Hormonal regulation and differential expression of neuropilin (NRP)-1 and NRP-2 genes in bovine granulosa cells

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Abstract

Although much is known about the biology of vascular endothelial growth factor (VEGF) and its receptors, little is known about the role of the VEGF receptors neuropilin (NRP)-1 and NRP-2 in the process of bovine follicle development. The aim of the present study was to examine the hormonal regulation of NRP-1 and NRP-2 mRNAs by real-time PCR in follicles from the bovine ovary and in cultured granulosa cells. The NRP-1 gene was expressed in the granulosa and theca cells in the post-selection (POF) and pre-selection (PRF) follicles in the bovine ovary. In contrast, the NRP-2 gene was expressed only in the theca cells in the POF and the PRF. The level of NRP-1 mRNA was significantly increased by treatment of the cultured granulosa cells with 10 ng/ml estradiol (E2). In contrast, the addition of progesterone (P4) to the culture medium decreased the expression of the NRP-1 gene. The level of NRP-1 mRNA was increased by 10 ng/ml E2 with or without 1 ng/ml P4, but the level of NRP-1 mRNA was decreased if the P4 level was increased to 10 ng/ml, even when 1 ng/ml E2 was also added. Follicle-stimulating hormone did not stimulate the expression of the NRP-1 gene. These results are the first data showing that NRP-1, but not NRP-2, is expressed in the granulosa cells of bovine follicles and that NRP-1 gene expression is regulated by sex steroids. Our findings suggest the involvement of NRP-1 in follicle development in the cow.

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Introduction

The impact of vascular endothelial growth factor (VEGF) as a central regulator of angiogenesis in the ovary was demonstrated in studies using the techniques of gene or protein injection (Hazzard *et al.* 2002, Shimizu *et al.* 2003, Xu *et al.* 2005). VEGF induces signal transduction in the cells via VEGF receptors that belong to the tyrosine-kinase receptor family. The *fms*-like-tyrosine kinase-1 (Flt-1) and fetal liver kinase-1 (Flk-1) bind to VEGF with high affinity. The Flt-1 (or VEGFR-1) and Flk-1 (or VEGFR-2) genes are expressed in the granulosa (Berisha *et al.* 2000) and theca (Shimizu *et al.* 2002) cells of follicles in many species in association with the activation of granulosa cell function and the process of follicle growth in the ovary.

The affinity purification approach revealed that the VEGF receptors in breast cancer MDA-MB-231 cells are encoded by neuropilin-1 (NRP-1) (Soker *et al.* 1998). In addition, the expression cloning approach led to the identification of another receptor for VEGF165, which turned out to be the product of the closely related gene, neuropilin-2 (NRP-2) (He & Tessier-Lavigne 1997, Soker *et al.* 1998). The neuropilins have a short intracellular

domain and are therefore unlikely to function as independent receptors. Indeed, no response to VEGF165 was observed when cells expressing NRP-1 but no other VEGF receptors were stimulated with VEGF165 (Soker *et al.* 1998). It is thus likely that NRP-1 is a VEGF165 coreceptor. This assumption is supported by studies showing that VEGFR-2 binds to VEGF165 more efficiently in cells expressing NRP-1, and that this potentiating effect is subsequently translated into a better migratory response to VEGF165 as compared with the migratory response of cells expressing VEGFR-2 but not NRP-1 (Soker *et al.* 1998).

NRP-1 and NRP-2 are expressed in the ovaries of cyclic (Pavelock *et al.* 2001) and immature (Miyabayashi *et al.* 2005) rats. These reports suggest the possibility that NRP-1 and NRP-2 take part in vascular formation in a manner dependent on follicular development. However, whether NRP-1 and NRP-2 are expressed in the granulosa or theca cells in the ovary is still unknown. In addition, it is still not known whether the expression of these factors is influenced by steroid hormones and follicle-stimulating hormone (FSH). Thus, we examined the expression of NRP-1 and NRP-2 in the granulosa and theca cells of

post-selection and pre-selection follicles in the bovine ovary and the effect of steroid hormones and FSH on the expression of these genes in cultured bovine granulosa cells.

Materials and Methods

Sample collection

Paired ovaries were obtained from 21- to 26-month-old nonparous Holstein × Japanese Black F1 cattle at a local slaughterhouse. Only ovarian pairs with a corpus luteum and apparently normal follicles were used in the present study. Follicular fluid (FF) was aspirated from selected follicles using a syringe fitted with a 20 gauge needle and stored at −20 °C. Theca cell layer and granulosa cells were harvested from aspirated follicles. The tissue samples were placed in the RNA^{later} (Ambion, Austin, TX, USA) and frozen at −30 °C. Follicles were classified into two groups based on the diameter: post-selection follicles (POF) >8.5 mm in diameter; pre-selection follicles (PRF) 7.0–8.5 mm in diameter.

Isolation and culture of bovine granulosa cells

Ovaries were obtained at a nearby slaughterhouse from cows and heifers just after slaughter. After transport to the laboratory at 30 °C, the ovaries were washed three times with pre-warmed McCoy 5A medium. Granulosa cells were collected from medium sized follicles (4–7 mm) by aspiration using a needle (18 gauge) and syringe (plastic, 10 ml) and washed in Dulbecco's modified Eagle's/F12 (DMEM/F12) medium. Then, the cell suspension was centrifuged, resuspended, and seeded at a density of 2- to 5 × 10⁵ cells per well (24-well culture plates) in 1 ml DMEM/F12 containing 10% fetal calf serum (FCS) and antibiotics. The cells were cultured for 24 h at 37 °C in a 5% CO₂ atmosphere and then the wells were washed with DMEM/F12 to remove unattached cells and remaining tissue debris. The culture medium was replaced with serum-free medium supplemented with estradiol-17β (E2, 1–100 ng/ml), progesterone (P4, 1–100 ng/ml) and FSH (1–10 ng/ml) at several concentrations and the culture was continued for 6 h. Treatments were terminated by aspirating the medium and rinsing the cells two times with phosphate-buffered saline, and the cells were used for RNA extraction.

RNA extraction, reverse transcription (RT) and quantitative PCR

Tissue samples were homogenized in denaturing solution containing 4 M guanidium thiocyanate (Wako Pure Chemical Industries, Ltd, Osaka, Japan), 25 mM sodium citrate (Wako), 0.5% sarkosyl (Sigma Chemical Co., St Louis, MO, USA) and 0.1 M β-mercapto ethanol (Kanto Chemical Co. Inc., Tokyo Japan). Total RNA was extracted with phenol-chloroform, further purified, and treated with DNase using a commercial kit (SV Total RNA Isolation System; Promega Co., Madison, WI, USA), and then frozen at −20 °C in RNA Storage Solution (Ambion). Single-strand cDNA was reverse transcribed from total RNA (0.5 μg–5 μg) using a 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics Co., Indianapolis, IN, USA) and random primer. The RT conditions consisted of 10 min annealing at 25 °C, 60 min cDNA synthesis at 42 °C, and 5 min inactivation at 99 °C.

Genes for NRP-1, NRP-2 and β-actin were quantified by real-time PCR with LightCycler (Roche Diagnostics Co.) using a commercial kit (QuantiTect SYBR Green PCR; QIAGEN GmbH, Hilden, Germany). PCR was performed with the following primers: NRP-1 (GenBank accession no. AF395335), 5'-CCA GAA GCC AGA GGA GTA CG-3', 5'-CTT TTC CGA TTT CAC CCT CA-3'; NRP-2 (GenBank accession no. AF 534636), 5'-TTC AGT GCA ATG TCC CTC TG-3', 5'-AGT CCA AGT TGG GTG TCC AG-3' and β-actin, 5'-CCA AGG CCA ACC GTG AGA AGA T -3', 5'-CAA CGT TCC GTG AGG ATC TTC A -3'. The PCR conditions were 2 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 58 °C, and 45 s at 72 °C. The amounts of the product at each step were monitored in real time.

Steroid hormone extraction and assay

Concentrations of E2 in the FF and culture medium, and P4 in the FF samples were estimated using an enzyme immunoassay (EIA) as described previously (Miyamoto *et al.* 1992, Wijayagunawardane *et al.* 1998). Steroid hormones were extracted with diethyl ether. The extraction efficiency was 85%. The ranges of the standard curves were 2–2000 pg/ml for E2 and 0.05–50 ng/ml for P4. The intra- and interassay coefficients of variation were 6.2 and 8.5% for E2, 4.5 and 7.4% for P4.

Table 1 Mean follicle diameter, and concentrations of estradiol (E) and progesterone (P) in follicular fluid, and E/P ratio in cattle. Values are means ± S.E.M.

Follicle	Mean follicle diameter (mm)	Follicular fluid		E/P ratio
		Estradiol (ng/ml)	Progesterone (ng/ml)	
Post-selection follicle (n=4)	15.6 ± 1.6	57.6 ± 13.4	18.7 ± 2.5	3.3 ± 1.1
Pre-selection follicle (n = 5)	7.6 ± 0.2	4.08 ± 1.5*	8.2 ± 1.4*	0.54 ± 0.2*

*P < 0.05 versus post-selection follicle.

Data analysis

All data are presented as means $\pm$ S.E.M. Differences in the expression of NRP-1 and NRP-2 between the POF and PRF were analyzed by Student's *t*-test. Values of NRP-1 and NRP-2 in treated bovine granulosa cells were tested for significant differences using ANOVA, followed by the Fisher's Least Significant Difference test as a multiple comparison test. Differences were considered significant at $P < 0.05$ or less.

Results

Follicle characteristics

Table 1 shows the characteristics of the ovarian follicles used in this study. The concentrations of estradiol and progesterone in follicular fluid were significantly higher in the POF than in the PRF. The ratios of E/P in the POF and PRF were 3.3 ± 1.1 and 0.54 ± 0.2 respectively.

Expression of NRP-1 and NRP-2 mRNA in granulosa and theca cells of POF and PRF

The NRP-1 gene was expressed in the granulosa and theca cells in the POF and the PRF in the bovine ovary (Fig. 1A). In contrast, the NRP-2 gene was only expressed in the theca cells in the POF and the PRF (Fig. 1A). The level of NRP-1 mRNA was significantly higher in the granulosa cells of the POF than in those of the PRF (Fig. 1B). The expression of the NRP-1 and NRP-2 mRNAs in the theca cells showed no significant change between the POF and the PRF (Fig. 1C and 1D).

Effect of estradiol or progesterone on the NRP-1 gene

The level of NRP-1 mRNA in the cultured granulosa cells significantly increased when the cells were treated with 10 ng/ml estradiol (Fig. 2A). In contrast, the addition of progesterone to the culture medium decreased the expression of the NRP-1 gene (Fig. 2B). The level of NRP-1 mRNA in cells treated with 10 ng/ml estradiol plus 1 ng/ml progesterone was the same as that in cells treated with estradiol alone and was significantly increased as compared with the control level (Fig. 2C). In cells treated with 1 ng/ml estradiol plus 10 ng/ml progesterone, the level of NRP-1 mRNA expression did not differ from that in cells treated with 10 ng/ml progesterone alone (Fig. 2D).

Effect of combination of estradiol and FSH on the NRP-1 gene

The expression of the NRP-1 gene in the granulosa cells was not stimulated by FSH (Fig. 3A). Analysis of estradiol production by granulosa cells cultured in serum-free medium showed that FSH did not stimulate estradiol secretion (223 ± 52 , 197 ± 24 , 269 ± 78 and 254 ± 34 pg/ml

Figure 1 (A) Expression of NRP-1 and NRP-2 genes in granulosa (G.C.) and theca cells (T.C.) and (B-D) levels of NRP-1 and NRP-2 mRNA in the granulosa (B) and theca (C and D) cells of post-selection (POF) ($n = 4$) and pre-selection (PRF) ($n = 5$) follicles in the bovine ovary. The mRNA levels were analyzed by a quantitative RT-PCR. The data are expressed as means $\pm$ S.E.M. β -Actin was used as a loading control.

for 0, 1, 5, 10 ng/ml FSH respectively). In cells treated with a constant concentration of 1 ng/ml estradiol, the expression of NRP-1 was not induced even if the FSH concentration in the medium was increased (Fig. 3B).

Figure 2 NRP-1 mRNA expression in cultured bovine granulosa cells is differentially regulated by estradiol and progesterone. The indicated concentration of estradiol (E2) or progesterone (P4) was added to the culture medium. The mRNA levels were analyzed by a quantitative RT-PCR. The data are expressed as means $\pm$ S.E.M. of results obtained in three experiments. Different superscripts denote significantly different values ($P < 0.05$).

Discussion

The present study provides the first evidence that NRP-1, but not NRP-2, is expressed in the granulosa cells of the antral follicle from the bovine ovary, and that the expression of the NRP-1 gene in cultured bovine granulosa cells is enhanced by estradiol and reduced by progesterone. Previous studies showed that NRP-1 and NRP-2 are expressed in the cyclic rat uterus (Pavelock *et al.* 2001) and immature rat ovary (Miyabayashi *et al.* 2005). Our data using the bovine ovary demonstrated that the NRP-1 gene is expressed in granulosa and theca cells, while the NRP-2 gene is only expressed in the theca cells. This result suggests that the functions may differ between NRP-1 and NRP-2 in the bovine ovary during the course of follicle development.

We found that the expression of the NRP-1 gene significantly increased when cultured granulosa cells were treated with 10 ng/ml estradiol. Estradiol is one of the major factors in follicle selection, which is a critical phenomenon in monovular species such as cattle (Ginther *et al.* 2001). Co-expression of NRP-1 and Flk-1 (or VEGFR2) in porcine aortic endothelial cells was reported to enhance VEGF165 binding to Flk-1 and the Flk-1-mediated chemotactic activity of VEGF165, suggesting that NRP-1 is a coreceptor for Flk-1 (Soker *et al.* 1998). VEGF and Flk-1/KDR mRNA and protein were both detectable in follicle tissue sections and in *in vitro* cultured granulosa cells (Greenaway *et al.* 2004). Moreover, the VEGF gene is highly expressed in the granulosa cells of large follicles (dominant) that possess high concentrations of estradiol (Berisha *et al.* 2000). Therefore, our data suggest that estradiol-stimulated NRP-1

Figure 3 NRP-1 mRNA expression in cultured bovine granulosa cells is not regulated by follicle-stimulating hormone (FSH). The indicated concentration of FSH with or without estradiol (E2) was added to the culture medium. The mRNA levels were analyzed by a quantitative RT-PCR. The data are expressed as means $\pm$ S.E.M. of results obtained in three experiments.

expression may be involved in the final growth of the preovulatory (dominant) follicle. On the other hand, the present study demonstrated that P4 reduced the expression of the NRP-1 gene in granulosa cells, suggesting that the expression of NRP-1 gene may be inhibited via the progesterone receptor. However, the mechanism of the regulation of NRP-1 expression by progesterone is currently unknown. Our data basically indicate that estradiol is stimulative and progesterone is suppressive towards NRP-1 expression in bovine granulosa cells.

We also observed that the expression of the NRP-1 gene in bovine granulosa cells was not stimulated by FSH alone. In addition, NRP-1 gene expression was not changed even if a low concentration of estradiol was added to FSH. In general, the expression of various factors such as growth factors is stimulated by the combination of FSH and estradiol (Voge *et al.* 2004). Our data suggest that, in the presence of FSH, estradiol might not affect the expression of the NRP-1 gene in bovine granulosa cells.

In summary, we have shown for the first time the differential expression and hormonal regulation of neuropilins in the bovine ovary. Notably, steroid hormone regulation of the NRP-1 gene in the granulosa cells may be associated with follicular angiogenesis during follicle development. Consequently, additional studies will be needed to examine the detailed expression pathway of the NRP-1 gene during follicle development in the bovine ovary.

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