Author's personal copy

ELSEVIER

Available online at www.sciencedirect.com
ScienceDirect

Theriogenology 75 (2011) 628–637

Theriogenology
www.theriojournal.com

Expression of melanocortin receptors *mRNA*, and direct effects of ACTH on steroid secretion in the bovine ovary

A.N. Amweg[a], A. Paredes[b], N.R. Salvetti[a,c], H.E. Lara[b], H.H. Ortega[a,c,*]

[a] *Department of Morphological Sciences, and Centre for Experimental Biology and Laboratory Animal Sciences, Faculty of Veterinary Sciences, National University of Litoral, Santa Fe, Argentina*
[b] *Department of Biochemistry and Molecular Biology, Faculty of Chemistry and Pharmaceutical Sciences, Universidad de Chile, Santiago, Chile*
[c] *National Research Council (CONICET), Buenos Aires, Argentina*

Received 19 July 2010; received in revised form 27 August 2010; accepted 14 September 2010

Abstract

Melanocortin receptors (MCRs) are involved in physiological responses to ACTH, as well as to α-, β- and γ-melanocyte-stimulating hormone (α-, β- and γ-MSH). Their expression has previously been analyzed in various bovine tissues; however, there are apparently no reports regarding their localization in the ovaries. In the present study, the expression of MCR mRNA in various bovine ovarian structures was characterized with reverse transcription polymerase chain reaction (RT-PCR). Furthermore, whether ACTH affected follicular components by affecting steroid secretion in fragments of ovarian follicular wall of medium and large antral follicles cultured in serum free medium with 1, 10, and 100 nM ACTH, was also determined. *Melanocortin receptors* mRNA was localized in the theca cells of various follicular stages, whereas only *MC3R* mRNA was weakly evident in granulosa cells. *Melanocortin receptors* 1, 2, and 3 mRNA were present in the CL, whereas in stroma, only *MC2R* mRNA was expressed. There were significant increases in estradiol and cortisol concentrations in response to ACTH in medium follicles, as well as increased concentrations of testosterone and cortisol in large follicles. These results confirmed earlier reports in other species, and demonstrated that MCRs were present in bovine ovaries. Since ACTH induced steroid secretion from the ovary *in vitro*, we inferred that melanocortin peptides could be involved in regulatory mechanisms related to ovarian functions, e.g. ovulation, steroidogenesis, and luteal function.
© 2011 Elsevier Inc. All rights reserved.

Keywords: Ovary; POMC; ACTH; Melanocortin; Cattle

1. Introduction

Adrenocorticotropic hormone (ACTH) and α-, β- and γ-melanocyte-stimulating hormone (α-, β- and γ-MSH) are derived from the post-translational processing of the precursor molecule proopiomelanocortin (POMC) [1]. These POMC products are collectively called melanocortin peptides or melanocortins. The principal source of melanocortins is the pituitary gland, but the POMC gene is also expressed in a variety of other brain regions, as well as a number of peripheral tissues, in particular skin [2].

Recognition and cloning of melanocortin receptors (MCRs) has greatly improved the understanding of peptide-target cell interactions. To date, five MCRs (termed MC1R to MC5R) have been cloned. Expres-

* Corresponding author. Tel.: 54-3496-420639-223; fax: 54-3496-428576-350.
E-mail address: hhortega@fcv.unl.edu.ar (H.H. Ortega).

0093-691X/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2010.10.003

Author's personal copy

sion of *MC2R* mRNA was reported in the ovaries of fish [3,4] and mice [5], and *MC5R* was identified in human ovaries [6]. Furthermore, expression of MCRs has been analyzed in various bovine tissues [7–15]. However, there are apparently no reports regarding expression of specific *MCR* mRNA or its localization in ovaries. It should, however, be considered that the interactions between melanocortins and reproduction have been clearly established, associating them with varioius processes related to metabolism, stress, immunity, and neuroendocrine signalling pathways [2,16].

Stress-associated stimulation of the hypothalamic-pituitary-adrenal/ovary axis disrupted reproductive function [17]. Poor ovarian follicular development, reduced ovulation rates, and ovarian cyst formation were reported following ACTH treatment and resulting increases in glucocorticoid concentrations [18–21]. Also, high glucocorticoid concentrations during the late follicular phase of the estrous cycle can disrupt gonadotrophin release [22,23].

Although these studies indicated an effect of glucocorticoids at the cellular level within the ovary, little research has been performed to understand the direct effects of ACTH on ovarian function, although there could be receptors for this hormone within the ovary of some species. Therefore, the objective of the current study was to examine the expression of *MCR* mRNA in bovine ovaries using reverse transcription polymerase chain reaction (RT-PCR). We postulated that these receptors, differentially expressed in ovarian components, could mediate the actions of melanocortins directly affecting follicular development and ovarian function. To confirm this, we analyzed *in vitro* the direct effects of ACTH on constitutive follicular wall steroideogenesis.

2. Materials and methods

2.1. Collection and preparation of tissues

Ovaries from 66 randomly cycling cattle with apparently normal follicular activity were collected at a local abattoir, within 20 min after death. These ovaries were from mixed breeds of *Bos taurus* cows, assessed visually as non-pregnant and with no apparent abnormality in the reproductive system. Intact ovaries were washed in sterile PBS, refrigerated, and transported immediately to the laboratory.

Each batch of ovaries was placed on ice, and antral follicles were removed using dissecting scissors. Prior to dissection of the ovaries, follicle diameter was measured with calipers, and follicular fluid from each follicle was aspirated and stored separately at −20 °C pending determination of progesterone and estradiol concentrations. Follicles with an obviously atretic appearance (debris in the antrum) were discarded. Large antral follicles were obtained only from ovaries without a visible, active CL.

For RT-PCR, follicles were classified into three categories according to their calculated diameters, as described previously [24]: small (<5 mm, n = 15), medium (5–10 mm, n = 15) or large (>10 mm, n = 15). To avoid cross contamination and damage due to manipulation, only one follicular structure was obtained from each ovary. Follicles were hemisected in PBS, and granulosa cells (GCs) were gently scraped into tubes containing approximately 20 mL of sterile PBS. The cell suspension was centrifuged at 400 × *g* for 10 min, the supernatant discarded, and the GC pellets resuspended in Trizol LS reagent (Invitrogen, Carlsbad CA, USA). Follicular walls were further washed several times with PBS to remove residual GC. The surrounding stroma was also removed from follicular walls and regarded as theca tissues [25]. Samples of CL (n = 5) were also obtained from cattle in the luteal phase. All samples were snap-frozen in liquid nitrogen and stored at −80 °C pending total RNA extraction. The adrenal cortex, liver, spleen and spinal cord tissue and white blood cells of a non-pregnant cow, which served as both positive and negative controls (Table 1), were obtained and conserved in the same manner.

2.2. Total RNA extraction

Total RNA was isolated from the samples after treatment with Trizol LS reagent (Invitrogen), according to the manufacturer's instructions with slight modifications. Briefly, 50 to 100 mg of tissue was homogenized with 750 μL of Trizol reagent (Invitrogen) and incubated for 10 min at 25 °C. Then, RNA was purified by vigorously homogenizing with chloroform and incubating for 15 min at 4 °C. After centrifugation at 12,000 × *g*, the aqueous phase was incubated with an equal

Table 1
Positive (+) and negative (−) controls used to analyze mRNA expression of melanocortin receptors in bovine tissues.

	MC1R	MC2R	MC3R	MC4R	MC5R
Liver	−	−	+	−	+
Spleen	−	−	+	−	−
Spinal cord	+	+	+	+	+
White blood cells	+	+	+	+	+
Adrenal cortex	+	+	+	+	+

For review see [1,30].

Author's personal copy

volume of isopropanol for 60 min at −80 °C and centrifuged at 12,000 × *g* to obtain the pellet of mRNA that was then washed with 75% ethanol for 10 min at 4 °C. Alcohol was replaced by DEPC-water pre-warmed at 55 to 60 °C. The extracted RNA was DNase treated with deoxyribonuclease I (amplification grade; Invitrogen) to eliminate contaminating DNA, assessed for quality and quantity using a fluoroscopic method (Qubit, Invitrogen), aliquoted, and stored at −80 °C pending further use.

2.3. PCR primer design

2.3.1. Melanocortin receptors specific primers

Bovine sequences for MC1R, MC2R, MC3R, MC4R and MC5R were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Entrez/index.htm). Specific primers were designed to obtain PCR amplification products between 400 and 500 bp, spanning two mRNA-splicing sites, using the PrimerSelect program in the LASERGENE software (DNAStar, Madison, WI, USA). The primers were purchased from Invitrogen, and the sequences are summarized (Table 2). Oligonucleotide primers and amplification products were tested using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) software to confirm gene specificity and determine exon locations, assuring that they were not designed from any homologous regions coding for other genes.

2.3.2. Other primers

The primer sequences for cytochrome P450 aromatase (*CYP19a1*) and cytochrome P450 17αhydroxylase/17,20-lyase (*CYP17a1*) were designed following the same rules as MCR primers, and validated to confirm bovine granulosa and theca cell mRNA purity (no cross-contamination) [26]. Primers for housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously [27].

2.4. RT-PCR

The first strand of cDNA was synthesized in a 20 μL reaction mixture using 1 μg of total RNA. The reaction tubes contained 0.25 μL random hexamers (1 μg/μL), 0.4 μL dNTPs (25 mM), 4 μL 5 × reaction buffer, 2 μL DTT (0.1 M), 0.5 μL ribonuclease inhibitor (40 U/μL), and 1 μL M-MLV reverse transcriptase (200 U/μL; all reactives were from Invitrogen). Reactions were incubated at 25 °C for 10 min, 37 °C for 50 min, and inactivated for 15 min at 70 °C.

Each PCR assay was optimized with regard to annealing temperature and magnesium ($MgCl_2$) concen-

Table 2
Primer sequences, regions of the target genes and conditions used for semi-quantitative RT-PCR of bovine tissues.

Name	Sequence (5′–3′)	Gene accession no.	Amplicon position	Amplicon size (bp)	Annealing temperature (°C)	No. cycles
MC1R						
Forward	CAGCCTGCTCTTCATCACCTACTA	NM_174108	808–1229	422 bp	58.8	30
Reverse	AGCACTGCAGCACCTCTTG					
MC2R						
Forward	GGCATTACCATCGTGACCTTC	NM_174109	609–1057	449 bp	57.2	30
Reverse	TATATCCCTGTGGCTCCTAAAATC					
MC3R						
Forward	CGATCCCCGTGGTCAAC	FJ433881	771–1213	443 bp	57.4	30
Reverse	GCAACTGCCCCCTTCATACA					
MC4R						
Forward	TCGATCGCGGTGGACAGGTA	NM_174110	637–1129	493 bp	58.9	32
Reverse	TCCGCAGGGCATAAATCAGAGG					
MC5R						
Forward	CCAGCTGCGGCACGGTCTT	NM_001015542	512–942	431 bp	57.6	32
Reverse	GCTCTGAAAACAAACAATCTCCTT					
CYP17						
Forward	GGAGGCGACCATCAGAGAAGTGC	NM_174304	1105–1423	319 bp	60.8	35
Reverse	CAGCCGGGACATGAAGAGGAAGAG					
CYP19						
Forward	TAAAACAAAGCGCCAATCTCTACG	BTCYP19	8–348	341 bp	55.4	35
Reverse	GGAACCTGCAGTGGGAAATGA					
GAPDH						
Forward	CACCCTCAAGATTGTCAGCA	BC102589	492–594	103 pb	52	31
Reverse	GGTCATAAGTCCCTCCACGA					

Author's personal copy

tration. Firstly, amplification of control samples was performed with annealing temperatures between 51 and 69 °C and $MgCl_2$ between 1 and 2 mM, using the gradient feature of a gradient thermal cycler Techne TC-3000G (Techne Inc, Princeton, NJ, USA). The number of PCR cycles was determined in preliminary experiments by performing 20 to 35 amplification reactions. Then, PCR was carried out in a final volume of 25 μL containing 75 ng cDNA (previously quantified by the Qubit method), 1.5 mM $MgCl_2$, 0.5 μM forward primer, 0.5 μM reverse primer, 0.2 mM dNTP, 2.5 μL Taq buffer 10X, and 2 U Taq polymerase (5 U/μL; all reactives from Invitrogen). For every sample, cDNA (volume, 3 μL) was added to the master-mix solution. Amplification conditions included 30 to 35 cycles of denaturation at 94 °C for 45 s, annealing at 52 to 60.8 °C (Table 2) for 30 s, and extension at 72 °C for 1 min 30 s. Finally, a final extension step at 72 °C for 10 min were performed.

The PCR products were resolved by electrophoresis alongside a 1 kb DNA ladder (BioLogicos, Buenos Aires, Argentina) through a 2% agarose gel containing GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA), and visualized and digitized under UV light to confirm the presence of a single band at the appropriate size. The mRNA levels of the housekeeping gene GAPDH were similar for all follicle samples. Only samples positive for GAPDH mRNA expression were used to investigate the mRNA expression of the other genes. In addition, only granulosa samples positive for *CYP19a1* mRNA and negative for *CYP17a1* mRNA and the theca samples positive for *CYP17a1* mRNA and negative for *CYP19a1* mRNA expression were used to detect MCR mRNA expression.

2.5. Image analysis

Agarose gel images were digitized using an Olympus digital camera and PCR products analyzed using the Image Pro-Plus 3.1 program (MediaCybernetic, Bethesda, MD, USA).xppFor these assessments, *GAPDH* mRNA was selected as an internal control, since expression of *GAPDH* mRNA remained constant in all tissues studied. In comparative PCR analysis, the absolute optical density (OD) values for each PCR product were obtained by densitometry and were normalized with *GAPDH* levels, whereas relative levels of the specific mRNA were expressed in arbitrary units.

2.6. Nucleotide sequencing

The specificity of PCR products was checked by direct sequencing, to verify amplification of the correct sequences, at the University of Maine (Orono, ME, USA) DNA sequencing facility. The resulting sequences were verified using the MegAlign Tool, in the LASERGENE software (DNAStar).

2.7. Secretion of steroids by the follicular wall

The size of bovine ovaries precluded the whole-ovary culture system used successfully with rodents [28,29], but these methods can be adapted to permit follicle wall culture. In a laminar flow hood, ovaries were rinsed, and medium and large antral follicles were taken using a scalpel and placed in Krebs-Ringer bicarbonate buffer pH 7.4 (in mM: NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 25; $NaHCO_3$, 2.5; glucose, 11.5; bovine serum albumin, 0.1%; and ascorbic acid, 100 ug/mL; all were obtained from Sigma Chemical Co., St Louis, MO, USA). The culture medium had previously been equilibrated for 2 h in a humidified incubator (Forma Scientific 3154 Water-Jacketed Incubator, Asheville, NC, USA) gassed with 95% O_2 and 5% CO_2 at 37 °C. Small follicles were not cultured because was technically impossible to obtain enough tissue samples without stromal contamination.

Each large antral follicle was sectioned into four fragments (one for basal and three for treatments, approximately 50 mg each). For each medium follicle, only one fragment was obtained for each follicle and different follicles were used for each treatment. The fragments were cultured on 24-well (1.5 mL medium/well) culture plate (Nunc plate, Applied Scientific, South San Francisco, CA, USA), and incubated in Krebs-Ringer bicarbonate buffer, pH 7.4 for 20 min. After pre-culture, tissues were placed in medium with 1, 10, or 100 nM ACTH (Laboratorios ELEA, Buenos Aires, Argentina) for 3 h at 37 °C. As a control (basal secretion), tissues were incubated in the same way without ACTH. Each set of cultures ($n = 4$) was performed under identical conditions. Then, the culture media was taken from each well and maintained at −20 °C until assayed.

2.8. Hormone assays

Estradiol, progesterone, testosterone, and cortisol secreted into the incubation medium were measured by ELISA kits (Estradiol EIA, DSL-10-4300; Progesterone EIA, DSL-10-3900; Testosterone EIA, DSL-10-4000; Cortisol DSL-1-200; Diagnostic Systems Labo-

Author's personal copy

Table 3
Mean ± SEM concentrations of estradiol and progesterone in the follicular fluid of small, medium, and large bovine antral follicles.

Steroid (ng/mL)	Small	Medium	Large
Estradiol	97.31 ± 6.65[a]	143 ± 12.48[a,b]	282.41 ± 22.18[b]
Progesterone	62.12 ± 9.01	66.75 ± 10.72	81.52 ± 9.48

[a,b] Within a row, means without a common superscript differed (P < 0.05).

ratories, Webster, TX, USA), according to the manufacturer's instructions. The concentrations of progesterone and estradiol were also determined directly in the follicular fluid. The assay sensitivity was 7 pg/mL for estradiol, 0.13 ng/mL for progesterone, 0.04 ng/mL for testosterone, and 0.10 μg/dL for cortisol. For incubation medium, results were normalized in relation to tissue weight and expressed as hormone concentration/mg of tissue.

2.9. Statistical analyses

A statistical software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used to perform all statistical tests. The relative levels of the specific MCRs mRNA in ovarian components and the effects of different concentrations of ACTH on the secretion of steroids in culture medium were compared by ANOVA, followed by Duncan's multiple range tests. The comparison of basal secretion between medium and large antral follicles was performed using a nonpaired two-tailed Student's *t*-test. Regression analysis was used to determine the dose-response effects. For all statistical analyses, $P < 0.05$ was considered significant. Results are expressed as mean ± SEM.

3. Results

3.1. Follicular characterization

Concentrations of estradiol and progesterone in the follicular fluid from various follicular sizes are shown (Table 3). All follicles were categorized as estrogen-active, with an increase in large antral follicles ($P < 0.05$). However, progesterone concentrations were not significantly different among follicle categories.

3.2. Melanocortin receptors mRNA expression

Expression of *MCRs* mRNA were compared among RT-PCR samples containing the same amount of *GAPDH* cDNA. Controls were performed in parallel using water (no cDNA) and RNA samples (without RT), and no PCR product was visible. All granulosa samples were positive for *CYP19a1* mRNA and negative for *CYP17a1* mRNA, whereas theca samples were positive for *CYP17a1* mRNA and negative for *CYP19a1* mRNA expression. Data obtained in representative RT-PCRs for all primers in ovarian and control tissues are shown (Fig. 1 and 2).

The identity of the MCRs PCR products was confirmed by sequencing (range 97–99% homology with bovine sequences). *Melanocortin receptor 1* mRNA was weakly detected in the theca of small and medium antral follicles and higher ($P < 0.05$) in CL. The theca in large antral follicles, granulosa cells and stroma showed no expression. *Melanocortin receptor 2* mRNA levels were moderate in the theca cells of small and medium follicles and CL, and low ($P < 0.05$) in the theca of large antral follicles and stroma. No specific MC2R mRNA was detected in the granulosa.

The expression of *MC3R* mRNA was moderate in the theca cells of small antral follicles, but weak in the theca of medium and large antral follicles. The GC had a low expression, but CL a strong expression of *MC3R* mRNA ($P < 0.05$). No *MC3R* mRNA was detected in stroma. *Melanocortin receptor* 4 mRNA was moderately expressed in the theca cells of small and medium antral follicles, whereas *MC5R* mRNA was only weakly expressed in the theca cells of small and medium antral follicles, with a decrease in the theca of large antral follicles ($P < 0.05$). No *MC4R* or *MC5R* mRNA was identified in the granulosa, CL, or stroma.

The comprehensive screening of *MCRs* mRNA in various organs and tissues was performed to check the specificity of the primers (Table 1, Fig. 1). In the spleen, only a moderate expression of *MC3R* was observed, whereas in the liver, a high expression of *MC3R* and low expression of *MC5R* were evident. In the spinal cord, the expression of all MCRs was weakly detected, except *MC2R* (strongly detected). In white blood cells, *MC1R*, *MC2R* and *MC3R* were moderately expressed, whereas *MC4R* and *MC5R* had a low expression. Finally, in the adrenal cortex a high expression of *MC2R*, a moderate expression of *MC1R* and *MC3R,* and a low expression of *MC4R* and *MC5R* were observed.

3.3. Effects of ACTH on the secretion of steroids by the follicular wall

After incubating for 3 h, basal levels of steroids secretion were detected in all samples, whereas secretion of estradiol, progesterone and testosterone were significantly lower in small antral follicles, and cortisol was higher in large antral follicles (Fig. 3).

Author's personal copy

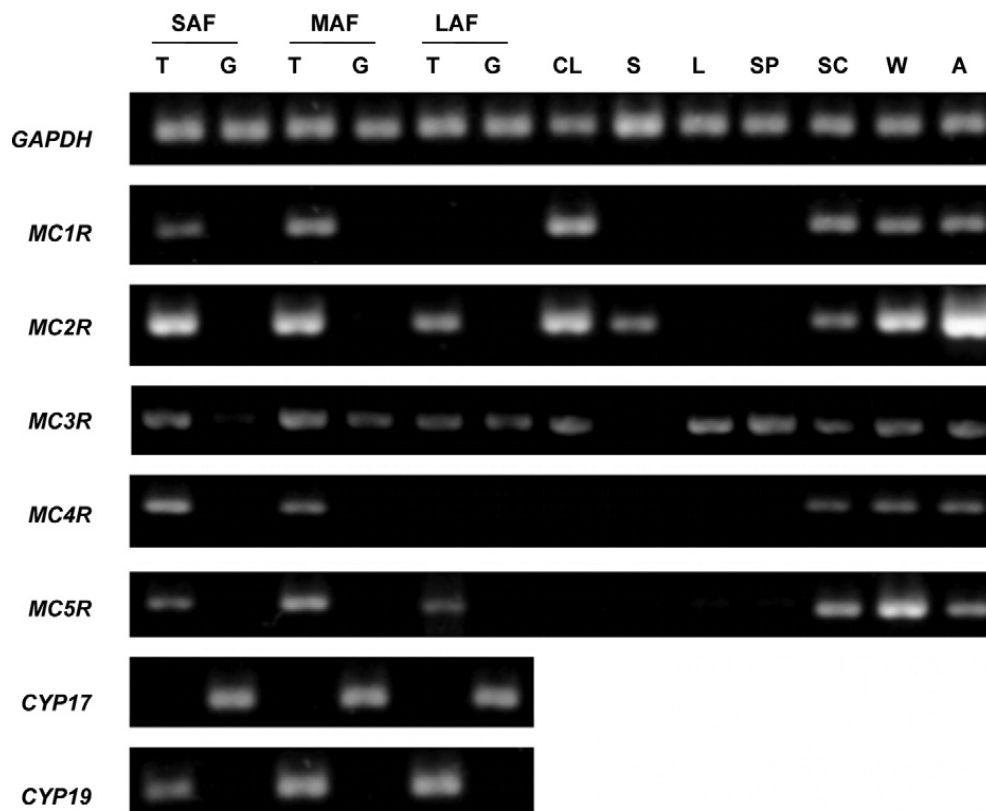

Fig. 1. Gel electrophoresis of specific RT-PCR products, showing the expression of specific primers in bovine ovarian components and control tissues.

SAF, small antral follicle; MAF, medium antral follicle; LAF, large antral follicle; G, granulosa; T, theca; CL, corpus luteum; S, stroma; L, liver; SP, spleen; SC, spinal cord; W, white blood cells; A, adrenal cortex.

In medium antral follicles, progesterone and testosterone secretion was not modified by a range of increasing doses of ACTH in the incubation medium. However there were dose-dependent increases for estradiol and cortisol, with a significant increase in response to 10 and 100 nM ACTH. In large follicles, progesterone and estradiol secretion was not modified by the presence of ACTH. A significant dose-dependent increase however was evident in testosterone and cortisol secretion. Significantly higher values of cortisol and testosterone secretion also occured in response to 10 and 100 nM ACTH.

4. Discussion

In this study, there was clear evidence that mRNA for all MCRs was expressed in bovine ovaries. The use of bovine primers and the results of controls tissues were in agreement with the expression patterns of MCRs previously described [1,30] and were also confirmed by sequencing. Based on semi-quantitative analyses, relative levels of mRNA for various MCRs changed at each stage of follicular development. Therefore, this study was undertaken to determine the gene expression of all components in a single study, to start to establish a relationship among various subtypes of the receptors in ovarian structures. Overall, the present results confirmed earlier reports in other species, and introduced new findings, including the existence of additional receptors in various ovarian components.

Unfortunately, there are no antibodies for MCRs for immunohistochemical or western blot studies with bovine tissues. Although we only demonstrated the presence of mRNA coding for the MCRs, not the protein, our findings confirmed previously published reports describing the presence of MC2R and MC5R in ovaries of various species [1,4]. To our knowledge, there has been no investigation into the expression and localization of MC1R, MC3R, and MC4R in reproductive organs. In this sense, although ACTH bound to all MCRs, it has been hypothesized that it exerts its effects on follicular steroidogenesis via MC2R and MC5R, thereby centralizing the studies on these receptors. Nonetheless, MC1R, MC3R and MC4R might also mediate actions related to other functions in ovarian physiology.

Author's personal copy

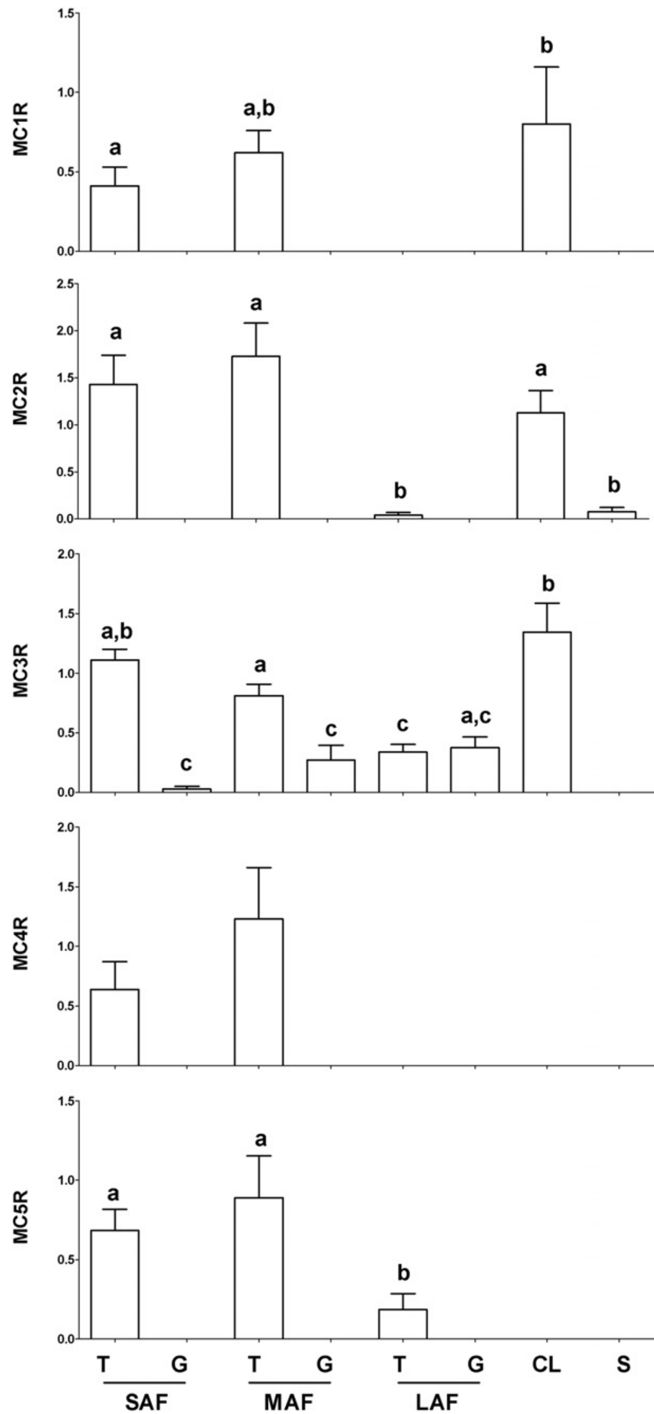

Fig. 2. Mean (± SEM) relative expression of melanocortin receptors (mRNA MCRs/mRNA GDPH) in bovine ovarian components and control tissues.

SAF, small antral follicle; MAF, medium antral follicle; LAF, large antral follicle; G, granulosa; T, theca; CL, corpus luteum; S, stroma; L, liver; SP, Spleen; SC, spinal cord; W, white blood cells; A, adrenal cortex.

[a,b]Means without a common superscript differed ($P < 0.05$).

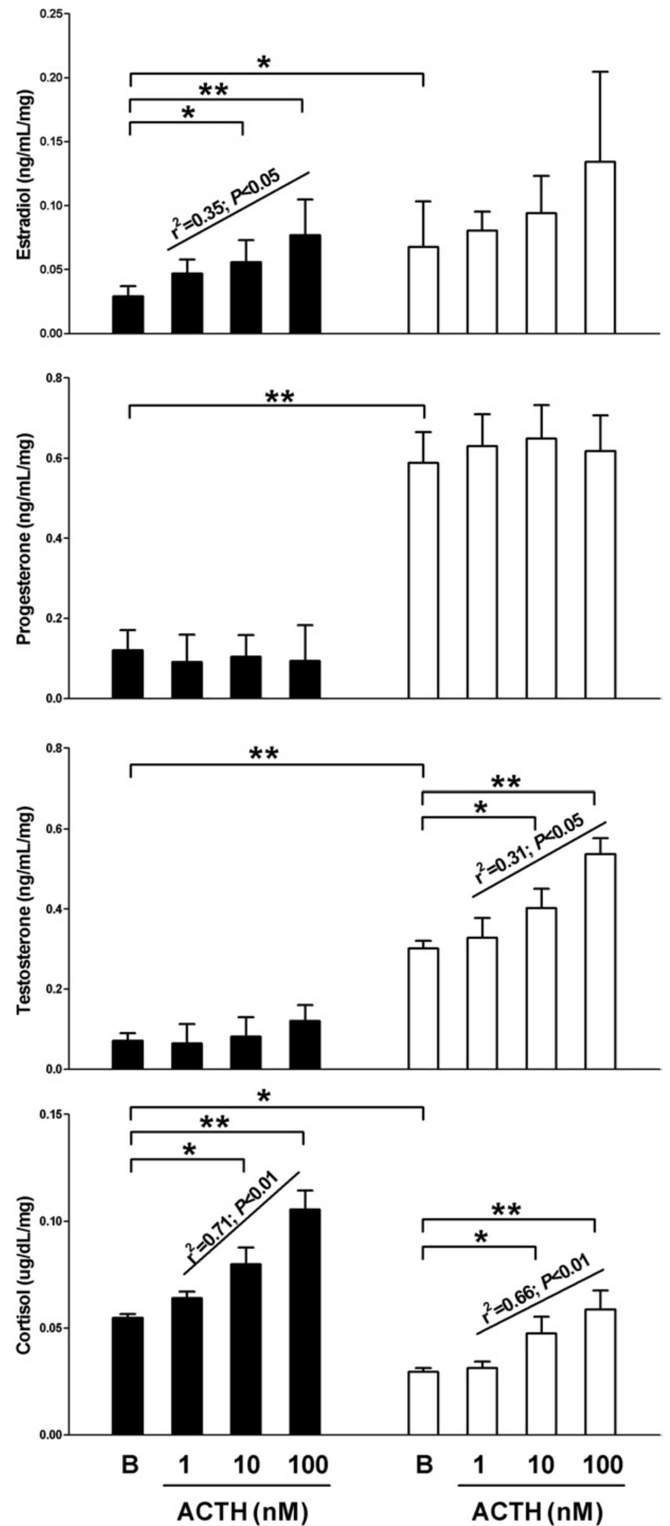

Fig. 3. Mean (± SEM) effects of ACTH on the secretion of steroids by the follicular wall of medium (black bar) and large (white bar) bovine antral follicles. Differences relative to basal secretion and differences in basal secretion between medium and large antral follicles are indicates: * $P < 0.05$ and ** $P < 0.01$.

Dose-response effects are also indicated.

Author's personal copy

Few studies have addressed the role of MCRs in mediating the effects of melanocortins on reproduction. It is well known that ACTH is acutely released from the pituitary gland in response to stressor stimulation and is the primary signal for adrenal cortisol biosynthesis. The stress-induced release of glucocorticoids is known to inhibit the immune [31], reproductive [20,32], and endocrine [33] systems. Furthermore, exogenous ACTH treatment in cattle, which increases endogenous cortisol secretion, induces follicular cysts [34,35]. An intraovarian effect of ACTH has been demonstrated in fish, where ACTH suppresses gonadotrophin-stimulated E2 production in ovarian follicles, and the expression of MC2R in ovaries and testes suggest a role for ACTH in modulating gonadal function throughout MCRs [4]. In agreement with this, in the present study, MC2R mRNA (the major signaling receptor for ACTH in the adrenal gland) and MC5R mRNA were expressed mainly in the theca cells of small and medium follicles, but not in the CL. Because theca cells express *CYP17a1*, a key enzyme in the steroidogenic pathway that produces the precursors of cortisol [4,36], it was expected that cortisol secretion could be stimulated by ACTH, by the presence of their receptors in theca.

In this study, basal secretion of estradiol, progesterone and testosterone were lower in medium than in large antral follicles, in agreement with previous results [37]. In contrast, basal cortisol secretion was higher in medium antral follicles than in large antral follicles. Giving ACTH to gilts during the luteal phase significantly elevated both plasma and intrafollicular cortisol concentrations [38] without disrupting gonadotrophin secretion [39]. Acosta et al [40] reported a temporal rise in follicular cortisol concentrations occurred around the preovulatory LH surge in cattle and that although concentrations of active glucocorticoids were suppressed during ovarian follicular development and maturation, they were increased during the ovulatory process triggered by an LH surge, suggesting a functional role for ACTH in the stimulation of ovarian cortisol secretion. In addition, that the follicular wall of medium antral follicles had higher levels of MCRs could be related to the stimulatory effect of ACTH on estradiol and cortisol secretion. The expression of MC2R could also be associated with the stimulatory effect of ACTH on testosterone secretion in large antral follicles, perhaps associated with a decrease in P450 aromatase activity. If we take into account the differentiation of follicular walls, the increased ACTH secretion previously reported during stress in cattle [41] could be responsible for the increase in testosterone that characterized cystic follicles during COD in cattle.

By contrast, evidence has accumulated to support the role of the immune system in reproduction. In these processes, cytokines act through either a paracrine or autocrine mechanism and are involved in follicle rupture and remodeling, leukocyte infiltration, angiogenesis, steroid hormone production, and oocyte maturation [42,43]. In addition, many features of the ovulatory process are common in the inflammatory process, and ovulation is often likened to an acute inflammation [43,44]. In these contexts, our findings contributed to the hypothesis that attributed anti-cytokine and anti-inflammatory effects to melanocortin peptides [45]. Virtually all cells responsive to the anti-inflammatory effect of melanocortins express MC1R, the receptor with the greatest affinity for α-MSH [1]. Consistent with the idea of multiple receptor involvement in the anti-inflammatory effects of melanocortins, we found the evident expression of MC1R and MC3R in the CL and some components of the follicular wall, which could indicate a close interaction between the immune and reproductive functions mediated by melanocortins. Also, although neither MC3R nor MC4R knockout mice have shown any obvious malfunction in reproduction [2], the CL was decreased in MC4R−/− mice [46], supporting a role in the ovarian physiology.

To conclude, we demonstrated for the first time that MCRs mRNA were present in bovine ovaries and that ACTH affected steroid secretion in bovine ovarian follicles. We inferred that melanocortins, through their stimulatory effect on steroid secretion, could be directly involved in the regulatory mechanisms of ovarian function.

Acknowledgments

We are grateful to Michael López Cepeda for technical support. This study was supported by a grant from the Argentine National Agency for the Promotion of Science and Technology (ANPCyT) (PICT 2007-01193) and MINCYT (Argentina)-CONICYT (Chile) International cooperation grant CH/08/04.

References

[1] Catania A, Gatti S, Colombo G, Lipton JM. Targeting melanocortin receptors as a novel strategy to control inflammation. Pharmacol Rev 2004;56:1–29.

Author's personal copy

[2] Schioth HB, Watanobe H. Melanocortins and reproduction. Brain Res Brain Res Rev 2002;38:340–50.
[3] Aluru N, Vijayan MM. Molecular characterization, tissue-specific expression, and regulation of melanocortin 2 receptor in rainbow trout. Endocrinology 2008;149:4577–88.
[4] Alsop D, Ings JS, Vijayan MM. Adrenocorticotropic hormone suppresses gonadotropin-stimulated estradiol release from zebrafish ovarian follicles. PLoS One 2009;4:e6463.
[5] Nimura M, Udagawa J, Hatta T, Hashimoto R, Otani H. Spatial and temporal patterns of expression of melanocortin type 2 and 5 receptors in the fetal mouse tissues and organs. Anat Embryol (Berl) 2006;211:109–17.
[6] Chhajlani V. Distribution of cDNA for melanocortin receptor subtypes in human tissues. Biochem Mol Biol Int 1996;38: 73–80.
[7] Raikhinstein M, Hanukoglu I. Mitochondrial-genome-encoded RNAs: differential regulation by corticotropin in bovine adrenocortical cells. Proc Natl Acad Sci U S A 1993;90:10509–13.
[8] Vanetti M, Schonrock C, Meyerhof W, Hollt V. Molecular cloning of a bovine MSH receptor which is highly expressed in the testis. FEBS Lett 1994;348:268–72.
[9] Liakos P, Chambaz EM, Feige JJ, Defaye G. Expression and regulation of melanocortin receptor-5 (MC5-R) in the bovine adrenal cortex. Mol Cell Endocrinol 2000;159:99–107.
[10] Haegeman A, Coopman F, Jacobs K, Mattheeuws M, Van ZA, Peelman L. Bovine melanocortin receptor 4: cDNA sequence, polymorphisms and mapping. Anim Genet 2001;32:189–92.
[11] Maudet C, Taberlet P. Holstein's milk detection in cheeses inferred from melanocortin receptor 1 (MC1R) gene polymorphism. J Dairy Sci 2002;85:707–15.
[12] Doghman M, Delagrange P, Blondet A, Berthelon MC, Durand P, Naville D, et al. Agouti-related protein antagonizes glucocorticoid production induced through melanocortin 4 receptor activation in bovine adrenal cells: a possible autocrine control. Endocrinology 2004;145:541–7.
[13] Valle E, Habermann FA, Moore SS, Crews DH, Benkel BF. Genomic localization and SNP discovery in the bovine melanocortin receptor 4 gene (MC4R). Anim Genet 2004;35:351–2.
[14] Gan HY, Li JB, Wang HM, Gao YD, Liu WH, Li JP, et al. [Relationship between the melanocortin receptor 1 (MC1R) gene and the coat color phenotype in cattle]. Yi Chuan 2007; 29:195–200.
[15] Mohanty TR, Seo KS, Park KM, Choi TJ, Choe HS, Baik DH, et al. Molecular variation in pigmentation genes contributing to coat colour in native Korean Hanwoo cattle. Anim Genet 2008; 39:550–3.
[16] Hohmann JG, Teal TH, Clifton DK, Davis J, Hruby VJ, Han G, et al. Differential role of melanocortins in mediating leptin's central effects on feeding and reproduction. Am J Physiol Regul Integr Comp Physiol 2000;278:R50–9.
[17] Moberg GP. A model for assessing the impact of behavioral stress on domestic animals. J Anim Sci 1987;65:1228–35.
[18] Kawate N, Inaba T, Mori J. Changes in plasma concentrations of gonadotropins and steroid hormones during the formation of bovine follicular cysts induced by the administration of ACTH. J Vet Med Sci 1996;58:141–4.
[19] Liptrap RM, McNally PJ. Steroid concentrations in cows with corticotropin-induced cystic ovarian follicles and the effect of prostaglandin F2alpha and indomethacin given by intrauterine injection. Am J Vet Res 1976;37:369–75.
[20] Liptrap RM. Stress and reproduction in domestic animals. Ann N Y Acad Sci 1993;697:275–84.
[21] Dobson H, Ribadu AY, Noble KM, Tebble JE, Ward WR. Ultrasonography and hormone profiles of adrenocorticotrophic hormone (ACTH)-induced persistent ovarian follicles (cysts) in cattle. J Reprod Fertil 2000;120:405–10.
[22] Barb CR, Kraeling RR, Rampacek GB, Fonda ES, Kiser TE. Inhibition of ovulation and LH secretion in the gilt after treatment with ACTH or hydrocortisone. J Reprod Fertil 1982;64: 85–92.
[23] Li PS, Wagner WC. In vivo and in vitro studies on the effect of adrenocorticotropic hormone or cortisol on the pituitary response to gonadotropin releasing hormone. Biol Reprod 1983; 29:25–37.
[24] Peter AT, Levine H, Drost M, Bergfelt DR. Compilation of classical and contemporary terminology used to describe morphological aspects of ovarian dynamics in cattle. Theriogenology 2009;71:1343–57.
[25] Sudo N, Shimizu T, Kawashima C, Kaneko E, Tetsuka M, Miyamoto A. Insulin-like growth factor-I (IGF-I) system during follicle development in the bovine ovary: relationship among IGF-I, type 1 IGF receptor (IGFR-1) and pregnancy-associated plasma protein-A (PAPP-A). Mol Cell Endocrinol 2007;264: 197–203.
[26] Lagaly DV, Aad PY, Grado-Ahuir JA, Hulsey LB, Spicer LJ. Role of adiponectin in regulating ovarian theca and granulosa cell function. Mol Cell Endocrinol 2008;284:38–45.
[27] Shibaya M, Matsuda A, Hojo T, Acosta TJ, Okuda K. Expressions of estrogen receptors in the bovine corpus luteum: cyclic changes and effects of prostaglandin F2alpha and cytokines. J Reprod Dev 2007;53:1059–68.
[28] Acuna E, Fornes R, Fernandois D, Garrido MP, Greiner M, Lara HE, et al. Increases in norepinephrine release and ovarian cyst formation during ageing in the rat. Reprod Biol Endocrinol 2009;7:64.
[29] Dorfman M, Ramirez VD, Stener-Victorin E, Lara HE. Chronic-intermittent cold stress in rats induces selective ovarian insulin resistance. Biol Reprod 2009;80:264–71.
[30] Getting SJ. Targeting melanocortin receptors as potential novel therapeutics. Pharmacol Ther 2006;111:1–15.
[31] Sapolsky RM. Stress hormones: good and bad. Neurobiol Dis 2000;7:540–2.
[32] Echternkamp SE. Relationship between LH and cortisol in acutely stressed beef cows. Theriogenology 1984;22:305–11.
[33] Vighio GH, Liptrap RM. Plasma hormone concentrations after administration of dexamethasone during the middle of the luteal phase in cows. Am J Vet Res 1990;51:1711–4.
[34] Ribadu AY, Nakada K, Moriyoshi M, Zhang WC, Tanaka Y, Nakao T. The role of LH pulse frequency in ACTH-induced ovarian follicular cysts in heifers. Anim Reprod Sci 2000;64: 21–31.
[35] Ortega HH, Palomar MM, Acosta JC, Salvetti NR, Dallard BE, Lorente JA, et al. Insulin-like growth factor I in sera, ovarian follicles and follicular fluid of cows with spontaneous or induced cystic ovarian disease. Res Vet Sci 2008;84:419–27.
[36] Garverick HA, Baxter G, Gong J, Armstrong DG, Campbell BK, Gutierrez CG, et al. Regulation of expression of ovarian mRNA encoding steroidogenic enzymes and gonadotrophin receptors by FSH and GH in hypogonadotrophic cattle. Reproduction 2002;123:651–61.
[37] Gregoraszczuk EL, Wojtowicz AK, Ptak A, Nowak K. In vitro effect of leptin on steroids' secretion by FSH- and LH-treated porcine small, medium and large preovulatory follicles. Reprod Biol 2003;3:227–39.

Author's personal copy

[38] Montgomery A, Viveiros M, Cummings E, Liptrap R. Rate of decline of cortisol concentrations in ovarian follicles following ACTH treatment in the sow. Can J Vet Res 1997;61: 309–11.
[39] Frautschy SA, Liptrap RM. Anovulation and plasma hormone concentrations after administration of dexamethasone during the middle of the luteal phase in sows undergoing estrous cycles. Am J Vet Res 1988;49:1270–5.
[40] Acosta TJ, Tetsuka M, Matsui M, Shimizu T, Berisha B, Schams D, et al. In vivo evidence that local cortisol production increases in the preovulatory follicle of the cow. J Reprod Dev 2005;51:483–9.
[41] Dobson H, Smith RF. Stress and reproduction in farm animals. J Reprod Fertil Suppl 1995;49:451–61.
[42] Kucharski J, Jana B. Immuno-endocrine mechanisms connected with the creation of corpora lutea persistent in animal ovaries. Pol J Vet Sci 2005;8:255–9.
[43] Bukovsky A, Caudle MR. Immune physiology of the mammalian ovary - a review. Am J Reprod Immunol 2008;59:12–26.
[44] Espey LL. Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction. Biol Reprod 1994;50:233–8.
[45] Lipton JM, Catania A. Anti-inflammatory actions of the neuroimmunomodulator alpha-MSH. Immunol Today 1997;18:140–5.
[46] Sandrock M, Schulz A, Merkwitz C, Schoneberg T, Spanel-Borowski K, Ricken A. Reduction in corpora lutea number in obese melanocortin-4-receptor-deficient mice. Reprod Biol Endocrinol 2009;7:24.