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Synergistic Effect of Dexamethasone and Prolactin on VEGF Expression in Bovine Mammary Epithelial Cells via p44/p42 MAP Kinase*

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ABSTRACT : Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis under various physiological and pathological conditions. We found that the VEGF isoforms VEGF120, VEGF164, and VEGF188 were expressed in the bovine mammary gland and bovine mammary epithelial cells (bMECs). Expression of VEGF in the mammary gland was significantly higher during the lactation period than during the dry period. Although dexamethasone or prolactin alone had little effect on the expression of VEGF, that in dexamethasone-treated cells was significantly induced after additional treatment with prolactin. Furthermore, the VEGF expression induced by the combination of dexamethasone and prolactin was reduced by PD98059 in a dose-dependent manner. This combination also stimulated the phosphorylation of p44/p42 MAP kinase in these cells. These results strongly suggest that the combination of dexamethasone and prolactin stimulates VEGF expression in bMECs via p44/p42 MAP kinase. (**Key Words** : p44/p42 MAP Kinase, Prolactin, Dexamethasone, VEGF, Bovine Mammary Epithelial Cells)

INTRODUCTION

The lactating mammary gland requires an adequate supply of nutrients and hormonal stimuli via the circulation system to sustain milk synthesis. Mammary blood flow in cows and goats controls milk secretion (Davis and Collier, 1985). Angiogenesis, the formation of capillaries from preexisting blood vessels, is accompanied by increased blood flow caused by vasodilatation and changes in vascular permeability (Hyder and Stancel, 1999). It has been reported that significant changes in microvascular density and architecture occur during pregnancy and lactation in rodents (Matsumoto et al., 1992; Pepper et al., 2000). These reports suggest that angiogenesis is essential not only for maintaining mammary blood flow during lactation but also for mammary development and differentiation during pregnancy and lactation.

Mammary development and differentiation are controlled to a great extent by hormonal stimuli such as estrogen, progesterone, and prolactin. Initial studies focused on hormones that cause mammary development (Sinha and Tucker 1969; Sejrsen et al., 1999), but more recent studies have suggested that mammary-derived factors may also be important (Purup et al., 2000). Angiogenic cytokines such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are candidates for the induction of mammary angiogenesis (Schweigerer et al., 1987; Carmeliet et al., 1996).

Accumulating evidence suggests that VEGF is a key regulator of angiogenesis under various physiological and pathological conditions, such as embryonic development, wound healing, and solid tumor growth (Ferrara 1999; Neufeld et al., 1999). VEGF is a multifunctional cytokine that stimulates the proliferation of endothelial cells and enhances microvascular permeability. VEGF improves the developmental competence of bovine oocyte and/or embryo *in vitro* (Luo et al., 2006). Five different VEGF transcripts encoding polypeptides of 121, 145, 165, 189, and 206 amino acid residues are generated by alternative splicing from a single human VEGF gene (Ferrara, 1999). cDNAs of the VEGF164 isoform (which corresponds to the human VEGF165 isoform) have been isolated from various mammalian species, including sheep and cow (Leung et al., 1989; Tischer et al., 1989; Cheung and Brace, 1998). The

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mRNA expression of both VEGF120 and VEGF164 is reported to be increased in the mammary gland during pregnancy and lactation in rodents (Pepper et al., 2000; Hovey et al., 2001). Furthermore, it has been reported that inactivation of VEGF in mammary gland epithelium in mice causes inadequate lobulo-alveologenesis and epithelial differentiation during lactation, which results in decreased milk secretion and malnourishment of pups (Rossiter et al., 2007). These reports suggest that VEGF is an essential factor for the development of the mammary gland and lactation. However, few studies have been conducted on VEGF in the mammary gland of dairy cows.

In the present study, we first determined the basal gene expression levels of VEGF isoforms in the bovine mammary gland and then performed molecular cloning of these VEGF isoforms. We further investigated whether lactogenic hormones affect VEGF expression in bovine mammary epithelial cells (bMECs), and if any effect was observed, we investigated the underlying mechanism.

MATERIALS AND METHODS

Materials

Dexamethasone, bovine insulin, and ovine prolactin were obtained from Sigma Chemical Co. (USA). PD98059, SB203580, and SP600125 were obtained from Calbiochem-Novabiochem (USA). Phospho-specific p44/p42 MAP kinase antibodies and p44/p42 MAP kinase antibodies were obtained from New England BioLabs (USA). Other materials and chemicals were obtained from commercial sources. PD98059, SB203580, and SP600125 were dissolved in dimethyl sulfoxide.

Preparation of bovine mammary tissue and mammary epithelial cells

The mammary glands from Holstein and Japanese Black cows were removed within 20 min of slaughter. bMECs were prepared as previously described with a few modifications (Ahn et al., 1995). In brief, mammary tissue obtained from lactating Holstein cows was dispersed in 120 U/ml collagenase (Sigma Chemical Co.). The cell suspension was filtered through a 150-μm nylon mesh filter to remove undigested tissue fragments and debris. The filtrate was then centrifuged at 80×*g* for 10 min. The centrifuged cells and cell clumps were washed twice with Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co.) containing 10% fetal calf serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The cells were then seeded into uncoated, 100-mm-diameter dishes in DMEM containing 10% fetal calf serum, 5 μg/ml of insulin, 100 U/ml of penicillin, and 100 μg/ml of streptomycin, and cultured at 37°C under a humidified atmosphere of 5% carbon dioxide and 95% air. The cells were seeded on 40-mm-diameter dishes (TPP, Switzerland) at a density of 5×10^4 cells/cm^2 for semiquantitative RT-PCR and western blot analysis. The medium was replaced with DMEM containing 5% fetal calf serum and 5 μg/ml of insulin, with or without 5 μg/ml of dexamethasone after reaching subconfluence. The cells were further cultured in the medium alone for 6 h and then stimulated with 5 μg/ml of ovine prolactin or the vehicle for the indicated periods. When indicated, the cells were pretreated with PD98059, SB203580, or SP600125 for 60 min before stimulation with prolactin. The experiments were performed on cells through passages 4 to 10.

Molecular cloning of bovine VEGF isoforms from mammary gland

Total RNA was isolated from mammary tissue using Isogen (Nippon Gene, Japan), after which a 1-μg sample was reverse-transcribed using M-MLV reverse transcriptase (Sigma Chemical Co.) according to the manufacturer's instructions. The primers bVEGF-f1 and bVEGF-r2 were designed from the nucleotide sequence of bovine VEGF164 cDNA (GenBank accession number M32976) to amplify the coding region of all VEGF isoforms. One microliter of cDNA was amplified using the Expand High Fidelity PCR system (Roche Diagnostics, Germany) in the presence of 10 pmol of each of the forward and reverse PCR primers in a 20-μl reaction mixture. A DNA thermal cycler (Takara, Japan) was used to repeat the following cycle 28 times: 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The PCR products were isolated by agarose gel electrophoresis, cloned into pGEM-T Easy vector (Promega, USA), and transformed into *Escherichia coli* DH5 alpha. Several independent clones harboring the amplified fragments were sequenced to eliminate the errors introduced during the PCR. The raw sequence data were assembled and corrected with Sequencher software (Genecodes, USA).

Semiquantitative analysis of mRNA expression

cDNA fragments of test genes were amplified within the linear range by PCR with GoTaq DNA Polymerase (Promega). The primer sets were bVEGF-f2 and bVEGF-r1 for VEGF and bactin-f and bactin-r for β-actin (Table 1). Amplification conditions were as follows: an initial denaturation of 94°C for 5 min followed by 22 cycles (for VEGF) of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s or 18 cycles (for β-actin) at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Aliquots of the PCR products were resolved on 1.5% agarose gels. The signal intensities were quantified using Scion Image software (Scion Corp., USA). The intensities of the VEGF transcripts were normalized by β-actin mRNA levels.

Table 1. Sequences of primers used in PCR

Name	Primer sequence (5′–3′)	Length
bVEGF-f1	ATGAACTTTCTGCTCTCTTGGG	22 mer
bVEGF-f2	CTTCACCATGCCAAGTGGTCC	21 mer
bVEGF-f3	ACGAAAGTCTGGAGTGTG	18 mer
bVEGF-r1	GCTCATCTCTCCTATGTGCTGG	22 mer
bVEGF-r2	TCACCGCCTCGGCTTGTCACA	21 mer
bactin-f	TGGACTTCGAGCAGGAGATG	20 mer
bactin-r	CCGCCGGACAGCACCGTGTT	20 mer

Western blot analysis of p44/p42 MAP kinases

The cells were washed twice with phosphate-buffered saline; resuspended in a lysis buffer containing 62.5 mM Tris/Cl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol; and boiled for 10 min. Equal amounts of total protein from each cell lysate were loaded, electrophoresed on an SDS-polyacrylamide gel (10%), and transferred onto PVDF membranes (Millipore, USA) by semi-dry blotting. Western blotting was performed using phospho-specific p44/p42 MAP kinase antibodies and p44/p42 MAP kinase antibodies. The signal intensities were quantified using Scion Image software. The intensities of prolactin-induced phosphorylation signals for each lane were normalized by those of the total expression of proteins. The histograms show the fold increase in levels of phospho-p44/p42 MAP kinase in each cell versus those of unstimulated cells.

Statistical analysis

The mean values were analyzed by unpaired *t*-test (Student's *t*-test) or one-way analysis of variance (ANOVA) followed by Bonferroni's test. Differences were considered significant at $p<0.05$.

RESULTS

Expression of VEGF mRNAs in bovine mammary gland

Analysis of the VEGF transcripts by RT-PCR with the bVEGF-f3 and bVEGF-r2 primers showed that mammary glands from both Holstein and Japanese Black cows

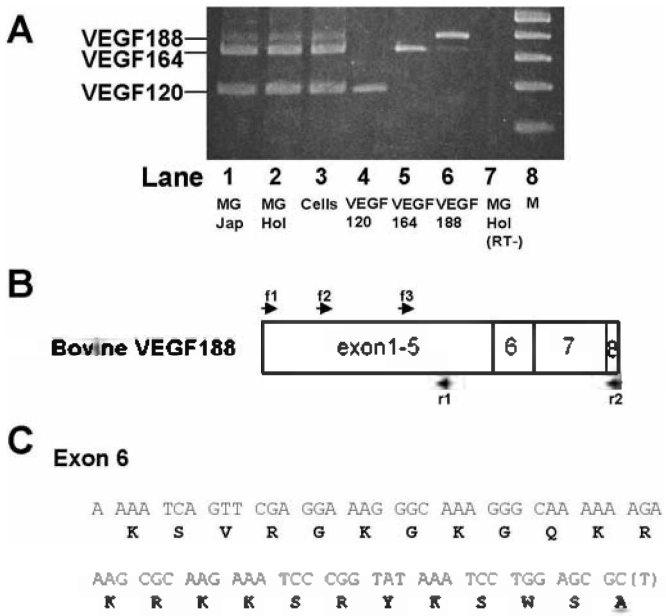

Figure 1. mRNA expression and molecular cloning of VEGF isoforms in bovine mammary gland. A: Expression analysis of VEGF isoforms in bovine mammary gland. All mammalian VEGF isoforms are known to share exons 1-5 and 8. RT-PCR was performed with primers (bVEGF-f3 and bVEGF-r2) that can amplify all VEGF isoforms. Aliquots of the PCR products were resolved on 4% acrylamide gels. Lane 1, RNA of mammary gland from Japanese Black cow; lane 2, RNA of mammary gland from Holstein cow; lane 3, RNA of cultured bovine mammary epithelial cells from lactating Holstein cow; lanes 4-6, plasmid DNA containing cloned bovine VEGF120, 164, and 188, respectively; lane 7, RNA of mammary gland from Holstein cow without RT (negative control); lane 8, molecular weight marker. B: Schematic representation of bovine VEGF188. The arrows represent the primers used for the PCR analysis. C: Nucleotide and deduced amino acid sequence of exon 6 of bovine VEGF188 (GenBank accession number AB450824). The amino acid residue different from exon 6 of human VEGF189 is underlined.

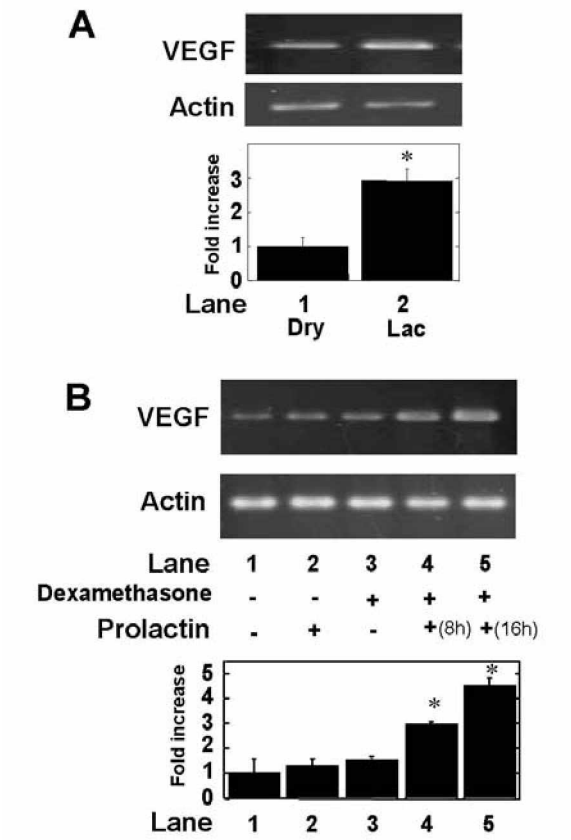

Figure 2. Alteration of VEGF mRNA expression in bovine mammary gland and bMECs. Semiquantitative RT-PCR was performed using the primers bVEGF-f2 and bVEGF-r1 (Table 1). A: Comparison of VEGF expression in mammary glands of Holstein cows during lactation and dry periods. Data are means± SD for 3 cows per group and expressed as the ratio of VEGF mRNA relative to β-actin mRNA (dry period = 1.0). * indicates a significant difference at $p<0.05$. B: Effects of lactogenic hormones on VEGF expression in bMECs. The cultured cells were pretreated with 5 μg/ml of dexamethasone or the vehicle for 48 h, and further cultured in the medium alone for 6 h. The cells were then stimulated with 5 μg/ml of prolactin or the vehicle for 8 (lane 4) or 16 h (lanes 1, 2, 3, and 5). Total RNA was isolated from dishes prepared in triplicate, and the relative expression of VEGF mRNA was determined by RT-PCR. * $p<0.05$ when compared with the value of the unstimulated cells.

predominantly expressed the VEGF120 and VEGF164 isoforms and weakly expressed the VEGF188 isoform (Figure 1A). bMECs from lactating Holstein cows expressed the same isoforms. No reaction product was seen in the negative control samples lacking cDNA.

We next performed molecular cloning of the entire coding regions of the VEGF isoforms expressed in the bovine mammary gland. Because all mammalian VEGF isoforms are known to share exons 1-5 and 8, the bVEGF-f1 and bVEGF-r2 primers were used in RT-PCR experiments with Holstein mammary gland RNA samples as the template. We isolated cDNAs for three VEGF isoforms: VEGF120, VEGF164, and VEGF188. Sequencing analysis showed VEGF164 to be identical to that cloned from folliculostellate cells, as reported by Leung et al. (1989) (accession No M32976). DNA sequences of the coding regions of bovine VEGF188 and VEGF120 were not found in public databases, and we deposited the nucleotide sequences in the DDBJ, GenBank, and EMBL databases under Accession Nos. AB450824 and AB455252, respectively. As reported for human VEGF189, bovine VEGF188 contained a highly basic 24-amino acid insertion derived from exon 6 (Leung et al., 1989), though with a Val to Ala substitution for the last amino acid (Figure 1B and C).

The results of the densitometric analysis of VEGF mRNA expression by RT-PCR in the mammary glands of cows during the lactation and dry periods are shown in Figure 2A. Compared with that during the dry period, the expression of VEGF was significantly higher during the lactation period (100-200 days after parturition).

Lactogenic hormones stimulated VEGF expression in bMECs

To determine the effect of lactogenic hormones on the expression levels of VEGF, bMECs were treated with prolactin and dexamethasone. Although dexamethasone or prolactin alone had little effect on the expression of VEGF, that in dexamethasone-treated cells was significantly increased after additional treatment with prolactin (Figure 2B).

Effects of PD98059, SB203580, or SP600125 on lactogenic hormone-induced VEGF expression in bMECs

To investigate the involvement of members of the MAP kinase family in lactogenic hormone-induced VEGF expression in bMECs, we examined the effect of specific inhibitors of MAP kinases or MAP kinase kinase on VEGF expression. Prolactin-induced VEGF expression after dexamethasone treatment was significantly reduced by PD98059, an inhibitor of upstream kinase that activates p44/p42 MAP kinase (MEK) (Alessi et al., 1995), but not by SB203580, an inhibitor of p38 MAP kinase (Cuenda et al., 1995), or SP600125, a specific inhibitor of stress-activated protein kinase (SAPK)/c-*Jun* *N*-terminal kinase (JNK) (Bennett et al., 2001) (Figure 3A). On the other hand, SP600125 significantly enhanced VEGF expression in the cells not treated with dexamethasone (Figure 3B).

Dose-dependent effect of MEK inhibitor on the VEGF expression induced by the combination of dexamethasone and prolactin in bMECs

To further investigate the involvement of p44/p42 MAP kinase in the VEGF expression induced by the combination

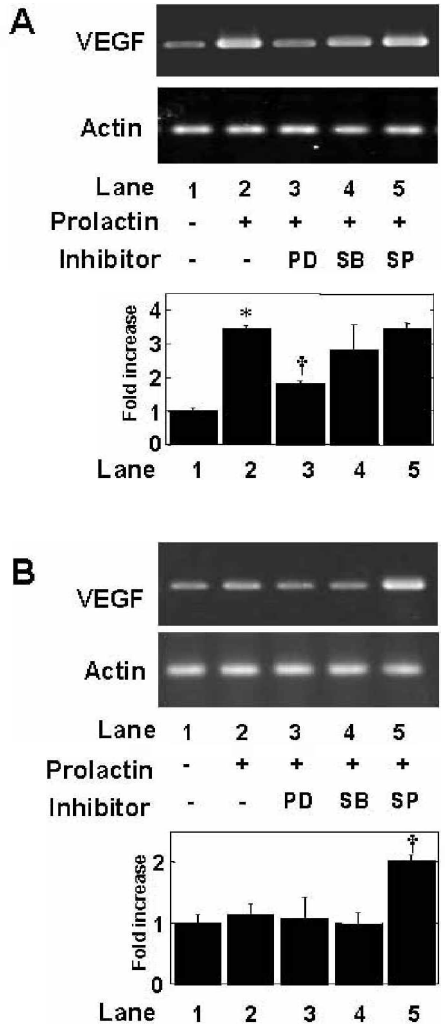

Figure 3. Effects of PD98059, SB203580, or SP600125 on prolactin-induced VEGF expression in bMECs. The cultured cells were pretreated with 5 μg/ml of dexamethasone (A) or vehicle (B) for 48 h and further cultured in the medium alone for 6 h. The cells were then incubated for 16 h in the presence of prolactin (5 μg/ml) or vehicle, with or without PD98059, SB203580, or SP600125 (each, 10 μM). One of the inhibitors was added to the culture 60 min before prolactin treatment. Total RNA was isolated from dishes prepared in triplicate, and the relative expression of VEGF mRNA was determined by RT-PCR. * $p<0.05$ when compared with the value of vehicle. † $p<0.05$ when compared with the value of prolactin alone.

of dexamethasone and prolactin in bMECs, we next examined the dose-dependent effect of PD98059. As shown in Figure 4A, PD98059 reduced the VEGF expression induced by this combination in a dose-dependent manner. Furthermore, we found that U0126, another inhibitor of MEK, also reduced VEGF expression by the combination of dexamethasone and prolactin (data not shown).

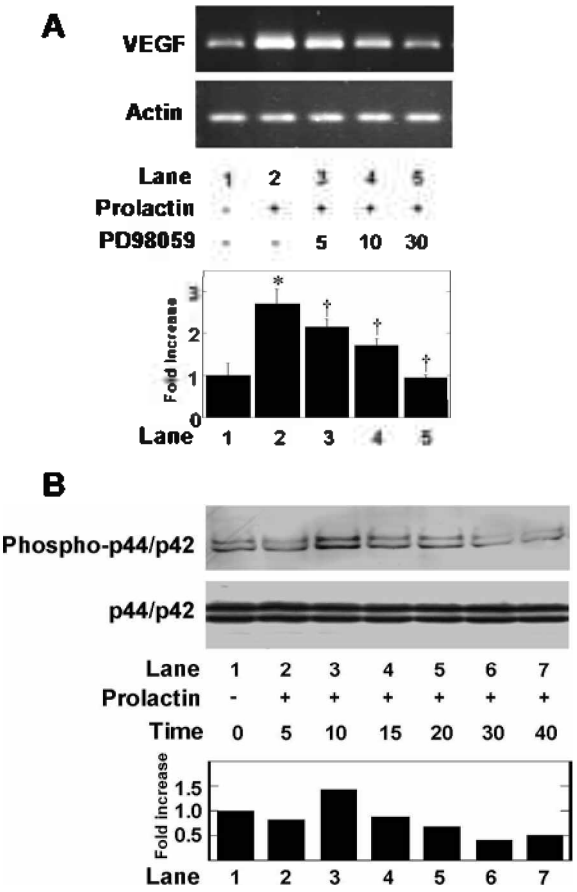

Figure 4. Involvement of p44/p42 MAP kinase in the VEGF expression induced by the combination of dexamethasone and prolactin in bMECs. A: Effect of PD98059 on mRNA expression of VEGF in bMECs. The cultured cells were pretreated with 5 μg/ml of dexamethasone for 48 h and further cultured in the medium alone for 6 h. The cells were then stimulated with 5 μg/ml of prolactin or vehicle for 16 h. Indicated doses of PD98059 or vehicle were added to the culture 60 min before prolactin treatment. Total RNA was isolated from dishes prepared in triplicate, and the relative expression of VEGF mRNA was determined by RT-PCR. Each value represents mean±SD of triplicate determinations. * $p<0.05$ when compared with the value of vehicle. † $p<0.05$ when compared with the value of prolactin alone. B: Effect of prolactin on phosphorylation of p44/p42 MAP kinase in bMECs. The cultured cells were incubated with 5 μg/ml of prolactin for the indicated periods. The cell extracts were subjected to SDS-polyacrylamide gel electrophoresis with subsequent western blot analysis with antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. Similar results were obtained with two additional and different cell preparations.

Effect of combination of dexamethasone and prolactin on phosphorylation of p44/p42 MAP kinase in bMECs

To confirm the involvement of p44/p42 MAP kinase in the VEGF expression induced by the combination of dexamethasone and prolactin, we investigated whether this

combination phosphorylates p44/p42 MAP kinase in bMECs. As shown in Figure 4B, prolactin induced rapid phosphorylation of p44/p42 MAP kinase in bMECs treated with dexamethasone after 10 min; the phosphorylation level was about 1.5-fold that of unstimulated cells. Moreover, p44/p42 MAP kinase was then rapidly dephosphorylated. The total amount of p44/p42 MAP kinase was not affected by prolactin.

DISCUSSION

It has been reported that cultured human MECs from colostrum express the VEGF121, 165, and 189 isoform mRNAs as shown here (Nishimura et al., 2002). In contrast, HC11 mouse mammary epithelial cells express only mRNA for VEGF120 and VEGF164. Furthermore, VEGF188 expression is decreased during pregnancy and is undetectable during lactation in mouse mammary glands (Hovey et al., 2001). The major functional difference between the various VEGF isoforms is their ability to bind to heparin. Although the three shorter isoforms are mainly diffusible, the two longer ones (VEGF189 and VEGF206) are sequestered in the extracellular matrix after secretion (Houck et al., 1992; Park et al., 1993) and are activated after proteolysis by plasmin and urokinase (Houck et al., 1992; Plouët et al., 1997). Thus, it is likely that the species-specific expression of VEGF isoforms reflects some differences in the angiogenesis system of mammary glands between humans, cattle, and mice.

In dairy cows, the serum concentration of prolactin increases dramatically a few days prepartum, peaks at parturition, and then gradually declines (Akers et al., 1981). The serum concentration of glucocorticoid also peaks at parturition (Edgerton and Hafs, 1973; Smith et al., 1973). However, serum glucocorticoid levels undergo little change as lactation advances (Koprowski and Tucker, 1973a). This is not the case for prolactin, the serum concentration of which increases during lactation in response to stimuli associated with milking; this response is largest at around 8 weeks of lactation, and gradually decreases thereafter (Koprowski and Tucker, 1973b). Synergistic effects of prolactin and glucocorticoid hormones are important for mammary development and differentiation, and for milk protein gene expression. For example, it has been reported that prolactin and cortisol synergistically induce α-lactalbumin secretion in bovine mammary tissue cultured *in vitro* (Goodman et al., 1983). Furthermore, while the accumulation of β-casein mRNA in bMECs is increased by prolactin, it is increased to a greater extent by a combination of prolactin and hydrocortisone (Choi et al., 1988). In the present study, we showed that the induction of VEGF expression in bMECs requires both prolactin and dexamethasone. This suggests that a synergistic effect of prolactin and glucocorticoid hormones will also be important for mammary angiogenesis.

Goldhar et al. (2005) have reported that prolactin stimulates VEGF gene expression in HC11 cells and Nb2 rat lymphoma cells. Similar to their results, we showed here by semiquantitative RT-PCR that prolactin stimulated VEGF expression in bMECs after treatment with dexamethasone. Prolactin signaling evoked mainly the signal transducer and activator of transcription (STAT) and MAP kinase pathways. In addition, it has been reported that the expression and secretion of VEGF is increased through the activation of MAP kinase in various cells (Berra et al., 2000; Kanno et al., 2004; Nakajima et al., 2006). Therefore, we next investigated the effects of specific inhibitors of MAP kinase or MAP kinase kinase on prolactin-induced VEGF expression. We showed that PD98059 significantly reduced the VEGF expression induced by the combination of dexamethasone and prolactin. We also found that this combination phosphorylated p44/p42 MAP kinase in bMECs. It is known that MAP kinases are activated by phosphorylation of tyrosine and threonine residues by dual specificity MAP kinase kinase (Raingeaud et al., 1995). Therefore, it is most likely that VEGF expression induced by this combination is mainly regulated by p44/p42 MAP kinase in bMECs. We also showed that SP600125 enhanced VEGF expression in bMECs without dexamethasone treatment, and that dexamethasone treatment altered the response of these cells to prolactin (Figures 2 and 3). It has been reported that prolactin stimulates SAPK/JNK activity in Nb2 cells (Schwertfeger et al., 2000) and PC12 pheochromocytoma cells (Cheng et al., 2000). In addition, it has been reported that prolactin stimulates rapid activation of SAPK/JNK in bMECs transfected with the long form of the prolactin receptor and that treatment of these cells with dexamethasone inhibits activation of SAPK/JNK (Olazabal et al., 2000). Taking these reports into account, it is likely that SAPK/JNK activation represses VEGF expression in bMECs and that this repression is suppressed by dexamethasone. Further investigation is required to clarify the in-depth roles of SAPK/JNK in the expression of VEGF in bMECs.

In summary, we found that the VEGF isoforms VEGF120, VEGF164, and VEGF188 are expressed in the bovine mammary gland and bMECs. After treatment with dexamethasone, prolactin induced VEGF expression in bMECs, and this effect was mainly mediated via p44/p42 MAP kinase.

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