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Direct actions of ACTH on ovarian function of pseudopregnant rabbits

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Abstract

The present study sought to assess whether the receptors for adrenocorticotrophic hormone (ACTH), MC2R, and for glucocorticoid (GR) are expressed in corpora lutea (CL) of pseudopregnant rabbits and whether ACTH and cortisol exert any direct action on luteal function. By immunohistochemistry, positive reaction for MC2R and GR was detectable within luteal cells of CL. The MC2R mRNA levels were five-fold less abundant in day 9 than in day 4 CL ($P<0.01$). At both stages, ACTH agonist (ACTH 1-24) increased progesterone and prostaglandin (PG) E_2 (PGE_2) ($P<0.01$), but reduced $PGF_{2\alpha}$ releases ($P<0.01$) *in vitro*. ACTH 1-24 injection increased plasma cortisol levels within 4h ($P<0.01$), but decreased ($P<0.01$) progesterone 24h later and for the following two days. ACTH administration to estrous rabbits caused a transitory increase in blood progesterone concentrations ($P<0.01$). Daily injections of ACTH did not modify progesterone profile following ovulation. In conclusion, ACTH directly up-regulates CL progesterone production *in vitro* via MC2R, but indirectly hampers luteal function via cortisol-GR associated mechanism.

1. Introduction

The long-held assumption that stress can impair reproduction in females of any species (Ferin M, 1998) based on empiric knowledge, only recently has been formalized on a scientific basis with the identification of a number of neuroendocrine signals within the hypothalamic-pituitary-adrenal system (HPA) that can disrupt the endocrine events controlling the estrous cycle and ovulation. The stress response is activated by corticotropin-releasing hormone (CRH) produced by neurons mainly localized in the hypothalamic paraventricular nucleus. These neurons project the median eminence to release CRH into the hypophyseal portal system for transport to the pituitary, where it stimulates the release of adrenocorticotrophic hormone (ACTH), which, in turn, stimulates glucocorticoid secretion from the adrenal glands. Thus, following activation of the HPA axis, CRH, ACTH, and glucocorticoid may cause infertility and modify sexual behavior by targeting, at different levels of the hypothalamic-pituitary-gonadotropic axis (HPG), both local hypothalamic pre-motor and/or gonadotropin-releasing hormone (GnRH) neurons responsible for GnRH tonic and pulse release (Pau et al., 1986) as well as gonadotropin release from the pituitary and sex steroid production from ovarian follicles and corpora lutea (CL). The question of how stress impacts negatively on the reproductive processes has relevant consequences given that, under current intensive livestock management, animals are repeatedly exposed to a wide range of stressful stimuli.

Although a variety of experimental manipulations have provided potential mediators for the stress-induced suppression of reproduction, the precise mechanisms by which hormones released during stress may inhibit reproductive processes have yet to be defined. Similarly, the potential role of ACTH in the direct regulation of ovarian function is poorly understood. Certainly, the effects of ACTH are mediated through the activation of the melanocortin receptor (MCR) type 2 (MC2R), a seven-transmembrane domains receptors coupled to G proteins belonging to the MCR family (Schioth 2001), which has five members identified to date (Chhajlani 1992; Chhajlani et al., 1993; Gantz et al., 1993; Mountjoy et al., 1992; Roselli-Reh fuss et al., 1993). Several studies have shown that ACTH action is mediated not only by cyclic adenosine monophosphate (cAMP), but also by calcium (Ca^{2+}), both interacting closely through positive feedback loops to enhance steroid secretion (Gallo-Payet and Payet 2003). In addition to the adrenal gland, MCR2R was also detected in the ovary, in the testis and in several other organs, including lung, brain and spinal cord of mouse embryos with specific temporal expression patterns (Nimura et al., 2006).

The rabbit is an ideal animal for studying luteal physiology because, compared to other animal models, its luteal stage can be precisely timed, given that ovulation is a neuroendocrine reflex that can be consistently induced by exogenous GnRH or human Chorionic Gonadotropin (hCG) administration (Mehaisen et al., 2005). On these premises, the main objectives of the present work focused on further improving our understanding of stress-linked mechanisms involved in the control of reproduction by assessing whether the receptor for ACTH, that is, the MC2R, and that for glucocorticoid (GR) are expressed in CL of pseudopregnant rabbits and whether ACTH exerts any direct action on luteal function.

2. Materials and Methods

2.1. Reagents

The following hormonal preparations were administered via i.m. injection: GnRH analogue (Receptal, Hoechst-Roussel Vet, Milan, Italy), equine Chorionic Gonadotropin (eCG, Folligon, Intervet, Milan, Italy), and synthetic ACTH 1-24 (Synacthen-depot, Novartis Pharmaceuticals Australia, North Ryde, Australia).

Tritiated hormones and [2,3-³H]L-arginine, having a specific activity of 30-40 Ci/mmol, were purchased from Amersham Biosciences (Amersham Biosciences Ltd, Little Chalfont, Bucks, UK), while progesterone, PGE₂, and PGF_{2α} antisera, and non-radioactive hormones came from Sigma (St Louis, MO, USA). The CORT kit for RIA of blood cortisol was bought from ICN Biomedicals Inc., Costa Mesa, CA, USA. The NOS detect™ Assay Kit was purchased from Alexis Corp. (Läufelfingen, Switzerland). The kit for the protein assay was purchased from Bio-Rad Laboratories (Segrate, Milano, Italy). Incubation wells were obtained from Becton Dickinson Co. (Clifton, NJ, USA), 96-well PCR plates from Bio-Rad Labs, medium 199 and Earles Balanced Salt Solution were from GIBCO (Grand Island, NY, USA). ACTH (1-24), protein kinase A (PKA) antagonist (H-89) AC inhibitor (2-O-methyladenosine), Ca²⁺ channel blocker (verapamil), HEPES, and BSA were purchased from Sigma, while all other pure grade chemical and reagents were obtained from local suppliers. Reagent for isolation of total RNA (TRIzol) was purchased from Invitrogen (S. Giuliano Milanese, Milano, Italy). iSCRIPT cDNA and iQ SYBR Green SuperMix were purchased from Bio-Rad Laboratories. The QIAquick PCR Purification Kit for sequencing PCR product was from Qiagen (Milano, Italy). Real-time PCR primers for MC2R and 18S were supplied by Invitrogen.

The primary rabbit polyclonal antibody anti-melanocortin receptor (MC2R) and mouse monoclonal antibody anti-glucocorticoid receptors (GR) used for immunohistochemistry were supplied by Alpha Diagnostic International (San Antonio, TX, USA) and Oncogene Research Products (San Diego, CA, USA), respectively. The biotinylated secondary antibodies, goat anti rabbit and goat anti mouse IgG used for IHC, were purchased from Vector Laboratories (Burlingame, CA, USA) as were the avidin-biotin complex (ABC, Vector Elite Kit) and the chromogen 3,3'-diaminobenzidine tetrachloride (DAB, Peroxidase Substrate Kit, SK-4100).

2.2. Animals and hormonal regimen

The protocols involving the care and use of the animals for these experiments were approved by the Bioethic Committee of the University of Perugia. For the experiment, sexually mature New Zealand White female rabbits, weighing 3.5-4 kg, were housed individually under controlled conditions of light (14h light/10h darkness) and temperature (18° C). Each animal had free access to food and water. Pseudopregnancy was induced with 20 IU eCG followed 2 days later by 0.8 µg GnRH analogue. Previous experiments in our laboratory showed that this hormonal protocol was consistently effective in inducing ovulation in does (Stradaoli et al., 1997). The day of GnRH injection was designated day 0. The rabbits were killed by cervical dislocation and reproductive tracts, promptly removed from each animal, were thoroughly washed with saline. For luteal gene expression analyses, the CL were excised from ovaries and, after careful dissection of non-luteal tissue, rinsed with RNase free phosphate buffered saline and frozen at -80° C. For the *in vitro* study, CL were harvested from three other does sacrificed at days 4 and 9 of pseudopregnancy as previously reported (Zerani et al., 2007). For the immunohistochemical detection of MC2R and GR, two additional animals for each luteal stage were sacrificed just prior to ACTH injection. The ovaries, excised immediately after sacrifice, were fixed by immersion in 4% (w/v) formaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 24h at room temperature, and subsequently processed for embedding in paraffin following routine tissue preparation procedures.

2.3. Immunohistochemistry of MC2R and GR

After 24-48 hours of fixation, the samples were dehydrated by passage through graded ethanol (70, 95, and 100%), embedded in paraffin wax and cut in 4 µm serial sections. Tissue sections were deparaffinized in xylene, rehydrated through graded ethanol and finally rinsed in distilled water according to protocols already described

(Brecchia et al., 2009). The specimens were dipped in 3% H₂O₂ in methanol for 1 hour to quench the endogenous peroxidase activity and rinsed in TBS (tris buffered saline). Background labelling was prevented by incubating the sections with normal goat serum diluted 1:10, for 30 minutes at room temperature. The slides were then incubated overnight at 4°C in a moist chamber with the following primary antibodies diluted in TBS containing 0.2% (v/v) Triton X-100 and 0.1% (v/v) BSA: rabbit polyclonal anti-MC2R (1:50) and mouse monoclonal anti-GR (1:100). The next day, the slides were rinsed in TBS, treated again with normal goat serum and then incubated with biotin goat anti-rabbit (for MC2R) and goat anti-mouse (for GR) secondary antibodies diluted 1:200, for 30 minutes at room temperature. After TBS washes, the slides were exposed to avidin-biotin complex (ABC kit) for 30 minutes and rinsed again with TBS. The peroxidase activity sites were visualized using the DAB kit as chromogen; for GR immunohistochemistry, the reaction site was visualized using a diaminobenzidine-nickel solution. The specimens were rinsed with distilled water, washed in running tap water and dehydrated by passing through graded ethanol (v/v: 70, 95, and 100%), cleared in xylene and mounted with Eukitt medium for light microscopy. Sections in which the primary antibodies were omitted or substituted by pre-immune rabbit or mouse gamma globulin were used for the negative controls of non-specific staining. A positive control for MC2R antibody was included using mouse adrenal gland. The intensity of immunostaining for MC2R in CL was assessed and compared microdensitometrically as previously described (Zerani et al. 2010). The image analysis system (IAAS 2000 image analyzer, Delta Sistemi, Rome, Italy) was calibrated by taking the background developed in sections incubated with non-immune serum as 'zero' and a conventional value of maximum intensity of staining as '100'. Data were expressed in arbitrary units. The luteal cells were considered positive for GR only if nuclear or cytoplasm staining was present. The mean number of luteal cells expressing positive signals was calculated and averaged with those obtained from different sections of CL to yield the mean number of cells per 0.01 mm² per rabbit as previously reported (Dall'Aglio et al., 2006).

2.4. MC2R and GR real-time RT-PCR

Total RNA was extracted from CL of three rabbits for each luteal stage as previously described (Boiti et al., 2005). Five µg of total RNA was reverse transcribed in 20 µl of iSCRIPT cDNA using random hexamer according to the protocol provided by the manufacturer. Genomic DNA contamination was checked by developing the PCR without reverse transcriptase. Serial experiments were carried out to optimize the quantitative reaction, efficiency, and CT values. The optimal 25 µL PCR reaction volume contained 12.5 µL of

iQ SYBR Green SuperMix, 1 μ L forward and reverse primers (stock concentration 10 μ M), and water to 25 μ L. The primers used are listed in Table 1. All reagents were mixed as a master mix and distributed into a 96-well PCR plate before adding 2 μ L of cDNA for each gene (diluted 10 fold with water). For every PCR run, reaction controls without template, as negative controls, and without reverse transcriptase in RT were included to ascertain that RNA was free of genomic DNA contamination. Samples amplification fidelity was also verified by agarose gel electrophoresis. PCR was performed on an iCycler iQ (Bio-Rad Laboratories) with an initial incubation at 95 °C for 1.5 min, followed by 40 cycles at 95° C for 15s, 53° C for 30s, during which fluorescence data were collected. The threshold cycle (Ct value) was automatically computed for each trace. PCR products were purified and sequenced by Qiaquick PCR Purification Kit according to manufacturer protocol. The 18S Ct housekeeping gene was determined to normalize samples variations in the amount of starting cDNA. Standard curves were generated by plotting the threshold value (Ct) against the log cDNA standard dilution (1/10 dilution) in nuclease-free water. The slope of these graphs was used to determine the reaction efficiency. Quantification of the standard curve was evaluated by iCycler system software while that of mRNA gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The melting curve analysis, performed immediately after the PCR end cycle, was used to determine the specificity of each primer set. A melt-curve protocol was performed by repeating 80 heating cycles for 10s, from 55° C with 0.5° C increments, during which fluorescence data were collected.

2.5. *In vitro* incubations

For the *in vitro* study, either day 4 or day 9 CL were randomly distributed (one CL/well) into incubation wells in 1 ml of culture medium 199 with Earles Balanced Salt Solution containing 2.2 mg/ml sodium bicarbonate, 2.3 mg HEPES, and 3% BSA, referred to here as M199. Before treatment, the CL were quartered inside each well using fine forceps. Each incubation set of wells was divided into 6 experimental groups of 5 wells as follows: (I) medium alone as control; (II) ACTH (ACTH 1-24, 1 μ M); (III) ACTH plus adenylyl cyclase (AC) inhibitor (2-O-methyladenosine, 2 μ M); (IV) ACTH plus PKA inhibitor (H-9, 3 μ M); and (V) ACTH plus calcium channel blocker (verapamil). The culture plates were incubated at 37° C in air with 5% CO₂ as reported elsewhere (Boiti et al., 2000). The medium of each well was collected after 4h of incubation and stored immediately at -20° C for later determination of progesterone, PGF_{2 α} , and PGE₂. Each CL was weighed and stored immediately at -20° C for later determination of NOS activity and protein concentration. Preliminary

experiments for the assessment of the dose-response curve (data not shown) led to our choice of incubation condition and of the minimum effective dose for ACTH used in the *in vitro* study.

2.6. *In vivo* ACTH treatments

The dosage for ACTH treatments *in vivo* was based on previous data (Boiti and Yalow, 1976). Three different experimental protocols were deployed. In each of the protocols, 30 µg/kg ACTH (ACTH 1-24, treated group) or saline (control group) was injected i.m. at 8:00 AM. The day before the experiment, a catheter was inserted into the central ear vein of each rabbit.

Experiment I. Two groups of rabbits (n=5/group for each luteal stage) were treated with ACTH or saline at days 4 or 9 of pseudopregnancy. Blood samples (2 ml) were collected from each of the unrestrained catheterized animal, free to move in their cages, just before (basal) and then 2, 4, 6, 8, 24, 48, and 72h after treatment.

Experiment II. ACTH or saline was injected i.m. in estrous rabbits (n=5/group). Blood samples (2 ml) were collected from each of the unrestrained catheterized animal just before (basal) and then 2, 4, 6, 8, 24, 48, and 72h after treatment.

Experiment III. ACTH or saline was injected i.m. daily in estrous rabbits (n=5/group) for seven days before GnRH administration (day 0). Blood samples were collected daily from the ear marginal vein of each rabbit just before ACTH treatments and for nine days after GnRH injection.

The blood samples, collected into EDTA-containing tubes, were immediately centrifuged at 3000 $\times$ g for 10 min and plasma stored frozen until assayed for progesterone and cortisol concentrations to assess the functional status of CL and the activation of the adrenal axis. For the purposes of this work, functional luteolysis was defined as a 50% drop of plasma progesterone from pre-treatment values, and complete luteolysis as the failure of CL to secrete progesterone so that blood levels fall below 1.0 ng/ml, the concentration found in estrous rabbits (Browning et al., 1980).

2.7. Hormone assays

Progesterone, PGE₂ and PGF_{2α} concentrations were determined by RIA, using specific antibodies according to the procedure reported elsewhere (Zerani et al., 2007). Lipid fraction was extracted from corresponding samples with ethyl ether. For extraction, 1 ml plasma or culture medium was used and each sample was assayed

in duplicate. Plasma cortisol was assayed in duplicate by RIA, using a CORT kit as previously described (Brecchia et al., 2009). The assay sensitivity and intra- and inter-assay coefficients were: progesterone, 8 pg/ml, 5.3% and 10.2%; PGE₂, 12 pg/ml, 8% and 12%; PGF_{2α}, 19 pg/ml, 7%, 14%; cortisol 150 pg/ml, 4.8% and 6.5%.

2.8. NOS activity determination

In rabbit CL, NOS activity was determined by monitoring the conversion of [³H]L-arginine into [³H]L-citrulline using a commercial NOS assay kit according to the procedure previously described (Boiti et al., 2000).

2.9. Statistical analysis

Data on gene and protein expressions, hormone levels, and enzyme activity were examined by ANOVA followed by Student-Newman-Keuls t-test. All values are means $\pm$ SD; differences were considered significant at P<0.05.

3. Results

3.1. Localization of MC2R and Glucocorticoid receptor

Independently of luteal stage, positive reactions for MC2R were detectable in the cytoplasm of luteal cells (Fig. 1, A) and interstitial cells. Within the follicles, MC2R signals were evidenced in the ooplasm and in both theca and granulosa cells (Fig. 1, B). Strong immuno-reaction was also found in the ovarian epithelial cells and stromal cells (Fig. 1, C). Additionally, immunosignals for were observed in endothelial cells of blood vessels (Fig. 1, D), but only during day 9 of pseudopregnancy. No staining was detectable ovarian sections (negative control) when the primary antibody was omitted or substituted with rabbit IgG (Figs. 1A insert, 1E). The cortical area of adrenal gland used as a positive control exhibited immunopositivity for MC2R in the cytoplasm of cells (Fig. 1, F). The intensity of MC2R immunostaining was lower in day 4 than in day 9 CL (33.56 ± 2.11 and 36.98 ± 2.86 respectively, P<0.05). Immuno-reactivity for GR was detected in luteal cells of both day 4 (Fig. 2, A) and day 9 CL (Fig. 3A). The number of GR positive cells was lower (P<0.01) in day 4 than in day 9 CL (2.5 ± 1.67 and 11.7 ± 3.01 , respectively). At both luteal stages, the immuno-reaction for GR was detectable also in the cytoplasm of some luteal cells (Figs 2A and 3A). Positive staining for GR was also visualized in the nucleus of

fibroblasts forming the external layer of day 9 CL (Fig 3A). Moreover, immuno-reactivity for GR was observed in the ooplasm of primordial and primary follicles (Figs 2B and 3B) and also in the nucleus of granulosa cells in day 9 ovary (Fig. 3B, arrows heads). Staining was absent when the corresponding primary antibodies were omitted or substituted with pre-immune serum (Figs 2A and 3A, inserts).

3.2. MC2R and GR gene expressions by real-time PCR

The standard curves were generated by plotting threshold value (CT) vs. log of cDNA standards diluted in nuclease-free water. Standard curves show an amplification efficiency greater than 96%, with $r = 0.95$. PCR amplification efficiency was determined using the slope of the standard curve: Efficiency = $(10^{-1/\text{slope}}) - 1$. Fit of linear regression, correlation coefficient, and slope of relative curves were used to assess quality of each real time primer and probe set. Conditions were optimized to obtain >95% PCR efficiency. The temperatures of the melting products were determined graphically by fluorescence change rate (d(RFU)/dT) vs. temperature. A single melting curve peak for each PCR product represents an unique band on 2% agarose gel electrophoresis.

The gene expression analysis was calculate by $2^{-\Delta\Delta C_t}$ method, corrected by subtracting Ct number of the 18S housekeeping sample. The MC2R transcript was expressed in rabbit CL independently of luteal stage, but its mRNA levels were 5-fold ($P < 0.001$) less abundant in day 9 than in day 4 CL (Fig. 4, Panel A). The GR mRNA levels were 1.5-fold higher in day 9 than in day 4 CL (Fig. 4, Panel B)

3.3. In vitro ovarian response to ACTH

Independently of luteal stage, ACTH increased both progesterone ($P < 0.01$) and PGE_2 ($P < 0.05$) releases by CL cultured *in vitro* (Fig. 5, A and B), but reduced ($P < 0.01$) $\text{PGF}_{2\alpha}$ production (Fig. 5, C). All these actions were counteracted by the co-incubation with the inhibitors of PKA and AC, and the Ca^{2+} channel blocker (Fig. 5, A-C). In contrast, ACTH did not affect NOS activity (Fig. 5, D).

3.4. In vivo ovarian response to ACTH

In experiment I, independently of luteal stages, ACTH treatment markedly increased ($P < 0.01$) basal cortisol levels, which peaked 20.0-35.0 $\mu\text{g/dl}$ 4-8h after its administration and then gradually declined to control values

within 24h and up to 72h later (Fig. 6, A). ACTH administered at day 4 of pseudopregnancy induced a partial luteolysis 24h after injection when plasma progesterone remained at values of 2-3 ng/ml for the next two days, lower ($P<0.01$) than those of controls (Fig. 6, B). At day 9, ACTH caused a marked decline ($P<0.01$) of progesterone profile 24h after its injection, whose concentrations remained lower than that of controls ($P<0.01$) for the following 72h (Fig. 6, B).

In estrous rabbits (experiment II), injection of ACTH increased basal cortisol levels ($P<0.01$), which peaked 29 $\mu\text{g/dl}$ 6h after ACTH administration and then gradually declined to control values 24h later and for the next 72h (Fig. 7, A); progesterone plasma levels were higher ($P<0.01$) until 8h with a peak at 2h, and then gradually declined to basal values during the next 72h (Fig. 7, B). In estrous rabbits (experiment III), daily injection of ACTH for 7 days did not modify cortisol and progesterone plasma levels either before or after induction of ovulation by GnRH (Fig. 8, A and B, respectively).

4. Discussion

The present study indicates for the first time on rabbit CL the intracellular mechanism responsible for ACTH-dependent direct up-regulation of luteal progesterone production *in vitro* as well as the expression and localization of MC2R and GR genes and proteins through RT-PCR and immunohistochemistry analysis. *In vivo*, exogenous ACTH administration causes a dual effects, with early stimulation of progesterone release by steroidogenic tissues followed by inhibition of its production, depending on the luteal stage of pseudopregnancy.

The relative abundance of MC2R mRNA levels in rabbit CL was markedly influenced by luteal stage, being higher in the early- than in mid-luteal stage. This finding suggests that MC2R expression is modulated by the endocrine milieu, although it is also possible that it simply reflects changes in cell composition occurring during CL development. By immunohistochemistry, MC2R was revealed in different ovarian compartments, but mostly in the cytoplasm of luteal cell. The MC2R mRNA levels in rabbit CL did not coincide with those of protein, as evidenced by immunostaining densitometry results. In fact, the former were higher in day 4 CL whereas the latter in day 9 CL, suggesting that post-transcriptional regulations modulate the expression of the receptor differently depending on luteal age. However, the present data suggest that this receptor is functionally expressed in steroidogenic cells of both CL, seeing that the *in vitro* ACTH steroidogenic effects were revealed in day 4 and 9 CL. Specific ACTH-binding sites were studied by Scatchard analysis in luteal cells isolated from pseudopregnant rats (Horváth et al., 1986). The MC2R has high affinity for ACTH, but it does not bind to the

MSH peptides (Catania et al., 2004). MC2R mRNA levels were relatively high in the mouse fetal testis, declining rapidly after birth (O'Shaughnessy et al., 2003). It has been reported recently that MC2R is expressed in both gonocytes and the interstitial tissue of the fetal mouse testis (Nimura et al., 2006). Other evidence showed MC2R expression restricted to mouse interstitial tissue and to Leydig cells (Johnston et al., 2007). *In situ* hybridization revealed that the MC2R was highly expressed in the cortex of the adrenal gland, consistently with the role of MC2R in mediating the effects of ACTH for steroid synthesis and secretion (Xia et al., 1996). MC2R was found not only in adrenal gland, but also in murine white adipose tissue (Fan et al., 1997), a fact that correlates with the lipolytic action of ACTH on adipocytes (Grunfeld et al., 1985). Expression for MC2R was also identified in the skin (Slominski et al., 1999) and in both human and murine mononuclear leukocytes (Johnson et al., 2001). Receptors for ACTH are also expressed ectopically in non adrenal cells, but its synthesis and proper plasma membrane insertion requires a melanocortin 2-receptor accessory protein (MRAP), a small single transmembrane protein that co-expresses and co-localizes with MC2R (Metherell et al., 2005; Sebag and Hinkle et al., 2007).

ACTH in mouse is a potent stimulator of fetal Leydig cells steroidogenesis and incubation of ACTH with neonatal testicular cells led to a time-dependent increase in testosterone production (Johnston et al., 2007). As mentioned, the hypothesis that ACTH acts directly on luteal steroidogenic machinery upon binding to MC2R is further confirmed by *in vitro* studies that showed an increase of progesterone production and release by CL following incubation with ACTH. These changes are likely due to the activation of the ACTH-MC2R intracellular signaling cascade, because the other MCR known subtypes are not involved in the steroidogenesis. Our *in vitro* results showed that the inhibition of the AC/cAMP/PKA pathway counteracted the *in vitro* effects of ACTH, thus strengthening the hypothesis that, upon engagement by ACTH, the MC2R activates the intracellular AC/cAMP/PKA downstream signaling pathway (Schimmer et al., 1972; Buckley and Ramachandran 1981). Recently, it has been demonstrated that the activation of the AC/cAMP second messenger system up-regulates the production of steroid hormones by modulating the steroidogenic acute regulatory (StAR) protein which, being involved in the transport of cholesterol into the mitochondria, is the rate-limiting step in steroidogenesis (Stocco DM, 2000; Gyles et al., 2001; Clark et al., 2001).

Interestingly, independently of luteal phase, ACTH increased PGE₂ release *in vitro* and inhibited PGF_{2α} synthesis via the AC/cAMP/PKA cascade and the intracellular Ca²⁺ release as inferred by the blocking actions of the corresponding inhibitors co-incubated with ACTH. Stimulation of progesterone production by ACTH in luteal cells harvested from pseudopregnant rats was inhibited by a cyclooxygenase inhibitor, indomethacin, and

enhanced by PGE addition (Horváth et al., 1986). Taken together, these observations suggest that ACTH may enhance luteal steroidogenesis through local modulation of prostaglandin synthesis. In fact, it is now widely accepted that prostaglandins play a key role in regulating the function of CL with opposing actions between $\text{PGF}_{2\alpha}$ and PGE_2 , having luteolytic and luteotrophic properties, respectively (Niswender et al., 2000). In rabbits, the CL themselves synthesize $\text{PGF}_{2\alpha}$ and PGE_2 (Gobbetti et al., 1999, Boiti et al., 2000, Zerani et al., 2005), the production of which is regulated by a large array of local and systemic factors, suggesting a paracrine and autocrine role for these two PGs (Boiti et al., 2005; Zerani et al., 2007). By converse, ACTH did not influence the activity of the NOS/NO system which has been found to regulate luteal steroidogenesis (Gobbetti et al., 1999) as well as luteal regression in rabbits (Boiti et al., 2003).

Daily injection of ACTH did not alter ovarian function, as reflected by progesterone concentrations in peripheral plasma before and after GnRH induction of ovulation that were comparable to those of control rabbits. During the estrous phase, the transient increase of plasma progesterone concentrations following exogenous administration of ACTH was likely due to the stimulation of the adrenal gland, given that no immuno staining for MC2R was observed in other ovarian steroidogenic structures besides CL and follicles. Similarly, the daily injection of ACTH for a week did not cause overproduction of cortisol by the adrenals as testified by the normal range of its peripheral plasma concentrations. Continuous infusion of ACTH in bovine on days 2-9 of the estrous cycle resulted in a decreased slope of the progesterone increase during the infusion period, but had no effect on both CL weight and progesterone content (Wagner et al., 1977). Continuous ACTH or hydrocortisone infusion throughout the estrous cycle of intact heifers, after a transient increase of plasma progesterone concentrations on days 3 to 4, caused severe suppression of CL function during the luteal phase (da Rosa and Wagner, 1981; Li and Wagner, 1983). The same effect was observed following infusion with cortisol to adrenalectomized heifers, but not with ACTH. These induced hyperadrenal conditions resulted in clear inhibition of progesterone secretion by the CL that was likely mediated by cortisol. In non-lactating, non-pregnant Friesian cows, prolonged adrenal stimulation at the time of induced corpus luteum regression delayed follicular development and impaired LH release (Gabai et al., 2006). In sheep, according to Phogat et al. (1999), exogenous ACTH administration during the breeding season reduced the GnRH self-priming effect *in vivo* and delayed the LH surge, at least partially by direct effects at the pituitary gland. ACTH treatment induced significant pathological effects on ovine ovarian structures, probably through treatment-induced increase in cortisol (López-Díaz and Bosu, 1997).

At both luteal stages of pseudopregnancy, ACTH caused a partial functional demise that became evident 24 hours after its administration and persisted for the following 72 hours. By converse, no changes in the peripheral

plasma progesterone concentrations were evidenced in the first few hours after ACTH injection at both days 4 and 9 of pseudopregnancy. These findings clearly indicates that the extra contribution of progesterone from the adrenals and from CL due to the direct action of ACTH as shown by the *in vitro* studies was quantitatively limited compared to the much higher steroidogenic capability of CL. Although the direct actions of glucocorticoids in the regulation of ovarian function are still poorly understood, they may influence ovulation as well as luteal development, function, and demise by virtue of their anti-inflammatory capability and through local interference with prostaglandins, growth and mitogen factors, and cytokines actions in a paracrine and/or autocrine way (Hillier and Tetsuka, 1998). Glucocorticoid receptors were expressed in the rabbit CL at both gene and protein levels independently of luteal stage. By immunohistochemistry, clear positive staining for GR was detected in the nucleus as well as in the cytoplasm of some luteal cells. Similar finding were also reported in other species (Andersen, 2002) as GR distribution within cellular compartments depends on its binding with hormone. Unbound GR is mainly localized within the cytoplasm, whereas bound GR is rapidly translocated to the nucleus (Heitzer et al., 2007). Apparently, the GR was more expressed in day 9 than in day 4 CL as judged by the number of positive luteal cells. Thus, it remains to be verified whether formation and function of the CL may benefit from a high local concentration of free cortisol.

In summary, the present data indicate that ACTH directly modulates rabbit ovarian functionality by means of the up-regulation of luteal progesterone synthesis *in vitro* and through its time dependent stimulation/inhibition *in vivo*. This steroidogenetic production regulation involves the MC2 type ACTH cognate receptor, AC/cAMP/PKA and Ca^{2+} intracellular mechanisms, and PGE_2 mediation.

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Table.1 Primers used for gene quantification by real-time RT-PCR.

Gene	Accession Number		bp	Primers
MC2R	BC069074 Homo sapiens	Forward	133	GCCATCACACTGACCATCC
		Reverse		CGTTCACCTGGAAGAGGAC
GR	NM_001082147.1 O. Cuniculus	Forward	70	CATTAAGTCCCCCGGGTAGAGAGGA
		Reverse		ATCACATTCCCGCGCTCCGC
18S rRNA	X03205.1 Homo sapiens	Forward	148	CGATCAGATACCGTCGTAGT
		Reverse		TTCCTTTAAGTTTCAGCTTTGC

Figure legends

Figs. 1, Panels A-C. Immunohistochemical identification of MC2R in the ovary explanted at 4 day of pseudopregnancy; the receptor is expressed (Panel A) in the cytoplasm of luteal cells, (Panel B) ooplasm (O), cytoplasm of follicular (F) and theca (T) cells, (Panel C) ovary epithelium (arrowhead) and stromal cells (arrow). Panel D: Ovary explanted at 9 day of pseudopregnancy; note MC2R signals in endothelial cells (arrow). Panel A-insert and E: Control sections in the absence of primary antibody. Panel F: Immunopositivity for MC2R in the cortical area of the mouse adrenal gland. Scale bars = 20 μ m.

Fig. 2. Immunohistochemical identification of glucorticoid receptors in ovary explanted at day 4 of pseudopregnancy. Positive reaction is evident in the cytoplasm of some luteal cells (LC, Fig 2A arrows) and in the ooplasm of a oocyte (Fig 2B, arrow). Scale bar=50 μ m

Fig. 3. Immunohistochemical identification of glucorticoid receptors in ovary explanted at day 9 of pseudopregnancy. GR-positive reaction is present in the nucleus of a great number of luteal cells (LC, Fig 3A arrows), in the cytoplasm of some luteal cells and in the nucleus of theca cells (T, Fig 3A). Moreover GR-immunoreaction is evident in the ooplasm of primordial and primary follicles (Fig 3B, arrow) and, in the same follicles, in the nucleus of granulosa cells (Fig 3B, arrows head). Scale bar=50 μ m

Fig. 4. Real-time PCR mRNA expressions for MC2R (panel A) and GR (Panel B) in CL collected at days 4 and 9 of pseudopregnancy. Data are represented as the fold changes of mRNA expression at day 9 compared to those at day 4 normalized to 18S housekeeping gene. The Relative abundances of target genes were calculated using the $2^{-\Delta\Delta CT}$ method. The means $\pm$ SD of MC2R and GR mRNA expression levels for the three mRNA measurements were calculated for 3 animals/group. An asterisk above the bar indicates a significantly different values at $P<0.01$ between days 4 and 9 of pseudopregnancy.

Fig. 5. *In vitro* effects of medium alone, ACTH, ACTH plus AC inhibitor (ACi), PKA inhibitor (PKAi), and Ca^{2+} channel blocker (Ca blk) on progesterone (Panel A), PGE_2 (Panel B), $PGF_{2\alpha}$ (Panel C) releases, and NOS activity (Panel D) by rabbit CL harvested at days 4 and 9 of pseudopregnancy. Results are the means $\pm$ SD of five replicates; significant difference from control at $P<0.05$ and at * $P<0.01$.

553

554 Fig. 6. Plasma cortisol (panel A) and progesterone (panels B) concentrations following ACTH (30 µg/kg i.m) or
555 saline treatments to pseudopregnant rabbits at different luteal stages (days 4 and 9). Values are means ± SEM for
556 5 animals/group; significant difference from control at * $P < 0.01$.

557

558 Fig. 7. Plasma cortisol (panel A) and progesterone (panels B and C) concentrations following ACTH (30 µg/kg
559 i.m) or saline treatments to estrous rabbits. Values are means ± SEM for 5 animals/group; significant difference
560 from control at * $P < 0.01$.

561

562 Fig. 8. Peripheral plasma cortisol (panel A) and progesterone (panel B) concentrations after daily injections of
563 ACTH (30 µg/kg i.m) or saline during 7 days before induction of ovulation by GnRH administration. Values are
564 means ± SEM for 5 animals/group; significant difference from control at * $P \leq 0.01$.

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570 Fig. 1

571

Fig. 2

Fig. 3

MC2R and GR mRNA levels at different luteal stages of pseudopregnancy

Fig. 4

Fig. 5

Cortisol and progesterone responses to ACTH at two different luteal stages of pseudopregnancy

Fig. 6 Modify 24 h Panel B

Cortisol and progesterone responses to single ACTH injection to estrous rabbits

Fig. 7

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Cortisol and progesterone responses to daily ACTH injections to estrous rabbits before GnRH induced ovulation

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618

619 Fig. 8