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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 adrenocorticotropic 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 4 h ($P < 0.01$), but decreased ($P < 0.01$) progesterone 24 h 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, 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 adrenocorticotropic 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 and Wikberg, 1992; Chhajlani et al., 1993; Gantz et al., 1993; Mountjoy et al., 1992; Roselli-Rehfuss 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 Chori-

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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

onic 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, PGE_2, and $PGF_2\alpha$ 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), Ca^{2+} 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 (14 h light/10 h 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 (Stradaioli 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 24 h 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 h 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% H_2O_2 in methanol for 1 h 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 min 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 min at room temperature. After TBS washes, the slides were exposed to avidin–biotin complex (ABC kit) for 30 min 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 mm^2 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 micrograms 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 μL forward and reverse primers (stock concentration 10 μM), and water to 25 μL. 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 μL 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 15 s, 53 °C for 30 s, 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 $2^{-\Delta\Delta Ct}$ 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 10 s, from 55 °C with 0.5 °C increments, during which fluorescence data were collected.