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Regulation and localization of vascular endothelial growth factor within the mammary glands during the transition from late gestation to lactation

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The vascular network within the developing mammary gland (MG) grows in concert with the epithelium to prepare for lactation, although the mechanisms coordinating this vascular development are unresolved. Vascular endothelial growth factor A (VEGF-A) mediates angiogenesis and vascular permeability in the MG during pregnancy and lactation, where its expression is upregulated by prolactin. Given our previous finding that late-gestational hyperprolactinemia induced by domperidone (DOM) increased subsequent milk yield from gilts, we sought to establish changes in vascular development during late gestation and lactation in the MGs of these pigs and determine whether DOM altered MG angiogenesis and the factors regulating it. Gilts received either no treatment ($n = 6$) or DOM ($n = 6$) during late gestation, then had their MG biopsied from late gestation through lactation to assess microvessel density, VEGF-A distribution and messenger RNA expression, and aquaporin (AQP) gene expression. Microvessel density in the MG was unchanged during gestation then increased between days 2 and 21 of lactation ($P < 0.05$). The local expression of messenger RNA for *VEGF-A*$_{120}$, *VEGF-A*$_{147}$, *VEGF-A*$_{164}$, *VEGF-A*$_{164}$*b*, *VEGF-A*$_{188}$, *VEGF receptors-1* and *-2*, and *AQP1* and *AQP3* all generally increased during the transition from gestation to lactation ($P < 0.05$). Immunostaining localized VEGF-A to the apical cytoplasm of secretory epithelial cells, consistent with a far greater concentration of VEGF-A in colostrum and/or milk vs plasma ($P < 0.0001$). There was no effect of DOM on any of the variables analyzed. In summary, we found that vascular development in the MG increases during lactation in first-parity gilts and that VEGF-A is a part of the mammary secretome. Although late-gestational hyperprolactinemia increases milk yield, there was no evidence that it altered vascular development.

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1. Introduction

Successful lactation relies on the coordinated processes of mammary epithelial growth and differentiation during gestation [1] that occur in close apposition to a complex vascular network [2]. This network forms throughout gestation via angiogenesis [3] to ultimately provide sufficient blood flow and the associated nutrients required for milk production [4]. A potent initiator of angiogenesis in all mammalian organs is vascular endothelial growth factor A (VEGF-A; [5]). This locally produced factor is critical for complete mammary gland (MG) development, where the conditional deletion of VEGF-A in the mammary myoepithelium and epithelium of mice decreased blood vessel density and function, and severely impacted milk production and pup growth [6]. In addition, VEGF-A regulates vascular permeability [5] that is essential for the regulated transfer of nutrients, oxygen, and metabolic waste during milk synthesis and secretion [7].

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Various factors modulate the expression and actions of VEGF-A and hence may alter angiogenesis and vascular permeability within the MG. The effects of VEGF-A are conferred via 2 tyrosine kinase receptors, VEGF receptors-1 and -2 (VEGFR-1 and VEGFR-2; [8]) that can have their effects enhanced by the coreceptor neuropilin 1 (NP1; [9]). Furthermore, there is considerable potential for hypoxia to occur within the MG during gestation and lactation given the increased growth and metabolic rate of mammary epithelial cells (MEC; [10]). As a result, increased abundance of hypoxia inducible factor 1α (HIF-1α) can upregulate VEGF-A expression [11,12]. Finally, sufficient vascular permeability within the MG is realized by the actions of developmentally regulated [13], water-permeable aquaporins (AQPs) that allow water to move from the microcirculation into milk [14]. Within the MGs, AQP1 localizes to the endothelium, whereas AQP3 localizes to the epithelium to contribute to milk synthesis [15,16].

The endocrine system that directs growth and differentiation of the mammary epithelium may also regulate angiogenesis within the MG [17]. Prolactin (PRL) directs the growth and differentiation of MEC during gestation in preparation for lactation [18,19], and it has been established that the MG of pigs are critically sensitive to PRL between days 90 and 110 of gestation [20]. As an extension of those findings, we recently used the dopamine type-2 receptor antagonist domperidone (DOM) to induce hyperprolactinemia in late-gestation gilts (average of 51.68 ± 9.2 ng/mL PRL at days 91 and 96 of gestation vs 10.6 ± 1.2 ng/mL, [21]) that resulted in DOM-treated gilts producing significantly more milk during the subsequent lactation. Because PRL also increases the production of VEGF-A by mouse MEC in vitro [22], the question arises as to whether the galactopoietic effect of late-gestational hyperprolactinemia is mediated by increased angiogenesis within the MG.

A resolution of the mechanisms underlying the regulation of lactation in sows is warranted, given that insufficient milk yield can limit the growth of piglets [23], whereas galactogogues such as DOM are often used to support human lactation, where the MG of pigs constitutes an authentic model for the breast [24]. Given these considerations, our objective was to investigate whether DOM-induced hyperprolactinemia in first-parity gilts altered various aspects of angiogenesis in their MG. Because we previously showed that late-gestational hyperprolactinemia significantly increased subsequent milk production [21] and milk yield is proportional to mammary blood flow [7], we hypothesized that late-gestational hyperprolactinemia would also promote angiogenesis and the expression of its regulatory factors within the MG.

2. Materials and methods

2.1. Animals and sample collection

All procedures were approved by the University of California, Davis Animal Care and Use Committee (Protocol #15217). In a previously described study [21], 12 first-parity Yorkshire × Hampshire gilts in 2 experimental blocks (3 gilts per treatment per block) were randomly assigned to receive either no supplement (CON, n = 6) or DOM (n = 6) from days 90 to 110 of gestation. DOM-treated gilts received Equidone (11% wt/vol DOM, 0.4 mg/kg body weight; Equi-tox Pharma, Central, SC, USA) twice daily in their feed from days 90 to 110 of gestation, where complete intake was confirmed at each feeding. Animals were fed at 8 AM and 8 PM and had ad libitum access to water via nipple. Gilts were individually housed in 3 × 3 m pens and moved to farrowing crates on day 111 of gestation. Lactating gilts nursed and weaned standardized litters of approximately the same size (n = 8.37 ± 0.16 piglets).

MGs were biopsied using a vacuum-assisted approach [25] on days 90, 100, and 110 of gestation and days 2 and 21 of lactation. For a given time point, biopsies were taken from the same MG in all animals, with gland position across time following a sequential pattern starting from the right posterior gland of the mammary chain, then moving contralateral, and thereafter, diagonally forward. Each gland was only biopsied once, and only lactating glands that were actively nursed were biopsied. Two or 3 tissue cores were collected at each biopsy, where 1 or 2 were flash frozen and stored at −80°C although 1 was fixed in 10% formalin overnight at 4°C.

Blood samples were collected by jugular venipuncture on days 90, 100, and 110 of gestation to confirm hyperprolactinemia as described [21]. Blood samples were also collected from 6 gilts from 1 experimental block (n = 3 CON and n = 3 DOM) on days 2 and 21 of lactation. Samples were collected into sodium heparin-coated Vacutainer tubes (10 mL; BD, Franklin Lakes, NJ, USA) at 8 AM before the feeding (12 h post-DOM ingestion). Plasma was collected after centrifugation at 3,700*g* for 15 min at room temperature, aliquoted, and stored at −20°C.

Corresponding colostrum and/or milk samples were also collected from these same 6 gilts on days 1 and 20 of lactation. Samples on day 1 were collected approximately 2 h after farrowing. On day 20 of lactation, gilts were given oxytocin (20 IU intramuscular; VetOne, Boise, ID, USA) approximately 1 h after the morning feeding to induce milk ejection. Each milk sample was a composite sample from the anterior, middle, and posterior suckled glands. Samples were collected into 15-mL conical tubes and immediately frozen at −20°C. The skim fraction was obtained after centrifugation at 3,500*g* for 10 min at room temperature.

2.2. Immunohistochemistry

2.2.1. Microvessel density

Mammary tissues were processed to paraffin followed by sectioning (5 μm). Sections were deparaffinized in xylene and rehydrated through graded ethanols before permeabilization using 0.3% Triton-X (Promega, Madison, WI, USA). Antigen retrieval was performed in citrate buffer (pH 6.0, 45 min; Diagnostic Biosystems, Pleasanton, CA, USA) in a vegetable steamer. Endogenous peroxidases were blocked with 3% hydrogen peroxide, and endogenous biotin was blocked with a commercial avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, USA). Blocking was achieved using 10% horse serum for 1 h at room temperature. The primary antibody against CD31 (PECAM-1, 1:200; Millipore, Billerica, MA, USA, #04–1074) was incubated

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