Original Article

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Possible Role of Hypoxia-Inducible Factor-1Alpha and Vascular Endothelial Growth Factor During Periovulation and Corpus Luteum Formation in Cows

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Abstract

The objective of the study was to characterize expression patterns of hypoxia-inducible factor-1alpha and vascular endothelial growth factor isoforms during periovulation in the cow. Ovaries containing follicles or corpora lutea were collected by transvaginal ovariectomy ($n=5$ cows/group) and timely defined (after gonadotropin-releasing hormone application) as follows: (I) 0 h; (II) 4 h; (III) 10 h; (IV) 20 h; (V) 25 h (follicles); and (VI) 60 h (early corpora lutea). The mRNA expression of genes encoding hypoxia-inducible factor-1alpha and vascular endothelial growth factor isoforms was quantified by reverse transcriptase quantitative polymerase chain reaction. The high mRNA expression of hypoxia-inducible factor-1alpha in the control follicle group (before gonadotropin-releasing hormone) was followed by a continuous and significant downregulation afterward with a minimum level in follicle group close to ovulation, and a significant increase just after ovulation. The mRNA of vascular endothelial growth factor isoforms was high during luteinizing hormone peak (4 h after gonadotropin-releasing hormone), decreased significantly thereafter to the lowest levels in follicle group 25 h after gonadotropin-releasing hormone, followed by a rapid and significant increase just after ovulation (newly corpora lutea). In conclusion, our results of hypoxia-inducible factor-1alpha and vascular endothelial growth factor regulation in follicle groups around ovulation indicate them to be necessary mediators of the luteinizing hormone-dependent ovulation and significant molecular mechanisms of angiogenesis during early corpora lutea formation.

Keywords: Angiogenesis, corpus luteum, follicles, HIF-1alpha, ovary, VEGF

Introduction

The ovarian gonadotropin surge initiates the regulation of specific factors and molecular mechanisms that lead to the final follicle growth, subsequent ovulation, as well as the formation of corpus luteum (CL) and its function (Kuo et al., 2011; Rico et al., 2014; Tsafriri & Reich, 1999; Zimmermann et al., 2003). These ovarian complex processes (especially the transition of follicular tissue to CL) seem to be dependent on the development of a new vascular network (angiogenesis), regulated by synchronous communication of endocrine and locally produced (paracrine) factors (Berisha et al., 2008; Curry & Smith, 2006; Schams et al., 2009; Schilffarth et al., 2009; Shirasuna et al., 2008; Thompson et al., 2015). Angiogenesis is considered to be crucial for the reproductive organ physiology and especially for the ovary function (Abulafia & Sherer, 2000; Amselgruber et al., 1999; Berisha et al., 2016a; Fraser & Lunn, 2000).

Besides gonadotropins and steroids, the dominant mediators of the ovarian angiogenesis (especially in follicles and CL) include angiogenic molecules like angiopoietins, vascular endothelial growth factor (VEGF), hypoxia inducible factor (HIF), fibroblast growth factor (FGF), etc. (Berisha et al., 2002a, 2002b, 2016b; Kobayashi et al., 2001, 2002; Neuvians et al., 2003; Tanaka et al., 2004). However, some researchers indicated the importance of hypoxia and its interface with diverse locally produced (paracrine) factors during angiogensis (Klipper et al., 2010; Nishimura & Okuda, 2015; Thompson et al., 2015).

Within this frame, the locally produced pro-angiogenic factors, hypoxia-inducible factor 1-alpha (HIF1A) and VEGF family members, seem to be very important regulators of the follicular development, subsequent ovulation, and formation of CL (Berisha et al., 2017; Li et al., 2020; Rico et al., 2014). The roles of HIF1 and its induced VEGF in the bovine early CL are well known, which is mainly documented by Klipper et al. (2010) and Nishimura and Okuda (2010). Hypoxia-inducible factor 1-alpha is one of the most important hypoxia-regulated pro-angiogenic factors for CL formation immediately after ovulation (Meidan et al., 2013; Van den Driesche et al., 2008).

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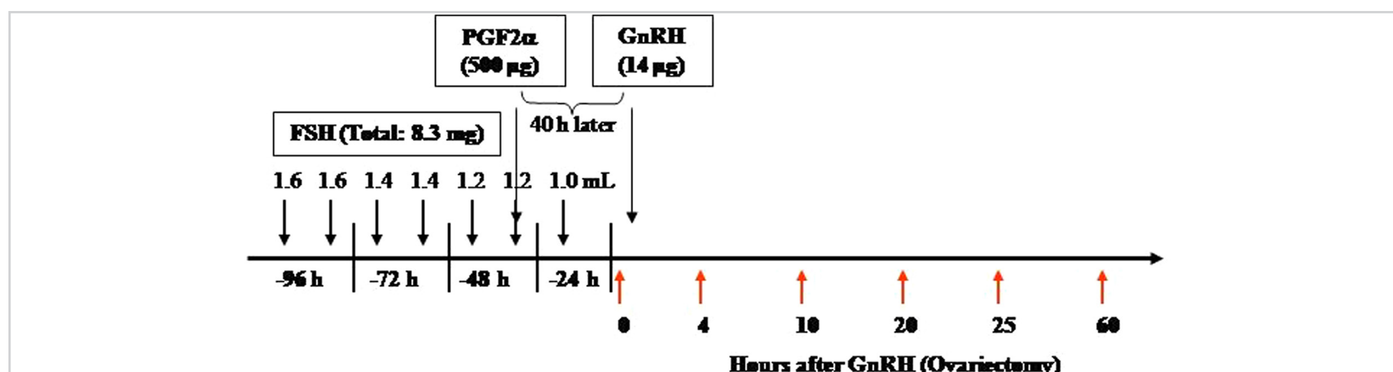

Figure 1.
Time Schedule of the Treatment for Multiple Ovulation and Ovary Collection in Cows. Ovaries Containing Preovulatory Follicles were Collected at 0 h (before LH peak), 4 h (during LH peak), 10 h (after LH peak), 25 h (before ovulation), and 60 h (after ovulation, early CL, day 2–3) Relative to Injection of GnRH to Induce an LH peak (n = 5 cows/group). PG2α = prostaglandin F2-alpha; FSH = follicle-stimulating hormone; GnRH = gonadotropin-releasing hormone; LH = luteinizing hormone.

In addition, the temporal HIF1A regulation in antral follicles during the periovulation confirms the very important role of this factor in ovulation process and subsequent CL formation (Rico et al., 2014). It is well known that VEGF has very potent mitogenic effects on endothelial cells (Berisha et al., 2020; Chouhan et al., 2013; Connolly, 1991; Ferrara & Davis-Smyth, 1997; Senger et al., 1993). The most important VEGF tyrosine kinases receptors are considered as VEGF-R1 and VEGF-R2 (Ferrara & Davis-Smyth, 1997).

With the present study, we aim to characterize and evaluate the expression pattern of HIF1A and VEGF isoforms in experimentally time-defined follicle groups, before and after gonadotropin-releasing hormone (GnRH) application, particularly in the time interval between LH peak, ovulation, and following CL formation in the cow.

Methods

The multiple ovulation procedure (German Fleckvieh cows) was conducted as described by Berisha et al. (2006). The ovaries ($n = 5$ cow/group) were collected relative to the application of GnRH at 0 h (before LH peak), 4 h (during LH peak), 10 h (after LH peak), 25 h (before ovulation), and 60 h (post-ovulation, early CL, day 2–3). The schematic presentation of the superovulatory experiment and the ovary collection by transvaginal ovariectomy is given in Figure 1.

The detailed information about the material and methods used in our experiments and evaluation of expression levels of researched factors (Table 1) were described by Berisha et al. (2000, 2020).

RNA Extraction and Real-Time Polymerase Chain Reaction

Total RNA from follicles and CL tissue (deep frozen by −80°C) was extracted as described by Berisha et al. (2020). The purity of isolated RNA was verified by optical density (OD) absorption ratio OD260 nm/OD280 nm between 1.8 and 2.0. The RNA integrity was measured with the Agilent 2100 bioanalyzer (Agilent Technologies, Deutschland Gmbh, Waldbronn, Germany) in conjunction with the RNA 6000 Nano Assay according to the manufacturer's instructions (Brisha et al., 2020).

Constant amounts of 1 µg of total RNA were reverse-transcribed to cDNA using the following master mix: 26 µL RNAse-free water, 12 µL 5 × Buffer (Promega, Mannheim, Germany), 3 µL Random Primers (50 µM) (Invitrogen, Carlsbad, Germany), 3 µL dNTPs (10 mM) (Fermentas, St. Leon-Rot, Germany), and 200 U of M MLV Reverse Transcriptase (Promega, Mannheim, Germany) according to the manufacturer's instructions as described by Berisha et al. (2019). The changes in mRNA expression of examined target genes were assayed by normalization to the stable expressed and internal Ubiquitin (UBQ) control gene. In order to obtain the CT (cycle threshold) difference, the data were analyzed using the well-established method described by Livak and Schmittgen (2001).

Statistical Analysis

The statistical significance of differences in mRNA expressions in follicle classes and CL tissue of the examined factors was assessed by one-way analysis of variance (ANOVA) followed by the Holm Sidak as a multiple comparison test. Data, which failed the normality or

Table 1.
Changes of mRNA for HIF1A and VEGF165 in Periovulatory Follicles Collected at (I) 0 h, (II) 4 h, (III) 10 h, (IV) 20 h, (V) 25 h (follicles), and (VI) 60 h (early CL, 2–3 days) After Injection of GnRH to Induce an LH Surge

	Follicle Classes During Experimentally Induced Ovulation					
	I	II	III	IV	V	VI
HIF1A	24.78 ± 0.35[a]	24.30 ± 0.23[ab]	23.64 ± 0.15[b]	23.36 ± 0.41[b]	21.82 ± 0.57[c]	24.67 ± 0.20[a]
VEGF165	25.11 ± 0.08[ab]	24.47 ± 0.09[b]	24.17 ± 0.29[b]	23.15 ± 0.25[c]	22.12 ± 0.42[c]	25.65 ± 0.09[a]

Note: The changes in mRNA abundance of examined factors were assayed by normalization to the Ubiquitin (UBQ) internal control. In order to obtain the CT difference, the data were analyzed using the ΔΔCT method described previously by Livak and Schmittgen (2001). Results are shown as 40–ΔCT ± SEM (***n*** = 5 follicle/class), so that a high 40–ΔCT value indicates a high-gene expression level and vice versa. Different superscripts denote statistically different values ($p < .05$).
HIF1A = hypoxia inducible factor 1alpha; VEGF165 = vascular endothelial growth factor; GnRH = gonadotropin-releasing hormone; LH = luteinizing hormone; CT = cycle threshold; CL = corpora lutea.

equal variance test, were tested by one-way ANOVA on ranks followed by the Kruskal-Wallis test (Sigma Stat 3.0). All experimental data are shown as means ± SEM ($n = 8$–12). The differences were considered significant if $p < .05$.

Results

The HIF1A mRNA expression level in the follicle tissue of our multiple ovulation experiment (before and after GnRH and after ovulation) is shown in Table 1.

The high expression of HIF1A in the control follicle group (before GnRH) was followed by a continuous downregulation afterward with a minimum level in follicle group close to ovulation (follicle group 25 h after GnRH). The expression of the examined VEGF isoforms (VEGF121, VEGF165, VEGF189) in follicle groups around ovulation showed identical expression patterns; however, we presented here VEGF165, considered the prototypical pro-angiogenic VEGF-A isoform. Transcripts of all VEGF ligand isoforms were high during LH peak (4 h after GnRH) and decreased thereafter to the lowest levels in follicle group 25 h after GnRH (close to ovulation). The highest HIF1A and VEGF mRNA expression was detected after ovulation (newly CL), considered as the typical period of angiogenesis (Table 1).

Discussion

Ovarian gonadotropin surge initiates regulation of specific factors (endocrine and paracrine) and molecular mechanisms leading to the follicle growth, subsequent ovulation, as well as formation and function of CL (Acosta et al., 2005; Kuo et al., 2011; Zimmermann et al., 2003). These ovarian complex processes seem to be dependent on the development of a new vascular network (angiogenesis), regulated by synchronous communication of endocrine and paracrine factors (Curry & Smith, 2006; Hayashi et al., 2004; Shirasuna et al., 2008; Thompson et al., 2015).

The angiogenic factors produced by granulosa cells play a crucial role in follicular growth via stimulating angiogenesis in theca follicle layers of preovulatory follicles (Berisha et al., 2016a; Chowdhury et al., 2010; Kuo et al., 2011). Angiogenesis in theca follicle cells and tissue is dependent on follicle-stimulating hormone (FSH) that induced the expression trends of HIF1A and VEGF isoforms in granulosa cells (Li et al., 2020; Zimmermann et al., 2003). There are obvious evidences that follicular angiogenesis is regulated by an interaction between LH surge, specific extracellular matrix-degrading proteases, and diverse pro-angiogenic factors (Berisha et al., 2008; Curry & Smith, 2006; Van den Driesche et al., 2008). Our previous studies demonstrated the interface of LH peak to extracellular matrix-degrading proteases, VEGF, and other pro-angiogenic factors (Berisha et al., 2006, 2019; Shimizu et al., 2007a, 2007b).

There are obvious indications showing that the pro-angiogenetic molecule HIF1A is a highly important mediator of follicular growth and development, ovulation, and subsequent CL formation (Berisha et al., 2017, 2020; Li et al., 2020; Meidan et al., 2013; Nishimura & Okuda, 2015). The present results of HIF1A expression in our experimental follicle groups around ovulation and after CL formation are shown in Table 1. The high expression level of HIF1A before GnRH (control follicle group) was followed by a continuous downregulation, reaching the minimum of expression in the group of follicles close to ovulation. The mRNA expression results of our experimental follicle classes correlate well with an increase of positive staining for HIF1A mainly in granulose cells (Berisha et al., 2017).

The high level of HIF1A in the follicle tissue before the LH peak in our study (Table 1) seems to regulate the VEGF expression during LH peak (Table 1). There are a lot of indications about the importance of HIF1A for follicular angiogenesis in different species and also in cattle (Boonyaprakob et al., 2005; Duncan et al., 2008; Meidan et al., 2013; Nishimura & Okuda, 2010). In addition, the inhibition of HIF activity could prevent ovulation of the follicles (Kim et al., 2009). It is demonstrated that the rapid follicle growth, especially before ovulation, establishes a local hypoxic condition resulting in the accumulation of HIF1A that is responsible for VEGF and angiogenesis stimulation in antral follicles (Li et al., 2020). The time-dependent downregulation of HIF1A and VEGF expression in follicle before ovulation and rapid upregulation closely after ovulation (in the newly formed CL, period of angiogenesis) is considered the novelty of our experiments.

Furthermore, angiogenesis in theca follicle layers is dependent on the gonadotropin surge that stimulates the expression of HIF1A and VEGF in granulosa cells (Zimmermann et al., 2003). To verify the role of gonadotropins during folliculogenesis, Li et al. (2020) reported blocking of both angiogenesis and follicle ovulation using specific HIF1A and VEGF inhibitors despite FSH administration.

The VEGF expression is regulated by different mediators and molecular mechanisms including hormones, growth factors, cytokines, hypoxia, etc. In addition, Ferrara and Davis-Smyth (1997) demonstrated that VEGF regulates most steps of the angiogenic process, starting with extracellular matrix degradation and finishing with capillary tube formation. In our present study, the expression of all VEGF isoforms showed an identical expression pattern; however, we presented here VEGF165, considered the prototypical pro-angiogenic VEGF-A isoform (Peach et al., 2018). Transcripts of all VEGF isoforms (VEGF121, VEGF165, and VEGF189) were upregulated during LH peak, decreased thereafter to lowest levels, followed by an increase again during CL formation. As shown by immunohist ochemistry, VEGF ligand was localized in granulosa cells of growth follicles closed to ovulation (Berisha et al., 2008, 2000). Together, the results of our present study demonstrate a specific expression pattern of HIF1A and VEGF, depending on the developmental stage of the bovine follicles especially during LH peak and before ovulation in the cow. In addition, we specifically report a downregulation of these factors before ovulation and an upregulation after ovulation underling the critical involvement of HIF1A and VEGF cross-talk in postovulatory cellular differentiation.

In contrast to the follicle, the HIF1A and VEGF expression is significantly upregulated after ovulation, involving postovulatory cellular differentiation and CL formation (Berisha et al., 2020; Rico et al., 2014; Thompson et al., 2015; Van den Driesche et al., 2008). The high HIF1A and VEGF expression level after ovulation (CL day 2–3) in our study (Table 1) agree with previous studies indicating that the HIF1A-VEGF pathway might be crucial for the follicle ovulation (Kim et al., 2009; Li et al., 2020; Rico et al., 2014). Hypoxia-inducible factor 1-alpha expression in bovine early CL was first reported by Nishimura and Okuda (2010), while Duncan et al. (2008) reported it in primate CL. The follicles immediately after ovulation remain under extreme hypoxic conditions, stimulating HIF1A (Amselgruber et al.,

2000; Nishimura & Okuda, 2010), which is considered one of the major regulators of luteal angiogenesis (Krock et al., 2011; Meidan et al., 2013; Nishimura & Okuda, 2015). The high HIF1A expression in early CL (the typical angiogenesis, Table 1) agrees with earlier results in porcine and bovine models (Boonyaprakob et al., 2005; Duncan et al., 2008).

Conclusion and Recommendations

The results of our experiments indicate that the hypoxic conditions in the early CL regulate the expression of HIF1A followed by VEGF upregulation as shown previously (Curry & Smith, 2006). In addition, the interaction between the most important angiogenic factors VEGF via HIF1A is suggested to be the key mechanism of angiogenesis during CL formation and function (Li et al., 2020; Zhang et al., 2011). The high expression level of HIF1A and VEGF (both significantly upregulated) immediately after ovulation (CL formation) in our study (Table 1) demonstrates the essential role of these pro-angiogenic factors in the ovulation process, luteinization, and CL formation. Furthermore, the hypoxic condition in the early CL modulates the regulation of other important pro-angiogenic factors during early luteal formation (Abulafia & Sherer, 2000; Berisha et al., 2009, 2016a; Schams & Berisha, 2002).

The time-dependent regulation of HIF1A and VEGF expression in periovulatory follicles just before ovulation and in subsequent CL formation (immediately after ovulation) is considered the novelty of our experiments. Specifically, the downregulation before ovulation and the rapid upregulation after ovulation (early CL) underlie the critical involvement of HIF1A and VEGF cross-talk in postovulatory cellular differentiation and CL formation (Table 1). In addition, the significant upregulation of HIF1A and VEGF in newly formed CL suggests them to be crucial for the angiogenesis process and optimal CL formation and function.

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