Animal Reproduction Science 159 (2015) 52–59

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Natriuretic peptides stimulate oocyte meiotic resumption in bovine

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ARTICLE INFO

Article history:
Received 14 November 2014
Received in revised form 5 May 2015
Accepted 13 May 2015
Available online 21 May 2015

Keywords:
Germinal vesicle breakdown
Forskolin
Cumulus-oocyte complex
Cattle

ABSTRACT

The aim of the present study was to evaluate the expression of mRNA encoding natriuretic peptides (NPs) and their receptors in the cumulus-oocyte complex in cattle, a monovular mammalian species, and also to investigate the role of NPs in oocyte meiotic resumption *in vitro*. mRNA was observed for the NP precursor type-A (*NPPA*), type-C (*NPPC*), NP receptor-1 (*NPR-1*), receptor-2 (*NPR-2*) and receptor-3 (*NPR-3*) in bovine cumulus cells, and *NPR-2* mRNA was observed in oocytes. These results are different from those obtained in mouse and pig models. The effects of NPPA, NP precursor type-B (NPPB) and NPPC on the resumption of arrested meiosis maintained by forskolin were studied at three different doses (10, 100 and 1000 nM) with a 12 h culture system. The germinal vesicle breakdown rates were greater ($P \leq 0.05$) in oocytes that were cultured in the presence of one or a combination of NPs (from 44% to 73%) than the negative control (from 24% to 27%). Additionally, it was demonstrated that the concentration of cyclic guanosine 3′,5′-monophosphate (cGMP) is increased by NPPA and NPPC in oocytes and cumulus cells after 3 h of *in vitro* maturation. However, in both groups, the concentration of cyclic adenosine 3′,5′-monophosphate (cAMP) in the oocyte did not increase between 3 and 6 h of culture, even when forskolin was used. In summary, we observed the presence of mRNA for NPs and their receptors in the bovine cumulus-oocyte complex and demonstrated that, *in vitro*, NPPA, NPPB and NPPC stimulate oocyte meiotic resumption in a monovular species.

1. Introduction

The natriuretic peptides (NPs) system consists of three endogenous peptides with high homology: NP precursor type-A (NPPA), type-B (NPPB) and type-C (NPPC), as well as three receptors (NPR-1, -2 and -3) that are located on the target cell surface (Gardner et al., 2007; Misono et al., 2011). These peptides have mainly been studied in the circulatory system and are critical in the development of female reproductive organs (Tamura et al., 2004). NPPA and NPPB have higher affinities for NPR-1, whereas the main receptor for NPPC is NPR-2 (Potter et al., 2009). Both NPR-1 and NPR-2 are bound to guanylate cyclase and, when stimulated, increase the synthesis of cyclic guanosine 3′,5′-monophosphate (cGMP) (Hsueh et al., 2015). NPR-3 has no guanylate cyclase activity and is responsible for internalization and degradation of the ligand (Potter et al., 2009; Potter, 2011).

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http://dx.doi.org/10.1016/j.anireprosci.2015.05.012
0378-4320/

NPPC is essential to block meiosis in mice (Zhang et al., 2010; Tsuji et al., 2012) and pigs (Hiradate et al., 2013; Zhang et al., 2015). Loss-of-function mutations in either NPPC or NPR-2 result in precocious resumption of meiosis in oocytes that are enclosed within antral follicles (Zhang et al., 2010; Tsuji et al., 2012; Geister et al., 2013). NPPC is mainly synthesized in the mural granulosa cells, and its cognate receptor (NPR-2) is present in large amounts in cumulus cells (Zhang et al., 2010, 2011; Kawamura et al., 2011). Besides stimulating follicular development (Sato et al., 2012), the main reproductive function of NPPC in rodents is preventing resumption of meiosis. In mice, NPPC binds to NPR-2, which stimulates the synthesis of cGMP that diffuses into the oocyte through gap junctions and inhibits phosphodiesterase 3A (PDE3A; also know as oocyte-specific phosphodiesterase), maintaining high levels of cyclic adenosine 3′,5′-monophosphate (cAMP) (Vaccari et al., 2009; Norris et al., 2010; Zhang et al., 2010). In addition, FSH/eCG and estradiol increase the expression of NPPC in mouse granulosa cells (Kawamura et al., 2011; Lee et al., 2013), whereas oocyte-derived paracrine factors and estradiol promote the expression of mRNA and maintain the functionality of NPR-2 in cumulus cells (Zhang et al., 2011; Lee et al., 2013). Conversely, through epidermal growth factor receptor (EGFR), LH/hCG reduces the expression of NPPC in granulosa (Kawamura et al., 2011; Tsuji et al., 2012) and NPR-2 activity in cumulus cells in rodents (Robinson et al., 2012; Wang et al., 2013). However, the expression and functional roles of natriuretic peptides in oocyte meiotic resumption are poorly reported in monovular species.

It has been demonstrated that angiotensin II (Ang II), acting through type-2 receptor (AT2), is essential in the early stages of the ovulatory process (Ferreira et al., 2007) and stimulates resumption of meiosis in cattle (Giometti et al., 2005; Stefanello et al., 2006; Barreta et al., 2008). In the circulatory system, Ang II (signaling through AT2) and NPs have similar functions (Paulis and Unger, 2010). Our hypothesis is that the role of NPs in the bovine reproductive system is to stimulate resumption of meiosis, similar to Ang II. For this reason, the objectives of the present study were to evaluate the expression of mRNA encoding NPs and their receptors in the cumulus-oocyte complex (COC) and to investigate their roles in bovine meiosis resumption *in vitro*.

2. Materials and methods

All experimental procedures were approved by the Federal University of Santa Maria Animal Care and Use Committee (23081.013597/2011—66 CCR/UFSM). All chemicals used were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise indicated.

2.1. Oocyte recovery and nuclear maturation

Bovine ovaries at various stages of the estrous cycle were obtained from an abattoir and transported to the laboratory in 30 °C saline solution (0.9% NaCl) containing 100 IU/ml penicillin and 50 μg/ml streptomycin sulfate. The COCs were aspirated from follicles 3–8 mm in diameter. Only COCs grades 1 and 2 were recovered and selected under a stereomicroscope according to Leibfried and First (1979). After selection, COCs were washed three times in TCM-199 containing Earle's salts and L-glutamine (Gibco Labs, Grand Island, NY, USA) supplemented with 25 mM Hepes, 0.2 mM pyruvic acid, 0.4% fatty acid-free bovine serum albumin (BSA), 100 IU/ml penicillin, and 50 μg/ml streptomycin (TCM wash). Subsequently, the COCs were transferred to four-well culture dishes (Nunc®, Roskilde, Denmark) containing 200 μl of maturation medium with the appropriate treatment. Then, the COCs were cultured at 39 °C in an atmosphere containing 5% CO_2 in air, at 95% relative humidity, for 12 h. The basic culture medium was TCM-199 with Earle's salts and L-glutamine (Gibco Labs, Grand Island, NY, USA) supplemented with 25 mM Hepes, 2.2 mg/ml sodium bicarbonate, 0.2 mM pyruvic acid, 0.5 μg/ml of FSH (Folltropin®-V, Bioniche, ON, CA), 100 IU/ml penicillin, 50 μg/ml streptomycin, and 0.4% BSA.

2.2. Analysis of nuclear maturation

At the end of the culture period, the cumulus cells of COCs were removed by repeated pipetting, and denuded oocytes (DO) were fixed in 4% paraformaldehyde for 15 min, followed by permeabilization of the nuclear membranes with 0.5% Triton X-100. For assessment of nuclear maturation, oocytes were exposed to 10 μg/ml of bisbenzimide (Hoescht 33342) for 15 min. After slides were mounted, stained oocytes were classified under UV light (filter cube +A, with a wavelength of 340–380 nm) with a fluorescence microscope (Leica, DMI 4000B) according to the characteristics of the chromatin: germinal vesicle (GV), GV breakdown (GVBD), and metaphase I (MI). Oocytes that resumed meiosis (GVBD or MI) were described and statistically analyzed as GVBD.

2.3. Nucleic acid extraction, RT-PCR and PCR

After selection, cumulus cells (CC) and denuded oocytes (DO) were separated by vortexing for 5 min. The CC were immediately stored in Trizol, and the DO were exposed to 0.5% proteinase K until the zona pellucida was completely removed. Total RNA from a pool of COCs, DO and CC, was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Total RNA was quantified at 260 nm wavelength using a spectrophotometer (NanoDrop1000, Thermo Scientific). Purity was assessed at an absorption ratio of 260/280 nm, and samples with values below 1.8 were not used.

Total RNA (1 μg) was first treated with 0.2 U DNase (Invitrogen) at 37 °C for 5 min to digest any contaminating DNA, followed by heating at 65 °C for 3 min. RNA was reverse-transcribed (RT) in the presence of 1 μM oligo (dT), 4 U Omniscript RTase (Omniscript RT Kit; Qiagen, Mississauga, ON, Canada), 0.5 mM dideoxynucleotide triphosphate (dNTP) mix, and 10 U RNase inhibitor (Invitrogen) in a volume of 20 μl at 37 °C for 1 h. The reaction was ended by incubation at 93 °C for 5 min. PCR was performed using StepOnePlus™ Real-Time PCR (Applied Biosystems, Foster City, CA, USA) with Platinum SYBR Green Master Mix (Applied Biosystems) and specific primers (listed