 1.0 mM EDTA, 1.0 mM [3H]cAMP (15,000 cpm), 25 mM Tris-HCL, pH 7.5, and a nucleoside triphosphate-regenerating system consisting of 20 mM creatine phosphate, 0.2 mg/ml creatine kinase and 0.02 mg/ml myokinase. Incubations were performed at 32˚C for 10 min. Termination of adenylyl cyclase activity was accomplished by addition of 100 μl of a "stop" solution that contained 40 mM ATP, 10 mM cAMP and 1% sodium dodecyl sulfate. Formed [α-32P]cAMP and [3H]cAMP (used as recovery marker) were isolated and separated from [α-32P]ATP by double chromatography using Dowex 50 columns and alumina columns according to a procedure previously described [28] and were collected in scintillation fluid for beta counting. Results were expressed in picomoles of cAMP generated/mg protein/minute. Adenylyl cyclase activities detected in membranes prepared from corpora lutea were used as internal controls in all assays performed.

2.5. Statistics and Other Procedures

To minimise contamination with guanine nucleotide-like endogenous compounds that can result in high basal AC activities, the components of the nucleoside triphosphate-regenerating system were subjected to purification as previously reported [29]. The results are presented as the mean $\pm$ SD of two independent experiments using separated batches of membranes, each carried out in triplicate. Statistical significance between experimental groups was evaluated using Student's t test.

3. RESULTS

3.1. Presence of hCG/LH Receptors and G Proteins in Human Endometrium

Immunoblot analysis demonstrated the presence of hCG/LH receptors and the GTP-binding stimulatory (Gs) protein in the endometrial membranes obtained from both hormone replacement cycles and stimulated cycles. The receptor antibody immunoreacted with a major protein migrating at about 68 kDa (**Figure 1**). The band-inten-

sity of the hCG/LH receptor blots as determined by densitometric scanning did not change with the histologic date of the endometrium. Membrane fractions of human and rat CL expressed a similar band of about 68 kDa, although other bands migrating at about 100 and 80 kDa were also present (**Figure 2**).

Expression of endometrial Gsα protein was detected in bands migrating at 48 and 42 kDa (**Figure 3**). Other proteins of the G family were also present, including Gi3α proteins which migrated at 40 kDa (**Figure 4**) and common β subunits with a molecular weight of 37 kDa (data not shown). Expression of hCG receptor and Gs proteins was maintained during cycles of ovarian hyperstimulation (**Figure 3**). In particular, endometrial expression of Gsα protein was found in early, mid- and late luteal phase cycle-samples although a strong variety in individual band-intensity was noted (**Figure 3**). Having established the presence of hCG/LH receptors and GTP-
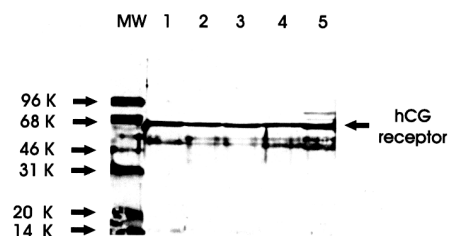

Figure 3. Controlled ovarian hyperstymulation cycles. Immunoblotting of human endometrium using polyclonal antibodies against Gsα protein. Lanes 1, 3, 5 and 6 represent human endometrium (50 μg) from 4 individuals (2 days after the hCG injection), lanes 2 and 4 represent human endometrium (50 μg) from other 2 individuals (9 days after the hCG injection) and lane 7 represents rat CL (50 μg). Western blot protocol was the same as for **Figure 1**.

Figure 1. Hormone replacement therapy cycles. Immunoblotting of human endometrium (30 μg) using polyclonal antibodies against hCG/LH receptors. Human endometrium exposed to oestradiol only (lanes 1 and 3) oestradiol plus day-6 (lanes 2 and 4) of progesterone supplementation. Lane 5 represents rat corpus luteum. A 12% gel under reducing conditions and Amersham ECL MW markers were used.
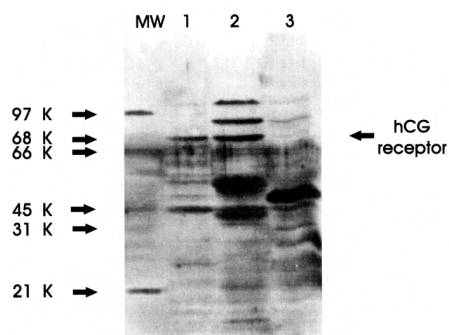

Figure 4. Expression and modulation by hormone replacement therapy of Gi3α protein in human endometrium. Immunoblotting results of six individual samples derived from 3 separate patients (lanes 1 and 2; lanes 3 and 4; lanes 5 and 6, respectively) undergoing hormone replacement therapy are presented. Immunoblotting protocol was the same as for **Figure 1**: 30 μg of human endoemtrial proteins were loaded onto the gel and polyclonal antibodies against Gi3α applied. Residual signs of previous experiments with repeated sequential stripping and probing using different antibodies are present. Human endometrium was exposed to oestradiol only (lanes 1 and 3) or oestradiol plus day 6 (lanes 2 and 4) of progesterone supplementation. Lane 7 represents rat corpus luteum.

Figure 2. Immunoblotting of human endometrium (pool of 5 individual samples from secretive phase) (lane 1), human corpus luteum (lane 2) and rat brain membranes (lane 3) using polyclonal antibodies against hCG/LH receptor. A 7.5% gel under not reducing conditions and Bio-rad Biotinylated MW markers were used.
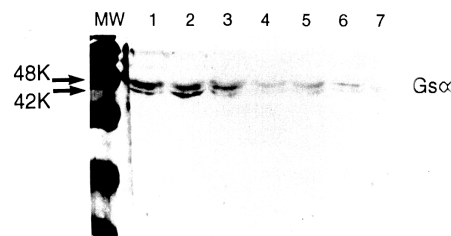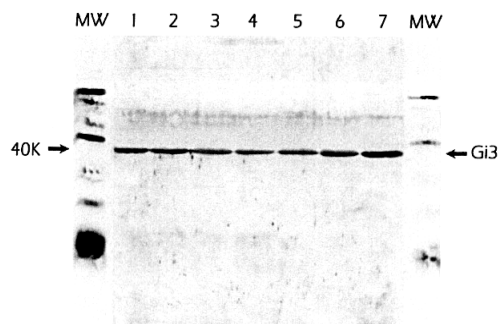
binding proteins, we then proceeded to evaluate the biological activity of adenylyl cyclase system in human endometrium.

3.2. Expression of AC Activity in Human Endometrium

AC expression was determined in endometrial membrane preparations derived from both hormone replacement therapy and controlled ovarian hyperstymulation cycles.

The enzyme activity was measured in the absence or presence of saturating concentrations of the enzyme activators GMP-P[NH]P (a hydrolysis resistant form of the guanine nucleotide, GTP), and the diterpene, forskolin (Fk). GMP-P(NH)P activates the Gs protein while forskolin directly activates the AC catalytic component. Membranes from human endometrium expressed high levels in AC activity (**Figure 5**). Compared to membranes prepared from corpora lutea used as internal controls, adenylyl cyclase activity in endometrial membrane fractions were 5 - 10 times higher (**Figure 5**). Sensitivity of endometrial AC to GMP-P(NH)P and forskolin indicated that human endometrium contain Gsα protein and the catalytic component of the effector system.

3.3. AC Responsiveness to hCG in Human Endometrium

Studies to evaluate AC sensitivity to hCG were carried out under conditions allowing maximal enzyme activation. Because the capacity of AC to respond to hormonal stimulation is dependent on guanine nucleotide [24,29], a saturating (100 μM) concentration of the hydrolysis-resistant guanine nucleotide, GMP-P(NH)P, was present in all the experiments. Also, a saturating (10 μg/ml) concentration of hCG was used to ensure maximal hormonal response. Stimulation by prostaglandin E2 (PGE2) and isoprotenerol were included as reference [29]. Initial experiments (n = 8) using endometrial samples obtained from controlled ovarian hyperstymulation cycles showed that hCG was not able to stimulate the adenylyl cyclase system. As for hCG, the catecholamine, isoproterenol, was also unable to stimulate endometrial adenylyl cyclase (**Figure 6**). In contrast, PGE2 significantly stimulated AC in human endometrium. A combination of all the data expressed as percentage of activation by guanine nucleotide alone is summarized in **Figure 6**. Notably, we found that the pattern of these results was the same, irespective of the steroid treatment (*i.e.*, stimulated or artificial cycles), or the phase of menstrual cycle (*i.e.*, proliferative or secretive) (data not shown). In control experiments performed with luteal membranes, hCG effectively stimulated adenylyl cyclase activity (**Figure 7**). Data from controls indicate that under the experimental conditions, hCG binds to the receptors and promotes activation of AC through Gs protein in a normally responsive AC system.

4. DISCUSSION

In this study we demonstrated the expression of hCG/LH receptors and G proteins in membrane fractions from human endometrium and measured the AC responsive ness to hCG. Using conditions that allow full AC expres-
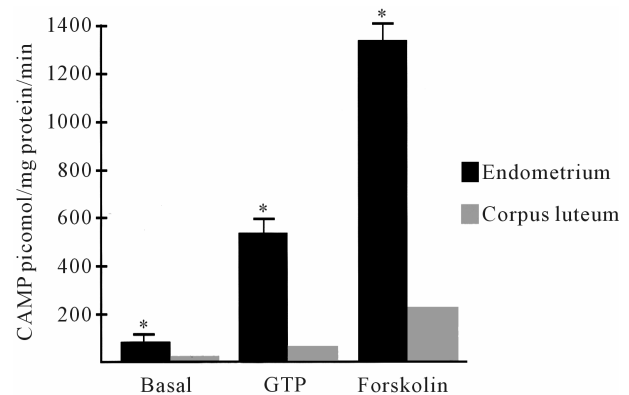

Figure 5. Comparison of adenylyl cyclase activity between human endometrium and human corpus luteum. Adenylyl cyclase activity was determined under basal conditions (no additions) and in the presence of either 100 μM GMP-P(NH)P or 100 μM Forskolin. *p < 0.05 compared to human luteal adenylyl cyclase activity.
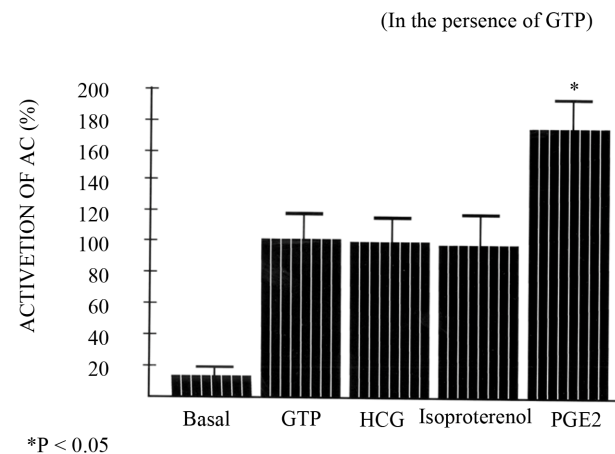

Figure 6. Responsiveness of endometrial adenylyl cyclase to 3 different hormones (hCG, isoproterenol, prostaglandin E2). Hormonal stimulation was carried out in the presence of guanyl nucleotide (100 μM) to ensure maximal enzyme responsiveness. Concentrations of hCG and PGE2 were both 10 μg/ml and that of isoproterenol was 100 μM. *p < 0.05 compared to stimulation with guanine nucleotide alone.

sion, we found that hCG does not stimulate the enzyme system. Our data indicates that hCG/LH receptors present in human endometrium, in great contrast to those described in gonadal tissue, are not coupled to the transmembrane effector adenylyl cyclase. The findings do not support the concept that hCG acts on human endometrium by a mechanism utilizing cAMP as second messenger [17-20]. It should be noted, nevertheless, that previous studies were performed on cell cultures and the proposed role of cAMP as second messenger was based on the determination of total cell production of cAMP. Herein we used membranes prepared from collected tissue, a model that provides more accurate information on the status of receptor-AC system *in vivo*. Moreover, we
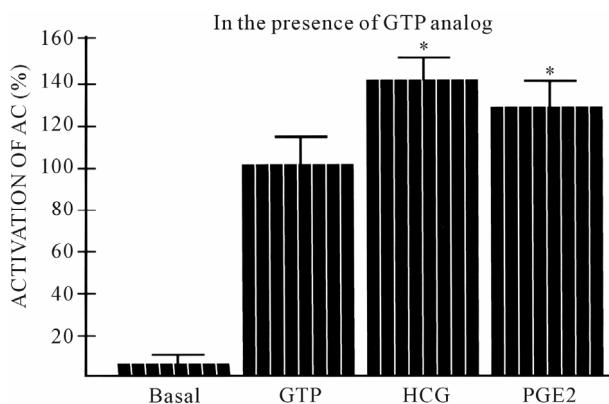
Figure 7. Responsiveness of rat corpus luteum adenylyl cyclase to hCG and prostaglandin E2 hormones. Hormonal stimulation was carried out in the presence of guanyl nucleotide (100 μM) to ensure maximal enzyme responsiveness. Concentrations of hCG and PGE2 were both 10 μg/ml and that of isoproterenol was 100 μM. *p < 0.05 compared to stimulation with guanine nucleotide alone.

determined AC activation by monitoring the direct conversion of the substrate ATP into the reaction product, cAMP. The approach is significantly different from the widely used method based on measurement of total accumulation of cAMP. In the latter, it is not possible to define whether increases in cellular cAMP are due to an enhanced cAMP synthesis by AC, a reduced cAMP degradation by the phosphodiesterase enzyme, or both. Our data, therefore, express true AC specific activity in membranes from human endometrium. By using internal controls (PGE2 and isoproterenol and luteal membranes) we have been able to demonstrate that lack of activation by hCG was not due to methodological artifacts but a property specifically related to the constitution of human endometrial hCG receptors. On the other side we must recognize that the use of a single hCG concentration is not an optimal situation. We were greatly limited in this study by the amount of tissue available. Human endometrial tissue is difficult to obtain; we just used the small amounts left following the clinical biopsies. Also, our experimental protocol required the homogenization of endometrial tissue and preparation of a cell membrane fraction which further reduced the material available for experimentation. It was thus not possible to conduct more elaborated studies including a wide range of different hormone concentrations at each data point. However, as mentioned above, we clarified that for studies regarding hCG stimulation a concentration of 10 μg/ml was shown to be saturating and to provide maximally stimulated hormone activation in a number of tissues [27, 29].

We also found that the AC system in endometrial membranes does not respond to the catecholamine, isoproterenol, but is responsive to PGE2. The findings agree

with previous studies performed on membrane fractions from human decidua parietalis demonstrating that AC is not sensitive to epinephrine, LH (10 μg/ml) nor hCG (25 IU/ml), while it responds to PGE2 [30]. Similar observations have been also reported for AC of bovine endometrium [31]. Taken together, the various lines of evidence strongly suggest that endometrial hCG/LH receptors are, at least under *in vivo* experimental conditions, not functional throughout the classic transmembrane AC system. Our results support the observation made *in vitro* on human endometrial epithelial cell lines that CG hormone activates a Gs/Gi/EGFR-independent and PI3-Akt-dependent Raf-ERK-Elk pathway, leading to mPTGES production [32]. The presence, as shown in our study, of common β and Gi proteins (known to be coupled to the Raf-ERK-Elk-MAPK pathway) is of note. Why the hCG/LH receptors couple to AC in gonads and myometrium and they don't in endometrium, it is unknown. Probably this system could be instrumental to avoid local receptor down regulation due to the large amount of binding hormone secreted by the embryo at the implantation site [32]. Perhaps the rapid production of prostaglandins produced by this unique system is responsible for the stimulation of the abundant AC present in human endometrium. The cAMP, which is the most important second cellular messenger regulating phosphorylation and gene transcription of most cellular enzymes and cytokines, is in turn the key candidate to promote the necessary changes for endometrial stromal maturation and decidua formation [33]. The high sensitivity to eicosanoids and abundance of adenyl cyclase in human endometrium both allow to explain in part the conflicting results obtained in experimental studies where increased cAMP production was obtained following in vitro incubation of endometrial cell with hCG [17-19].

In our study the pattern of hCG/LH receptors in the endometrial tissues as determined by immunoblot analysis, was not altered during the menstrual cycle, nor by the administration of a very high (10,000 IU) dose of hCG used to induce ovulation. This disagrees with immunocytochemical studies showing a differential expression of these receptor during the menstrual cycle and in response to exogenous administration of hCG [6,34,35]. This could be partially explained by the use of different polyclonal antibodies against the receptors and also by the type of studies (most immunohystochemical signals were noted in subnuclear or internal regions of the cells rather than in cell membranes) [34,35]. In addition, our immunoblot analysis indicated significant differences between receptors present in corpus luteum and endometrium. Thus, while endometrial hCG/LH receptors showed a single band of lower molecular weight (68 kDa), human and rat luteal hCG/LH receptors showed multiple subunits of higher molecular weight. It is likely that the differences reveal an abnormal expression of

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endometrial hCG/LH receptors. Studies from our laboratory as well as from others indicate that the endometrial hCG/LH receptors may represent an incomplete, non-mature form of receptor protein derived from an abnormally truncated mRNA transcript. Accordingly, we have found that compared to gonadal receptors, the endometrial hCG/LH receptor genes express a normal transmembrane region, but an abnormal extracellular region [36]. Similar observations have been reported by Stewart *et al.* [37] who detected presence of mRNA for the highly conserved transmembrane portion of the hCG receptor but not for the extracellular ligand-binding portion that recognizes and specifically binds hCG/LH. These forms of receptors may be due to a difference in the alternative splicing process during gene expression. Inefficient LHR maturation is a natural condition in rat tissues and there is a clear age-, tissue-, and sex-dependent variation in the relative amount of mature receptors to immature ones [38].

At the current time we know that the interaction between hCG ligand and hCG/LH receptor undergo an high degree of variability due to the presence, in nature, of multiple variants of hCG molecule having different glycosylation [10]. Therefore, in experimental studies, the use of purified or recombinant hCG may impact on the results [10,39]. The issue is even more complicated by the natural presence of receptors at different status of maturity (splice variants) [38,40] and mechanisms used by these to transfer signals into the cell. Most G protein-coupled receptors can couple to several G proteins within a single cell and several cells express more than one subtype of a particular receptor each potentially able to stimulate different G proteins. It is well established that common $\beta\gamma$ subunits of G proteins play a major role in mediating signal transmission to Ras and ERK/MAPK cascades but quantitative regulation of this pathway can also proceed through cooperation of $G\alpha_{q/11}$ and $G\alpha_i$ signals [41]. Cooperative signaling by multiple G proteins might represent a novel concept implicated in the regulation of different cellular responses induced by G protein-coupled-receptors [41]. Conflicting literature data can be explained by this and other considerations including whether studies are *in vivo* or *in vitro*, type and source of cells (epithelial, stromal, cloned cancer cell lines, monocytes) [7,42], additions to culture media (insulin vs steroids), timing and way of the end product measurement (*i.e.*, cAMP) [43], and also primers set used for PCR or polyclonal antibodies used for western blotting or immunohistochemistry studies.

5. CONCLUSION

In conclusion we believe that hCG, the main embryonic signal of embryo implantation, binds to quite special endometrial receptors frequently undergoing natural post-

trascription (splice variants) and post-translation changes [40,44]. These receptors respond to hCG with prostaglandins release by means of an uncommon signal transduction pathway. Prostaglandins are certainly coupled to AC in cell membranes of endometrium (glandular and stromal cells). The endometrium is particularly rich in AC and besides eicosanaoids this enzyme is ready to be activated and directly coupled to receptors for also other embryonic signals (*i.e.*, interleukin 1) [13]. In any case, this allows intracellular accumulation of enormous amounts of cAMP which is the internal messenger responsible for the activation of all enzymes and cytokines necessary to promote the condition of adequate endometrial receptivity (initiation of neo-angiogenesis and decidua process). Our opinion agrees with the notion that early in the luteal phase a complex molecular machinery is present in human endometrium ready to over-respond to initial amounts hCG secreted by the embryo [45]. On this regard other investigators have demonstrated by immuno-histochemical studies a change in hCG/LH receptors expression during the menstrual cycle similar to that reported by us for $Gs\alpha$ and AC during the very first days of progesterone supplementation using HRT cycles [2]. This short time window of endometrial receptivity-potential disappears during the course of luteal phase and it is probably anticipated and disrupted whenever external stimulation of the receptors or enzymes is altered by high dosages of exogenous hCG injection (as it is done during cycles of COH). This implies that during COH gonadotropin hormones may induce a release of endometrial cAMP with a subsequent acceleration of the process of stromal maturation and impairment of uterine receptivity. We have previously reported modification of AC function associated with precise histological dating of endometrium [2] and recent clinical and molecular studies support this contention [34,46-49].

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