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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Tumor necrosis factor receptor type-1 is not expressed on granulosa cells of pre-ovulatory follicles in sheep

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Abstract

This study was conducted to examine the expression of tumor necrosis factor receptor type1 (TNFR-I) on granulosa cells of pre-ovulatory follicles in sheep. In all ewes (n=15), estrus was synchronized with the Controlled Internal Drug Release CIDR (12 day). The granulosa cells were collected from pre-ovulatory follicle 48 hours after CIDR withdrawal. Expression of mRNA was investigated using a reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from cells and then reverse-transcribed to synthesize cDNA and PCR-amplified using specific oligonucleotide primers. Result of present study indicates TNFR-1 was not expressed at the mRNA level. In general, this finding provides further insight into the proposed physiologic roles of TNFα in ovarian function.

Keywords: Sheep; TNFR-I; Pre-ovulatory Follicles; granulosa cells; gene expression

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Introduction

There is an increasing body of evidence that tumor necrosis factor alpha (TNFα) is involved as regulatory factor in follicular development (Sakumoto et al, 2004). TNFα has been found in the granulosa cells, theca cells and corpus luteum of ovaries of various species, e.g., the rats (Sancho-tello et al, 1995), mice (Chen et al, 1993), rabbits (Bagavandoss et al, 1990), pigs (Wajant et al, 2003), cows (Sakumoto et al, 2000), sheep (Murdoch et al, 1997) and humans (Roby et al, 1990). There ovarian processes regulating expression of ovarian TNFα are follicular development, ovulation and luteal regression. Potential role of TNFα in follicular development is reduction of the number of LH receptors, adenylate cyclase activity, cAMP, protein kinase A and cholesterol side chain cleavage (CSSC) mRNA expression (Terranova, 1997). TNFα act via two distinct cell surface receptors, TNFR-I (55–60 kDa) and TNFR-II (75–80 kDa). Although the extracellular domains of these receptors exhibit high sequence homology, their intracellular domains are distinct indicating different signaling pathways are activated by ligand-receptor binding (Wajant et al, 2003). TNFR-I contains an intracellular death domain, which is required for signaling pathways associated with apoptosis. In contrast, TNFR-II can induce gene transcription for cell survival, growth and differentiation clearly, functional receptors must be present if TNFα is to play a role in ovarian function or development (Sakumoto et al, 2004). TNFα receptor are present in the Cow granulosa and theca cells (Sakumoto et al, 2003), pig (Richards et al, 1994), human (Rajesh et al, 1997), rat (Balchak et al, 1999). In bovine granulosa from small (1-5mm) follicle, TNFα via TNFR-I inhibit FSH-induced estradiol production.

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Similar results have been observed in human granulosa cells (Terranova, 1997). Inverse in the follicle of pig TNFα (via TNFR-II) acts as a survival factor (Nakayama et al., 2003). Although the presence, modulation and possible role of TNFR has been extensively explored in ovarian function in various species, the expression of TNFα and its receptors has not been investigated in the granulosa cell of sheep. The present study was conducted to examine the expression of TNFR-I in granulosa cells (GC) of pre-ovulatory follicles in sheep.

Materials and Methods

Granulosa cell preparation

The trial, performed during the breeding season, involve a total 15 ewes. All the sheep were 2-4 years old. They were maintained outdoors with access to indoor facilities. Estrus was synchronized in all animals, with the Controlled Internal Drug Release CIDER (12 day). Ewes were also injected a dose of 500 IU PMSG (Pregnant Mare's Serum Gonadotropin) after CIDER removal and estrous detection was performed with adult rams at 24 h after. The granulosa cells were collected from pre-ovulatory follicle 48 hours after CIDR withdrawal. Briefly ewes were surgically operated and the follicular fluid of pre-ovulatory follicles was aspirated from the surface of ovary using a 5 ml syringe and an 18 G needle. The Granulosa cells were separated of follicular fluid with the method described by Zheng et al (Zheng et al., 1992). Cumulus-Oocyte Complex (COC) and additional tissue were separated from follicular fluid via mouth pipette and then granulosa cells were collected, washed in PBS (phosphate-buffered saline) and centrifuged (250 g, 10 min) twice. The supernatant was discarded and the granulosa cells were used for RT-PCR analyses.

RT-PCR Analysis

For RT-PCR analysis, total RNA was extracted from granulosa cell samples using an RNeasy mini kit (Qiagen). cDNA was constructed from total RNA extracted. 11 µl of total RNA and 0.5 µg (100 pmol of oligo d(T)15 were mixed and incubated at 65°C for 5 min. Then 4 µl 5X reaction buffer, 0.5 µl (20 u) RiboLock™ RNase Inhibitor, 2 µl dNTP Mix and 2 µl (40 u) M-MuLV Reverse Transcriptase was added to primary Mixture. Reverse transcription was allowed to occur at 37°C for 1 hr. The reaction mixture was then incubated at 70°C for 10 min and immediately chilled on ice. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was carried out with the housekeeping gene, β-Actin, as an internal standard. The RT-PCR amplification was calibrated in order to determine the optimal number of cycles that would allow detection of the appropriate mRNA transcripts while still keeping amplification for these genes in the log phase. Primers for the amplification of partial cDNA sequences of β-Actin and TNFR-I were as follows: β-Actin sense, 5'- Tggg ACgA CATg gAgA AgAT CTgg C -3' and antisense 5'- CAgC ACAg CCTg gATg gCCA CgTA C -3'; and TNFR-I sense, 5'- ATgA AATg TTCC AggT ggAg -3' and antisense 5'- ATCT TTAC CAgT TgAA ggTC g -3'. The expected PCR product sizes of βactin and TNFR-I were 189 and 290 bp, respectively. Each polymerase chain reaction (PCR) contained cDNA, 1µl of each primer (10pmol/ µl), 3IU of Taq DNA polymerase, 2µl of PCR buffer, /4µl of dNTP mixture (10mµ/µl)./6µl of $Mgcl_2$ (50mµ/µl) in a final volume of 20µl. The mixture was subjected to PCR in a thermal cycler (Thechne, TC-512). Hot start-PCR conditions for TNFR-I were 94°C for 2 min, 27 cycles at 94°C for 30 sec, 60°C for 60 sec, 72°C for 2 min, and then 1 cycle at 72°C for 10 min. those for β-actin were 94°C for 2 min, 27 cycles at 94°C for 30 sec, 54°C for 30 sec, 72°C for 45 sec, and then 1 cycle at 72°C for 10 min. Seven microliters from each reaction were run on a 1/2% (w/v) agarose gels and stained with ethidium bromide. A ready-load 100 bp DNA ladder (Gibco) was used as a molecular weight marker for electrophoresis. The resultant bands under UV light were documented with the Eagle Eye video system. All RT-PCR reactions were performed twice for each RNA sample. To ensure that reactions did not reach a plateau in synthesis.

Results

Figure 1 and 2 show the results of TNFR-I mRNA and β-Actin mRNA expression in granulosa cells of pre-ovulatory follicles. PCR amplification of the reverse-transcribed RNA from granulosa cells did not show any band in the ethidium bromide-stained gel. In contrast, β-actin PCR amplification of reverse-transcribed RNA showed the expected 189-bp band in the ethidium bromide-stained gel.

Discussion

Our result indicates that receptors type I (p60, TNFR-I) was not expressed in granulosa cells of pre-ovulatory follicles at the mRNA level. Here, we consider for the first time expression of TNFR-I in granulosa cells of antrum follicle in sheep, therefore, this finding is interesting. TNFR-I has been found in cells/tissues of the reproductive system including theca cells, granulosa cells and corpus luteum of various species, e.g., the rat (Sakumoto et al., 2004), pig (Richards et al., 1994), human (Rajesh et al., 1997) and cow (Sakumoto et al., 2003). The negative result obtained for TNFR-I in the RT-PCR was not due to RNA degradation in the samples since β-actin was

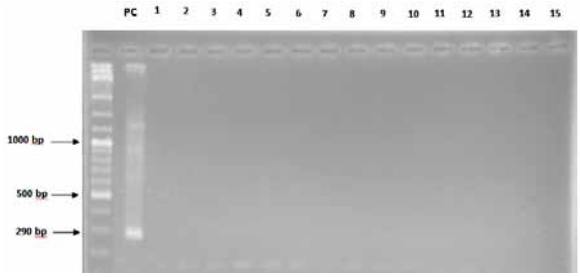

Fig. 1: Agarose gel electrophoresis (1/2%) image for RT-PCR product of the TNFR- mRNA from 15 samples and positive control (PC)

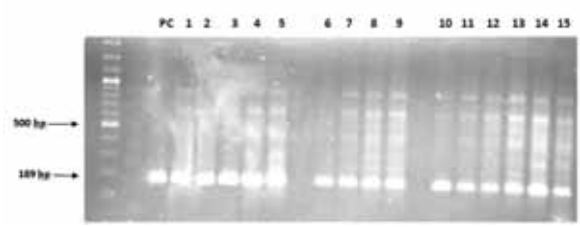

Fig. 2: Agarose gel electrophoresis (1/2%) image for RT-PCR product of the β-Actin mRNA as a control from 15 samples and positive control (PC)

expressed in all RNA preparations. There are several possible reasons for this discrepancy. Many findings demonstrated that TNFα and its receptors gene expression occurs within the ovarian cells at a specific developmental stage of the follicle. Veldhuis et al. (1991) found an increase in binding capacity of TNFα to TNFR-I at porcine granulosa cells after FSH/insulin treatment, suggesting an increase of receptor expression in granulosa cells during luteinization (Veldhuis et al., 1991). In human, Rajesh et al. (1997) showed that TNFR-I was not expressed in mature oocytes and cumulus cells at either the mRNA or protein level, whereas, these cells express TNFα as well as TNFR-II. Balchak et al. (1999) demonstrated that rat ovarian cells at critical stages of development (Embryonic Day 19, day of birth (Day 0), and Days 2, 5, 10, and 20) contained mRNA for both the p60 and p80 TNFα receptors but Messenger RNA for TNFR-I was significantly greater for the 60-kDa receptor on Day 20 (on Day 20 ovaries contain many large antral/pre-ovulatory follicles that contain a highly developed thecal and granulosa layer). Nakayama et al. (2003) detected expression of TNFR-II in granulosa cells of healthy follicles of pig ovaries, and TNFR-II disappeared during follicular atresia. They proposed that TNFα and its receptor system play an important role in induction of survival/proliferating signals in granulosa cells during follicular growth in porcine ovaries. In mouse Chen et al. (1993) indicated that TNFα gene expression occurs within the oocyte at a specific developmental stages of the follicle (type 4 follicles through type 7-8) and also during the later stages of follicular atresia. Similar to these reports you can refer to Terranova (1997) review. In this study, we chose pre-ovulatory antral follicles and at this stage follicles can grow and progress to the next stage (Scaramuzzi et al., 1993). Our results are similar to the findings obtained by Nakayama et al. (2003). In two studies presumably TNFα plays an important role in induction of survival/proliferating signals in granulosa cells during follicular growth because follicular development may be a consequence of suppression of death genes and/or over-expression of survival genes. In this study (*in vivo* study), we separated granulosa cells from ovary of alive animals 48 hours after CIDR withdrawal whereas in other studies conducted in cows (Spicer et al, 1994) and pigs (Prange et al, 2001), ovaries were obtained from the slaughtered animals. In such situation, we do not know the exact stage of estrous cycle of animals. This is significant in the results of RT-PCR analysis because presences of TNFα and its receptors mRNA in ovarian cells was stage-specific, synchronized and transient (Chen et al., 1993). Presumably, the expression and function of TNFα and its receptors could be affected by different levels of ovulation rate. The fate of the follicle is dependent on a delicate balance in the expression and actions of factors promoting follicular cell proliferation, growth and differentiation and of those inducing programmed cell death (Jang et al., 2003). The physiologic role of TNFα in ovary will depend on a balance between apoptosis-inducing and cell survival signaling (Fas/FasL, XIAP, FLIP, TGF-alpha and FSH) (Jang et al., 2003). Therefore, in species with high ovulation rate (for example sheep and pig) that over-expression of survival genes are dominant, presumably TNFR-II will express. No expression of TNFR-I in this study could be due to this reason. Nakayama et al. (2003) demonstrated TNFα and its receptor (TNFR-II) system play an important role in induction of survival/proliferating signals in granulosa cells during follicular growth in porcine ovaries (Nakayama et al., 2003). However, in bovine granulosa cells from small follicle, TNFα inhibited FSH-induced estradiol production (Terranova, 1997). Similar result has been observed in human granulosa cells (Terranova, 1997).

Conclusion

In conclusion, our findings indicate that sheep pre-ovulatory granulosa cells do not express TNFR-I. These findings provide further insight into the proposed physiologic roles of TNFα in ovarian function, especially in modulation of steroidogenesis. This finding also advances our knowledge of physiological mechanism of ovulation rate in sheep.

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