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Molecular Regulation of Noradrenaline in Bovine Corpus Luteum

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Noradrenergic stimulation increases progesterone, oxytocin and prostaglandins in the bovine luteal tissue. Better understanding of noradrenaline (NA) role in bovine the corpus luteum (CL) can advance our current knowledge on the regulatory mechanism of CL function. The present study was conducted to explore the source of noradrenaline and further to investigate whether nerve growth factor (NGF), insulin like growth factor 1 (IGF1) and transforming growth factor β1 (TGFβ1) influence the expression of dopamine-β-hydroxylase (DBH), biosynthetic enzyme of NA in cultured bovine luteal cells. Corpora lutea were collected and classified into stages of early, developing, mid, late, and regressed. Messenger RNA (mRNA) and protein expression of DBH were studied throughout the estrous cycle. Additionally, DBH protein expression was examined in cultured mid luteal cells after tumour necrosis factor alpha/interferon gamma (TNFα/IFNγ)-induced apoptosis or after treatment with NGF, IGF1, and TGFβ1. DBH mRNA and protein expressions were detected throughout the cycle without significant changes in the protein level while mRNA showed a decrease at the developing stage ($P < 0.05$). Interestingly, NGF, IGF1, and TGFβ1 increased DBH expression in cultured luteal cells ($P < 0.05$). The overall findings suggest non-neural source of noradrenaline in the bovine CL which appears to be regulated by NGF, IGF1, and TGFβ1 indicating intraluteal molecules play an important and unrecognized role in the CL function.

Key words: noradrenaline, apoptosis, bovine, corpus luteum

INTRODUCTION

The corpus luteum (CL) lifespan and function are regulated by luteotropic and luteolytic hormones, growth factors, cytokines, and neurotransmitters (see reviews, Niswender *et al*. 2000; Kotwica *et al*. 2002; Schams & Barisha 2004). Neurotransmitters are believed to play a special role in the neuron-endocrinotrophic stimulatory complex in the ovary (Tsafriri & Adashi 1994). Better understanding of noradrenaline (NA) role in the CL is physiologically important and can advance our current knowledge on the regulatory mechanism of CL function. The ovarian source of catecholamines is believed to be originated from sympathetic nerve fibers or from the adrenal gland via the blood stream. In human and nonhuman primate species, extra source of catecholamines has been reported, so-called, neuron-like cells (Aguado 2002). Since the bovine ovary is innervated by networks of adrenergic nerves which are located in close vicinity to primordial and primary follicles and around the blood vessels (Kaleczyc *et al*. 1995). There is no direct evidence to show that the bovine CL is capable of *de novo* synthesis of catecholamines, or that it has the ability to take up and retain NA from ovarian sympathetic neurons, fibers or the peripheral circulation. The lack of information on the source of endogenous NA raises question to its physiological significance in luteal progesterone and oxytocin stimulation *in vivo and in vitro* (Kotwica *et al*. 1991, 1994).

Noradrenaline is synthesized by a metabolic pathway common to all catecholamines. Amino acid tyrosine, the common precursor of catecholamines, is converted to L-dihydroxyphenylalamine (L-DOPA) by tyrosine hydroxylase (TH). L-DOPA afterwards is converted to dopamine by aromatic L-amino acid decarboxylase (AADC). Both enzymes TH and AADC are cytosolic and dopamine is taken up into vesicles by an uptake mechanism in the vesicular membrane. The enzyme dopamine-3-hydroxylase (DBH), converts the stored dopamine to NA (Parmer & Zinder 2002). There are clear data for insulin-like growth factor (IGF1) (Woad *et al*. 2000; Neuvians *et al*. 2003) and transforming growth factor (TGFβ1) (Maroni *et al*. 2011) in bovine luteal tissue, with regard to specific expression of mRNA, secretion of peptid and receptors. Transforming growth factor (TGF) family members, in particular, nerve growth factor (NGF) and its p57 receptor are believed to be involved in the regulatory mechanism of sympathic innervation (Thoenen & Barde 1980). In the ovary, NGF and both of its receptors, p75 NGFR and the high affinity TrkA tyrosine kinase receptor, are synthesized in thecal cells (Dissen *et al*. 1991, 1996), a prominent terminal field of sympathetic nerve fibers in the ovary (Dissen *et al*. 1991). Neonatal immunoneutralization of NGF blocks the development of ovarian sympathetic innervation and delays follicular growth (Lara *et al*. 1990), highlighting both the critical importance of the trophic factors in supporting the innervation of the ovary and the facilitatory role of sympathetic nerves on follicular development.

Denervation of porcine ovaries during the early luteal phase resulted in anestrous, absence of CLs and reduction in steroidogenic activity suggesting important role of local

innervation in the ovarian function (Jana *et al*. 2005). Several lines of evidence showed the influence of NA on bovine luteal function (Battista *et al*. 1989; Skarzynski & Okuda 2000; Miszkiel & Kotwica 2001). NA stimulates progesterone and oxytocin production by increases the activity of 3β-HSD and cytochrome P-450scc, as well as the activity of peptidylo-glycine-α-amidase a terminal enzyme for oxytocin release (Miszkiel & Kotwica 2001). Moreover, noradrenergic stimulation leads to lipolysis and increases the amount of serum-derived lipoprotein as a source of cholesterol for luteal steroidogenesis (Williams 1989). NA has been reported to increase the ovarian blood flow by up to 60% in guinea pigs (Martensson & Carter 1982). The above actions of NA are mediated by adrenergic receptors. The total number of β-receptors was highly correlated with progesterone concentration throughout the luteal phase in cattle (Pesta *et al*. 1994) while the proportion of β_1/β_2-receptors varied in the luteal phase in sheep (Payne & Cooke 1994). Adrenergic receptors are not only restricted on luteal cells. α-adrenergic receptors were also found in the ovarian and luteal blood vessels (Itoh *et al*. 2005).

The lack of direct and robust evidence on the source of endogenous noradrenaline in the luteal tissue is puzzling in respect to reports indicated the physiological significance of noradrenaline in stimulating luteal progesterone and oxytocin (Battista *et al* 1989; Kotwica *et al*. 1991, 1994, Kotwica & Rekawiecki 2007; Payne & Cooke 1998; Rekawiecki *et al*. 2010), as well as the presence of α-adrenergic (Re *et al*. 2002) and β-adrenergic receptors (Pesta *et al*. 1994) in the bovine CL. In fact, whether the CL receives noradrenergic innervation remains unclear. To our knowledge, the expression of NA biosynthetic enzyme, DBH, has not been reported in the CL of any species. Therefore, in the present study, we studied the expression of DBH mRNA and protein in order to identify the source of noradrenaline in bovine luteal tissues. We also investigated the effect of tumour necrosis factor alpha/interferon gamma (TNFα/IFNγ)-induced apoptosis on DBH protein expression in cultured luteal cells in order to clarify whether luteal regression would influence the expression of DBH. To explore whether locally synthesized NA is regulated by growth factors, we examined the effects of growth factors such as nerve growth factor (NGF) insulin-like growth factor (IGF1) and transforming growth factor (TGFβ1) on DBH protein expression in cultured mid luteal cells.

MATERIALS AND METHODS

Collection of Bovine CL. CLs were collected from Holstein cows at a local slaughterhouse within 10-20 min after exsanguination. The stage of the estrous cycle was defined as described in previous study (Ireland *et al*. 1980) are classified as early (Days 2-3 after ovulation, n = 4), developing (Days 5-6, n = 4), mid (Days 8-12, n = 4), late (Days 15-17, n = 4), and regressed (Days 19-21, n = 4) luteal stages. After determination of these stages, CLs were immediately separated from the ovaries and frozen rapidly in liquid nitrogen, and then stored at -80 °C until processed for studies of mRNA and protein expression. For cell culture, ovaries with mid luteal CLs were submerged in ice-cold physiological saline and transported to the laboratory.

RNA Isolation and cDNA Synthesis. Total RNA was prepared from luteal tissue using TRIZOL Reagent according to the manufacturer's directions (Invitrogen, Carlsbad, CA, No. 15596-026). Total RNA (1 μg) was reverse transcribed to cDNA using a ThermoScript™ RT-PCR System (Invitrogen, No. 11146-016).

Semi-Quantitative Polymerase Chain Reaction. Gene expression analysis was carried out using RT-PCRs with the housekeeping gene *β-actin* as an internal standard. One-tenth of the reaction mixture of cDNA synthesis was used in each PCR using primers for bovine *DBH* and *β-actin* gene. The sequence of *DBH* primers were forward 5'-TGGAGCTGTCCTGGAACAT-3' (19 mer) and reverse 5'- GCCTTCTGGAGTCCTCTGTG -3' (20 mer). These primers generated a specific 252 base pair (bp) product. The sequences of bovine *DBH* primers were based on a report by (Lewis *et al*. 1990). PCR product corresponding to position 161-412 (GenBank accession number NM-180995). The primers for *β-actin* were forward 5'-CGGCATTCACGAAACTACC-3' (19 mer) and reverse 5' ATCAAGTCCTCGGCACAC-3' (18 mer). These primers generated a specific 536 bp product (GenBank accession number NM-173979). The RT-PCR was conducted using a TaKaRa Taq (R001A; TAKARA Co., Tokyo, Japan) and a thermal cycler (iCycler Thermal Cycler 170-8720; Bio-Rad, Hercules, CA). The conditions for the PCR were as follows: after activation of the DNA polymerase by incubating for 2 min at 94 °C, 38 (*DBH*) and 18 (*β-actin*) cycles of reactions including denaturation for 1 min at 94 °C, annealing for 1 min at 58 °C, and extension for 2 min at 72 °C were performed. A portion (40%) of each reaction mixture was electrophoresed on a 1.5% agarose gel with a known DNA standard (100 bp ladder, N3231S; New England BioLabs, Beverly, MA), stained with ethidium bromide, and photographed under UV illumination. The relative band intensities were analyzed by computerized densitometry using NIH image software (National Institutes of Health, Bethesda, MD).

Protein Detection by Western Blot. CL tissues and cultured luteal cells were homogenized on ice in homogenization buffer (25 mM Tris-HCl, 300 mM sucrose, 2 mM EDTA, Complete (protease inhibitor cocktail; Roche Diagnostics GmbH, Mannheim, Germany, 1697498, pH 7.4) by a tissue homogenizer (Physcotron; Niti-on Inc., Chiba, Japan; NS-50), and then frozen in liquid nitrogen, and stored at -80 °C. For DBH protein cytoplasm were isolated from the tissue homogenates by centrifugation at 600 x g for 30 min. Protein concentration was determined by the method of Osnes *et al*. (1993), using BSA as a standard. The proteins were then solubilized in SDS gel-loading buffer (50 mM Tris-HCl, 2% SDS [Nacalai Tesque, Kyoto, Japan, 31607-94], 10% glycerol, 1% β-mercaptoethanol [Wako Pure Chemical Industries Ltd, Osaka, Japan, 137-06862], pH 6.8), and heated at 95 °C for 10 min. Samples (50 μg protein) were subjected to electrophoresis on a 17% SDS-PAGE for 1.5 h at 200 V. The separated proteins were

electrophoretically transblotted to a 0.2 μm nitrocellulose membrane (Invitrogen, LC2000) at 100 V for 2 h in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3). The membrane was then washed in TBS-T (0.1% Tween 20 in TBS [25 mM Tris-HCl, 137 mM NaCl] pH 7.5), and incubated in blocking buffer (4% skim milk in TBS-T) overnight at 4 °C. After the blocking incubation, the membranes was incubated with rabbit anti- bovine DBH (Biosensis, Flagstaff Hill, Australia, R-108-100) diluted 1:2000 for 1 h at room temperature and with *β-actin* antibody (Sigma-Aldrich, MO, USA A2228; 1:1000 in TBS-T). After incubation, the membrane was washed three times for 10 min in TBS at room temperature, and then incubated with 1:20000 in TBS-T secondary antibody (anti-Rabbit IgG, HRP, Amersham Biosciences, NJ, USA, RPN2108) for DBH and with anti-mouse Ig, HRP-linked (Amersham Biosciences, NA931) for *β-actin* (1:20,000 in TBS-TS) for 1.5 h and then washed three times in TBS for 10 min at room temperature. The signal was detected by ECL Western Blotting Detection System (Amersham Biosciences, RPN2109). The intensity of the immunological reaction in the samples was estimated by measuring the optical density in the defined area by computerized densitometry using NIH Image (National Institutes of Health, Bethesda, MD, USA).

Luteal Cell Culture. Only those CLs classified in the mid luteal stage were collected for the cell culture. Luteal tissue was enzymatically dissociated and luteal cells were cultured as described previously (Okuda *et al*. 1992). Dissociated luteal cells from three CLs collected from three different cows were pooled. The luteal cells were suspended in a culture medium, DMEM, and Ham's F-12 medium [D/F; 1:1 (vol/vol); Sigma-Aldrich, Inc.,St. Louis, MO, USA, No.D8900] containing 5% calf serum (Life Technologies, Inc., Grand Island, NY, USA, No. 16170-078) and 20 mg/ml gentamicin (Sigma,No. G1397). Cell viability was greater than 85% as assessed by trypan blue exclusion. The cells in the cell suspension consisted of about 70% small luteal cells, 20% large luteal cells, 10% endothelial cells or fibrocytes, and no erythrocytes. Experiments with isolated cells were performed 3 times each with separate cell preparations.

Effect of TNFα/IFNγ-Induced Apoptosis and NGF, IGF1, and TFGβ1 on DBH Expression in Cultured Luteal Cells. Dispersed luteal cells were cultured at 2.0 x 10^5 viable cells in 100 μl D/F medium containing 5% calf serum in plastic flasks (Greiner Bio-One GmbH, Frickenhausen, Germany, 658-170) for western blot. After 12 h of culture, the medium was replaced with fresh medium containing 0.1% BSA, 5 ng/ml sodium selenite and 5 μg/ml transferrin. The cultured cells were treated with 50 ng/ml TNFα (kindly donated by Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) and 50 ng/ml IFNγ (kindly donated by Dr. S. Inumaru, NIAH, Ibaraki, Japan) to induce apoptosis. After 24 h culture, the total protein was prepared for DBH immunobloting.

After 12 h of culture, the medium was replaced with fresh medium containing 0.1% BSA, 5 ng/ml sodium selenite and 5 pg/ml transferrin. The cultured cells were treated with 100 ng/ml nerve growth factor (NGF) extracted from murine submaxillary gland (Sigma, N6009), 100 ng/ml human recombinant transforming growth factor-β1 (TGF) (Sigma, T7039) or 100 ng/ml human recombinant insulin like growth factor-1 (IGF-1) (Sigma, I 3769). After 24 h cultures, the total protein was prepared for DBH immunobloting.

Statistical Analysis. Statistical significance of differences in the expressions of mRNA and protein throughout the luteal phase or in cultured luteal cells between control and treated groups were assessed by ANOVA followed by the Fisher protected least-significant difference procedure (PLSD) as a multiple comparison test. All experimental data are shown as the mean ± SEM.

RESULTS

mRNA Expression of *DBH* in Luteal Tissue. Specific transcripts for *DBH* mRNA were detected throughout the luteal phase. Semi-quantitative PCR analysis (*DBH* mRNA/*β-actin* mRNA ratio) showed lowest level of *DBH* mRNA expression at the developing stage compared with early luteal stage ($P < 0.05$). Moreover, there were no significant changes in *DBH* mRNA expression among the early, mid, late and regressed luteal stages (Figure 1).

Protein Expression of DBH in Luteal Tissue. DBH protein appeared at the predicted size of 74 kDa. The relative level of DBH protein was steady and there were no significant changes throughout the estrous cycle (Figure 2).

Effect of TNFα/IFNγ-Induced Apoptosis and NGF, IGF1, and TFGβ1 on DBH Expression in Cultured Luteal Cells. Relative protein expression of DBH in cultured luteal cells showed no significant change when apoptosis was induced by TNFα/IFNγ in comparison with the control (Figure 3). The relative levels of DBH protein expression in cultured mid luteal cells increased when cells were treated with NGF, IGF1, and TFGβ1 for 24 h in comparison with the control (Figure 4).

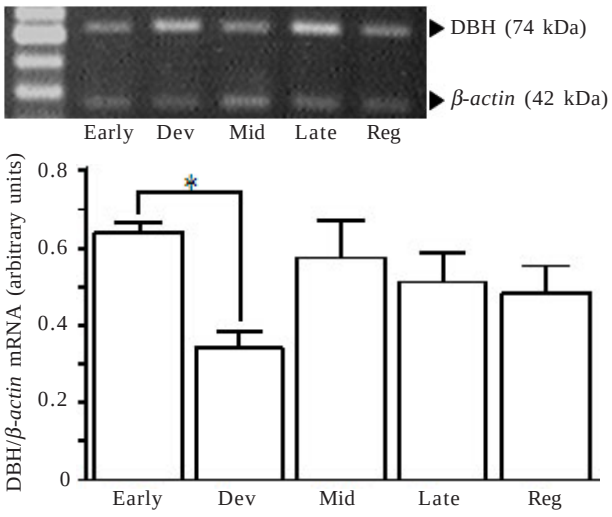

Figure 1. Changes in relative amount of *DBH* mRNA in the bovine luteal tissue throughout the estrous cycle. Representative sample is shown in the lower panel. The ratio of RT-PCR (*DBH* mRNA/*β-actin* mRNA) was presented mean ± SEM. Three separate experiments were carried out, asterisk indicates significant difference ($P < 0.05$) within the luteal phase, as determined by ANOVA followed by a Fisher PLSD as a multiple comparison test.

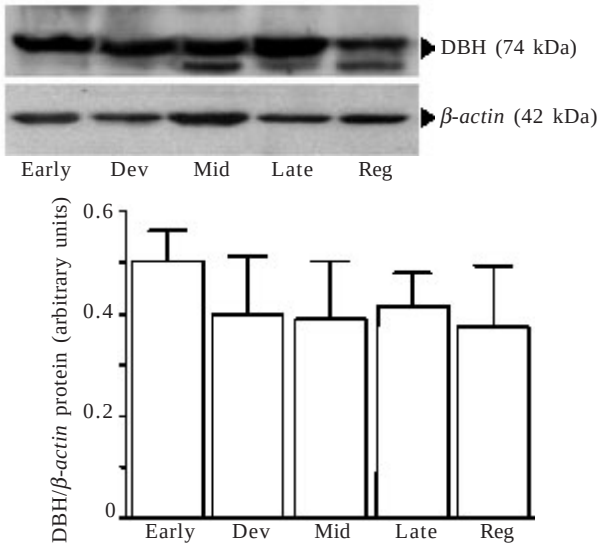

Figure 2. Changes in relative amount of DBH protein in bovine luteal tissue throughout the estrous cycle. The signal was detected by chemiluminiscence and quantified by computer-assisted densitometry. DBH protein appeared at the predicted size of 74 kDa. Representative western blot samples are shown in the upper panel. Four separate experiments were carried out and all values are expressed as mean ± SEM of the densiometric analysis of DBH protein levels in luteal tissues as a relative to the amounts *β-actin* protein. There was no significant change in the relative protein level, as determined by ANOVA followed by a Fisher PLSD as a multiple comparison test.

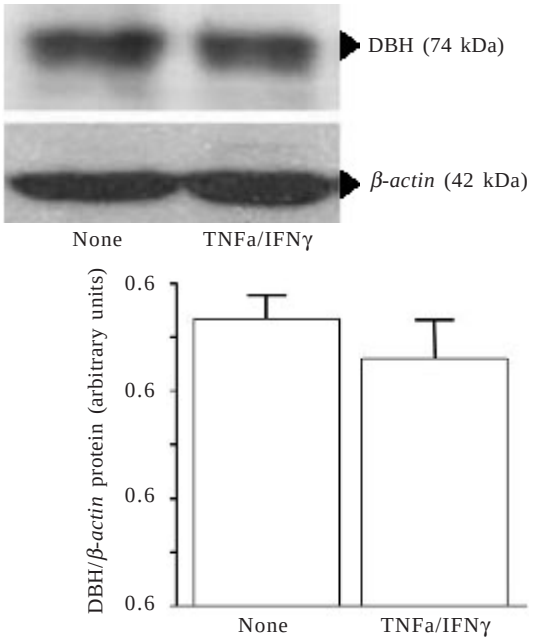

Figure 3. The effect of TNFα/IFNγ on DBH protein expression in 24 h cultured mid luteal cells. Representative western blot samples of DBH protein expression in control and after TNFα/IFNγ-induced apoptosis are shown in the right panel. Detected immunobloting signal was quantified by computer-assisted densitometry. Three separate experiments were carried out. All values expressed as mean ± SEM of the densiometric analysis of DBH protein levels in luteal tissues as a relative to the amounts β-actin protein. No significant changes were detected, as determined by ANOVA followed by a Fisher PLSD as a multiple comparison test.

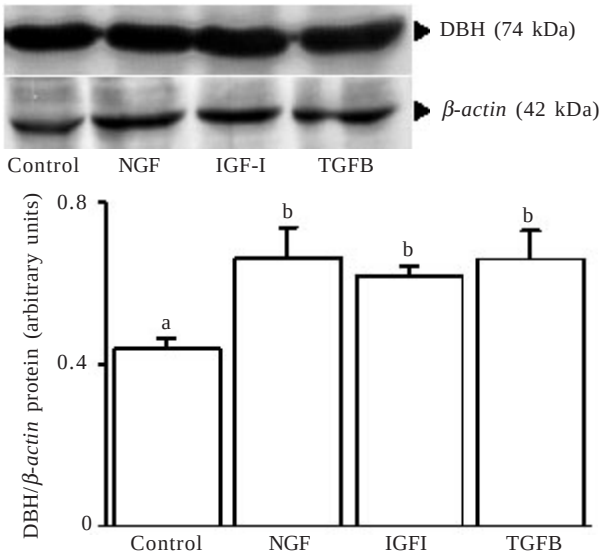

Figure 4. The effect of NGF, IGF-1, and TGFβ1 on DBH protein expression in 24 h cultured mid luteal cells. Representative western blot samples of DBH protein expression of untreated cultured luteal cells or treated cells with NGF, IGF-1, and TGFβ1, respectively are shown in the upper panel. Detected signal was quantified by computer-assisted densitometry. Four separate experiments were carried out.All values are expressed as mean ± SEM of the densiometric analysis of DBH protein levels in luteal cells as a relative to the amounts *β-actin* protein. Different letters indicates significant differences, as determined by ANOVA followed by a Fisher PLSD as a multiple comparison test.

DISCUSSION

The present study demonstrated for the first time DBH expression in non-neural source in the bovine CL. The presence of DBH expression in luteal cells in both luteal tissue sections and cultured mid luteal cells provide clear indication of *de novo* synthesis and non-neural source of NA. The specificity of the antibody was previously demonstrated its reactivity with adrenal chromaffin cells and noradrenergic fibers in the bovine tissues (Varndell *et al*. 1982; Kannisto *et al*. 1986). Earlier study indirectly demonstrated the presence of DBH (Battista *et al*. 1989; Kotwica *et al*. 1996) using DBH inhibitor in cultured bovine luteal cells to block the conversion of dopamine to NA. Non-neural source of NA has been demonstrated in various tissues and cells. The presence of the biosynthetic enzyme of DBH and/or NA production were demonstrated in lymphocytes in human and mouse (Bergquist *et al*. 1994), human oocyte (Mayerhofer *et al*. 1998), amnion epithelial cells (Elwan & Sukaragawa 1997), keratinocytes (Schallreuter *et al*. 1992), gastrointestinal and pancreas cells (Oomori *et al*. 1994a,b). The above studies suggest that NA is produced by non-neural tissue, in addition to its role as a neurotransmitter, NA serves as a local mediator, regulating various physiological functions including energy metabolism and the oxygen consumption (Lünemann *et al*. 2001). Furthermore, cholinergic innervation system in the bovine CL revealed non-neural source acetylcholine in the corpus luteum (Al-Zia'bi *et al*. 2009).

NA in the bovine CL was measured throughout the luteal phase and during pregnancy with highest levels in the newly formed CLs (Miszkiel *et al*. 1999). Our current data showed no changes in DBH expression throughout the luteal phase. These findings may lead us to hypothesize that NA is a multifunctional molecule involved in luteal development, maintenance and regression. Several lines of evidence have shown luteotropic role of NA in the CL by stimulating the production of progesterone and oxytocin (Kotwica *et al*. 1996; Payne & Cooke 1998; Kotwica *et al*. 2002) as well as prostaglandin E (Skarzynski & Okuda 2000). NA has been reported to affect angiogenesis in the CL by controlling the blood flow and pressure in the mature CL (Skarzynski *et al*. 2001) via a-adrenergic receptor (Itoh *et al*. 2005) and also to upregulate the expression of vascular endothelial growth factor in other tissues (Weil *et al*. 2003). In contrast, several studies indicated pro-apoptotic role of NA in various cell types such as endothelial cells (Fu *et al*. 2004) and alveolar epithelial cells (Dincer *et al*. 2001). Since DBH mRNA and protein were expressed in regressed luteal tissue in the present study and NA is released in the regressed CLs (Miszkiel *et al*. 1999). We used our *in vitro* model to explore whether induced apoptosis by TNFα/IFNγ (Taniguchi *et al*. 2002) could affect the expression of DBH in cultured mid luteal cells. Interestingly, there was no effect of TNFα/IFNγ-induced apoptosis on DBH protein expression, confirming our present data in luteal tissue in which DBH mRNA and protein did not change during luteal regression compared with the late and mid luteal stages. On the other hand, NA has been reported to boost prostaglandin F2α production in the cultured luteal cells (Skarzynski & Okuda 2000). Based on our current data and previously reported studies, one could postulate an extra role for NA in luteal regression, in addition to its well known luteotropic roles. Further studies are needed to clarify this assumption.

Several lines of evidence revealed the importance of transforming growth factor (TGF) family members, in particular, nerve growth factor (NGF) in the regulatory mechanism of ovarian sympathic innervation (Lara *et al*. 1990). Neonatal immunoneutralization of NGF blocks the development of ovarian sympathetic innervation and delays the follicular growth (Lara *et al*. 1990). NGF and its receptors have been detected in the luteal cells in hamsters (Shi *et al*. 2004) and in the granulosa cells and theca cells of ovarian follicles (Dissen *et al*. 2000). Since various types of growth factors are expressed in the bovine CL and believed to regulate CL function directly or indirectly by synergistic or antagonistic mechanisms (Schams & Berisha 2004). We hypothesized, therefore, that locally synthesized noradrenaline in the luteal cells is regulated by growth factors. Of our interest, NGF, IGF1, and TGFβ1 were selected due to their well known effect on DBH expression and activity in neural and adrenal chromaffin cells. To test this hypothesis, cultured mid luteal were treated with the above growth factors for 24 h. Our data revealed an increase in DBH expression in cultured luteal cells after growth factors treatment. These findings are in agreement with previous studies demonstrated DBH increasing activity or expression in neural cells and/or adrenal chromaffin cells in response to NGF (Badoyannis *et al*. 1991), IGF1 (Dahmer *et al*. 1990; Hwang & Choi 1995), and TGFβ1 (Howard & Gershon 1993; Combs *et al*. 2000). The upregulation of DBH by locally produced tropic factors support the notion of intraluteal regulation of the CL function (Nisweder *et al*. 2007). In fact, the presence of IGF1 (Schams & Berisha 2004) and TGFβ1 (Hou *et al*. 2007) have been detected in the bovine CL and suggest to influence CL function. The current data as well as the findings of the above studies highlight the importance of tropic factors in the regulatory mechanism of intraluteal function.

In summary, the present study demonstrated for first time the expression of DBH in non-neural source (luteal cells) and re-innervation phenomenon in degenerated and absorbed atretic follicles and corpus albicans. In addition to the established luteotropic role of NA, luteolytic role cannot be excluded since DBH expression did not change during luteal regression and TNFα/IFNγ-induced apoptosis in cultured luteal cells. Locally synthesized NA appears to be regulated by tropic factors in the bovine CL.

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