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Intrafollicular expression and potential regulatory role of cocaine- and amphetamine-regulated transcript in the ovine ovary

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ABSTRACT

Follicular growth is regulated by a complex interaction of pituitary gonadotropins with local regulatory molecules. Previous studies demonstrated an important role for cocaine- and amphetamine-regulated transcript (CART) in regulation of granulosa cell estradiol production associated with dominant follicle selection in cattle. However, intraovarian expression and actions of CART in other species, including sheep, are not known. The objective of this study was to investigate the expression of CART in sheep follicles and determine the effects of CART on indices of ovine granulosa cell function linked to follicular development. Results demonstrated the expression of CART messenger RNA and prominent intraovarian localization of CART peptide in granulosa cells of sheep follicles. Granulosa cell CART messenger RNA was lower, but follicular fluid estradiol concentrations were higher in large (>5 mm) follicles vs smaller 3- to 5-mm follicles harvested from sheep ovaries of abattoir origin. CART treatment inhibited follicle stimulating hormone-induced estradiol production by cultured ovine granulosal cells and also blocked the follicle stimulating hormone-induced increase in granulosa cell numbers. Results demonstrate expression of CART in sheep follicular tissues and suggest potential biological actions of CART, which are inhibitory to ovine follicular growth and development.

1. Introduction

Ovarian follicular development is a cornerstone of the reproductive process because only mature ovarian follicles release eggs to be fertilized. Granulosa cell production of estradiol is essential for follicular growth and triggers the preovulatory gonadotropin surge that triggers resumption of meiosis and ovulation [1,2]. Although the key role of pituitary gonadotropins, such as FSH, in regulation of follicular growth and estradiol production is well established, the potential influence and/or role of local intrafollicular regulatory molecules is not completely understood.

Evidence supports a novel local role for cocaine- and amphetamine-regulated transcript (CART) in regulation of antral follicle growth and development in cattle. Concentrations of CART in follicular fluid of healthy follicles are

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higher before than after dominant follicle selection, and *CART* messenger RNA (mRNA) is greater in atretic vs healthy follicles collected before and early after initiation of follicle dominance [3]. Furthermore, the CART peptide is a strong inhibitor of FSH-induced estradiol production and aromatase mRNA [4,5] and can also inhibit estradiol production induced by IGF1 [6] and BMP2 [7] in vitro. CART can also inhibit follicular estradiol production after intrafollicular injection in vivo [3]. Collectively, results support a potential regulatory role for CART in dominant follicle selection in cattle.

In mammals, CART is an important neuropeptide and is mainly found in the central nervous system [8–11], peripheral nervous system [12], gut [13], adrenal medulla [9,14,15], and stomach [16]. The previously described local role of CART in regulation of follicular development in cattle does not appear to be conserved across mammalian species. To our knowledge, a role for CART in the mouse ovary has not been reported. Studies reported by Murphy et al [17] indicated CART is undetectable in mouse ovaries and CART mutant mice are fertile [18]. We previously proposed that CART may be a species-specific regulator of antral follicle development during follicular waves. Although many sheep breeds typically ovulate more than a single ovulatory follicle, sheep do exhibit wavelike characteristics of follicular growth and development [19]. Hence, the objectives of described studies were to determine whether CART is expressed in sheep ovaries, the intrafollicular cell types expressing CART and the relationship of CART expression with steroidogenic capacity and/or follicle size, and to determine the effects of CART treatment on granulosa cell numbers and FSH-induced estradiol production in vitro.

2. Materials and methods

2.1. Animals and sample collection

All experiments were performed under protocols approved by the Animal Care and Use Committee at the Shanxi Agricultural University. Sheep ovaries of sexually mature ewes were collected from a local slaughterhouse (Jinzhong area, Shanxi, China) and transported to the laboratory on ice in sterile phosphate-buffered saline. On return to laboratory, pieces of stroma containing ovarian follicles were snap-frozen and stored at −80°C for total ovary RNA isolation. Antral follicles were dissected from remaining ovarian tissue surrounded by 2- to 3-mm ovarian stroma and processed for immunohistochemistry. Remaining ovaries were subjected to follicle dissection. All visible antral follicles were dissected from each pair of ovaries, measured with a caliper, and divided into 2 size classifications (3–5 mm and >5 mm). Follicular fluid was harvested from individual follicles 3 to 5 mm and >5 mm for measurement of follicular fluid estradiol by radioimmunoassay and granulosa cells processed from same follicles for RNA isolation. Granulosa cells from remaining follicles were also harvested and subjected to in vitro culture as described in the following sections.

2.2. RNA isolation and complementary DNA (cDNA) synthesis

Total RNA from sheep ovarian tissue or granulosa cells was isolated using Trizol (TaKaRa, Dalian, China), and dissolved in RNase-free H_2O. The genomic DNA was removed by DNAse digestion, and cDNA was synthesized using PrimeScript RT reagent Kit (TaKaRa) according to the manufacturer's instructions. The cDNA was stored at −20°C until use.

2.3. Cloning of ovine CART partial cDNA

Sequences in the conserved regions sequences of human, pig, and bovine nucleotide sequence found in National Center for Biotechnology Information GenBank Nucleotide database were used to design a forward primer (5′- CTG GAC ATC TAC TCT GCC GT -3′) and a reverse primer (5′- GAA GCG TGG GTG CCT CAT A-3′) for reverse transcription-polymerase chain reaction analysis of CART mRNA expression in the sheep ovary (samples of stroma were collected from ovaries of 3 different animals). A partial CART cDNA fragment was amplified from sheep ovary cDNA using the TaKaRa Ex Taq kit (TaKaRa). The thermal cycler program used consisted of 35 cycles at 98°C for 10 s, 57°C for 30 s, and 72°C for 1 min. The amplified cDNA was cloned into pMD19-T vector (TaKaRa), and plasmids containing inserts were sequenced.

2.4. Immunohistochemical localization of CART peptide in sheep ovary

Samples of stroma were collected from ovaries of 3 different animals. Immunohistochemical localization of CART peptide was conducted as described previously with minor modifications [20]. For immunohistochemistry, follicles were fixed in 4% paraformaldehyde and embedded in paraffin. The paraffin sections were gradually hydrated using a gradient of ethanol, and deparaffinized sections were then treated with 3% H_2O_2 at room temperature for 20 min to block the endogenous peroxidase activity. After blocking with 5% bovine serum albumin for 30 min at room temperature, the sections were incubated with rabbit anti-rat CART (55–102) polyclonal antisera (Phoenix Pharmaceuticals, Belmont, CA) at a 1:1,000 dilution. After 16 h of incubation at 4°C, the sections were incubated with biotinylated mouse anti-rabbit Immunoglobin G (Boster, Wuhan, China) for 30 min followed by detection with Streptavidin-Biotin Complex (Boster). Immunoreactivity was detected using diaminobenzidine substrate (Boster), and slides coverslipped and examined using a microscope.

Parallel controls were performed, including sections incubated with normal rabbit serum (Sijiqing, Hangzhou, China) instead of primary antibody or rabbit anti-rat CART (55–102) polyclonal antisera that had been preincubated overnight at 4°C with 2-μM rat CART (55–102) peptide (American Peptide, Sunnyvale, CA).

2.5. Measurement of the concentration of estradiol in follicular fluid

Follicular fluid was harvested from individual follicles (3–5 mm and >5 mm) for measurement of follicular fluid