**Multiple roles of hypoxia in ovarian function: roles of HIF-related and -unrelated signals during luteal phase.**[1]

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**Running head:** ROLES OF HYPOXIA DURING LUTEAL PHASE

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**1. Abstract.**

There is increasing interest in the role of oxygen conditions in the microenvironment of organs, since the discovery of a hypoxia-specific transcription factor, hypoxia-inducible factor-1 (HIF1). Ovarian function has several phases that change day by day, including ovulation, follicular growth, corpus luteum formation and regression. These phases are regulated by many factors, such as pituitary hormones and local hormones including steroids, peptides and cytokines, as well as oxygen conditions. Hypoxia strongly induces angiogenesis because transcription of a potent angiogenic factor, vascular endothelial growth factor, is regulated by HIF1. Follicular development and luteal formation are accompanied by a drastic increase of angiogenesis assisted by the HIF1-VEGF signaling. Hypoxia is also one of the factors for inducing luteolysis by suppressing progesterone synthesis and by promoting apoptosis of luteal cells. This review focuses on recent studies for hypoxic conditions as well as HIF1-regulated genes and proteins-in the regulation of ovarian function.

**Additional keywords**

Hypoxia, hypoxia-inducible factor

**Introduction**

Ovarian function has several phases that change day by day, including follicular growth, ovulation, luteal formation and regression. Follicles progress to ovulation through the proliferation of granulosa and theca cells. After ovulation, the corpus luteum (CL) develops accompanied by active angiogenesis, and when conception does not occur, regresses with the

decrease of progesterone (P4) synthesis and apoptosis of luteal cells. During the ovarian cycle, blood flow to the ovary changes, and affects the regulation of ovarian cycles (Nett *et al.* 1976; Niswender *et al.* 1976; Ford and Chenault 1981; Wise *et al.* 1982; Magness *et al.* 1983; Acosta *et al.* 2002). Changes in ovarian blood flow result in the changes in the transport of nutrients, hormones and gases, including $O_2$ to the ovary. In cows, ovarian blood flow has been reported to decrease during luteal regression, and to be kept at low levels during luteal formation after ovulation (Wise *et al.* 1982). Since a dominant follicle progress to ovulation during luteal regression (McCracken *et al.* 1999), follicular growth before ovulation occurs in parallel with the decrease of blood flow to the ovary. Furthermore, the oxygen content in ovarian venous blood begins to decrease at the late luteal stage (Wise *et al.* 1982). These findings indicate that the low oxygen condition (hypoxia) caused by the decreased blood supply is a characteristic part of the ovarian environment during follicular growth, ovulation and luteal formation. Therefore, understanding the role of oxygen conditions in the ovarian cycle should provide a better understanding of ovarian function. Cellular response to hypoxic conditions are strongly influenced by hypoxia-inducible factors (HIFs). HIFs are hypoxia-specific transcription factors that have roles in inducing several physiological processes including angiogenesis, erythropoiesis and glycolysis (Wiesener and Maxwell 2003; Hellwig-Burgel *et al.* 2005). This review focuses on studies of the roles of hypoxic conditions in the regulation of ovarian function, with emphasis on luteal formation and regression.

**Hypoxia-inducible factors (HIFs)**

Mammals have cellular mechanisms to adjust to low oxygen conditions. These

mechanisms are conserved and expressed in almost every mammalian cell type (Lee *et al.* 2004). The transcription factors activated during low oxygen conditions are called hypoxia-inducible factors (HIFs). They are hetero-dimers consisting of two subunits, HIF-α and aryl hydrocarbon receptor nuclear translocator (ARNT; also called HIF1β) (Chen *et al.* 2009). Both subunits contain basic helix-loop-helix (bHLH)-Per Arnt-Sim (PAS) domains that mediate heterodimerization and DNA binding (Dunwoodie 2009). HIF1β is constitutively expressed whereas the activity and expression of HIF1α depends on cellular oxygen concentrations (Jiang *et al.* 1996; Wenger 2002; Bruick 2003). Mammalian cells have three HIF-α genes (HIF1α, 2α, and 3α) (Jiang *et al.* 1996; Wenger 2002; Bruick 2003). Each contains an oxygen-dependent degradation domain (ODD) (Huang *et al.* 1998), which interacts with the von Hippel-Lindau (pVHL) E3 ubiquitin ligase complex (Maxwell *et al.* 1999; Cockman *et al.* 2000; Kamura *et al.* 2000; Ohh *et al.* 2000; Tanimoto *et al.* 2000) that targets HIF-α for proteasomal destruction under normoxia (Salceda and Caro 1997; Huang *et al.* 1998; Kallio *et al.* 1999; Ivan *et al.* 2001; Jaakkola *et al.* 2001). HIF1α is expressed ubiquitously, while the expression of HIF2α and HIF3α are more restricted (Chen *et al.* 2009). HIF1α and HIF2α dimerise with HIF1β forming HIF1 and HIF2, both of which activate key transcription factors (Chen *et al.* 2009; Dunwoodie 2009). HIF3α is found in three isoforms (HIF3α, neonatal and embryonic PAS (NEPAS) and inhibitory PAS protein (IPAS)) (Chen *et al.* 2009; Dunwoodie 2009). HIF3α isoforms dimerise with HIF1β forming HIF3 and HIF3NEPAS (Chen *et al.* 2009; Dunwoodie 2009). In general, HIFs bind to Hypoxia Response Elements (HREs) on DNA, which leads to the regulation of some 200 genes, of which 70 have been studied in detail (Chen *et al.* 2009; Dunwoodie 2009). HIF1 induces transcription of homeostasis-related genes such as vascular endothelial growth

factor (VEGF) and erythropoietin (EPO), whereas HIF2 and HIF3 have more specialized and tissue-specific regulatory roles (Carroll and Ashcroft 2005; Ietta *et al.* 2006; Chen *et al.* 2009; Dunwoodie 2009).

HIF1 initiates the defense against hypoxia by a variety of mechanisms. In kidney and liver, hypoxia induces the synthesis of EPO (Semenza and Wang 1992; Beck *et al.* 1993), which stimulates erythropoiesis, thereby increasing the $O_2$ capacity in the blood (Jelkmann 2004). In virtually all tissues, hypoxia induces the synthesis of proteins controlling local blood flow, such as VEGF (Forsythe *et al.* 1996; Kimura *et al.* 2001), endothelial nitric oxide synthase (eNOS) (Coulet *et al.* 2003), and heme oxygenase-1 (HOX-1) (Lee *et al.* 1997). VEGF stimulates angiogenesis and increases the permeability of blood vessels (Ferrara *et al.* 2003). eNOS and HOX-1 generate NO and carbon monoxide, which are potent vasodilatory substances that augment perfusion of the hypoxic tissue. At the cellular level, hypoxia induces the expression of virtually all glycolytic enzymes, including phosphoglycerate kinase-1 (PGK-1), enolase 1, and lactate dehydrogenase-1 (Firth *et al.* 1994; Semenza *et al.* 1996). Furthermore, the expression of membranous glucose transporters (primarily GLUT-1) is increased under hypoxic conditions, thereby increasing glucose uptake for glycolysis (Behrooz and Ismail-Beigi 1997; Gleadle and Ratcliffe 1997). To promote gene expression of all of these proteins, HIF1 binds to HREs present in the promoter and enhancer regions (Wenger 2002). HIF1 also induces transcription of an apoptosis-regulatory gene, nineteen kilodalton interacting protein-3 (BNIP3) (Bruick 2000). Apoptosis has important roles in ovarian physiology, especially occurs during follicular atresia (Matsuda *et al.* 2012) and luteal regression (McCracken *et al.* 1999). In cultured bovine luteal cells, the expression of HIF1 and BNIP3 are increased by incubation under hypoxic conditions

(Nishimura *et al.* 2006; Nishimura *et al.* 2008).

**Roles of hypoxic signaling in follicular development and ovulation**

The oxygen concentrations in the follicular fluid (FF) are lower in large follicles than in small follicles in cows (de Castro *et al.* 2008), sows (Basini *et al.* 2004) and women (Redding *et al.* 2008). The oxygen concentration in the FF decreases with the oocyte maturation (Fischer *et al.* 1992). Oxygen concentrations in the FF were first investigated in the 1970s. VEGF was found to have roles in the follicular development in 1992 by Ravindranath et al. (Ravindranath *et al.* 1992). HIF1 was recently found to have a role in the follicular development in pig (Basini *et al.* 2004; Boonyaprakob *et al.* 2005) and monkey (Duncan *et al.* 2008). The lower $pO_2$ in the FF in the large follicles than in the small follicles has been suggested to promote VEGF production via HIF1 in granulosa cells (Basini *et al.* 2004). On the other hand, in the primate ovary, nuclear immuno-staining of HIF1$\alpha$ is mostly absent in growing preantral and antral follicles, and is up-regulated in the granulosa cells at ovulation (Duncan *et al.* 2008). After ovulation, follicles rapidly change their functions and form CL with active angiogenesis (Redmer and Reynolds 1996), so that the HIF1-VEGF-induced angiogenesis system would be expected to be present in the later period of follicular development before ovulation and in the beginning of luteal formation immediately after ovulation.

**Roles of hypoxic signaling in luteal formation**

Angiogenesis during luteal formation was first observed in early 1990s' (Reynolds *et al.* 1992), and has been the subject of several reviews (Reynolds *et al.* 1994; Redmer and Reynolds

1996; Reynolds *et al.* 2000; Meidan *et al.* 2013). VEGF, a potent angiogenic factor, has been first identified in 1989 by Ferarra and Henzel (Ferrara and Henzel 1989), and found to be related to the angiogenesis in the luteal formation in cows (Grazul-Bilska *et al.* 1993) and in women (Kamat *et al.* 1995). Soon after the discovery of HIF1 (Wang and Semenza 1995), HIF1 has been found to be the most potent transcription factor for VEGF (Forsythe *et al.* 1996). The early luteal tissue just after ovulation is thought to be under hypoxic conditions, because of the destruction of vasculature by ovulation. Therefore, we planned to test the hypothesis that the luteal angiogenesis after ovulation is induced by hypoxia. In bovine luteal endothelial cells, the mRNA expressions of HIF1α and VEGF were not significantly different in normoxic (20% $O_2$) and hypoxic (1% $O_2$) culture (Tscheudschilsuren *et al.* 2002). On the other hand, mRNA expression of HIF1α in porcine CL was found to be high at the early luteal stage, which suggested that HIF1 assists in luteal formation (Boonyaprakob *et al.* 2005). To confirm the participation of HIF1 in luteal formation, analyses of HIF1α at the protein level are needed because the activity of HIF1 is more strongly regulated at the protein level than at the mRNA level (Salceda and Caro 1997). Under normoxic conditions, the HIF1α subunit is rapidly degraded by ubiquitin-proteasome pathway, whereas under hypoxic conditions, it becomes concentrated by down-regulation of the degradation, and becomes functional after dimerises with the other subunit HIF1β (ARNT) (Salceda and Caro 1997). Immunostaining shows that HIF1α protein is strongly expressed in the primate early CL (Duncan *et al.* 2008). In addition, HIF1α protein expression in bovine CL is higher at the early and developing luteal stages than at the other luteal stages (Nishimura and Okuda 2010). Hypoxia also induced that the expression of HIF1α protein, VEGF mRNA and protein increased under hypoxia in cultured

bovine developing luteal cells (Nishimura and Okuda 2010). During the peri-ovulatory period, the blood flow to the ovary is low (Wise *et al.* 1982). Recently, low oxygen conditions (10% $O_2$) have been shown to increase P4 synthesis by up-regulating steroidogenic acute regulatory protein (StAR) expression in the bovine luteinized granulosa cells, indicating the importance of hypoxia in luteinization (Fadhillah *et al.* 2014). These findings suggest that hypoxic conditions induced by decreased blood supply and degraded vasculature immediately after ovulation is important for forming the new CL under hypoxic conditions, and for promoting angiogenesis via HIF1 and VEGF activation (summarized in Figure 1).

**Roles of hypoxic signaling in luteal regression**

Ovarian blood flow in ruminants undergoes dynamic changes. Classical studies with electromagnetic probes have shown that ovarian blood flow is low just after ovulation, gradually increases toward the luteal stage, and then decreases during luteal regression in ewes (Nett *et al.* 1976; Niswender *et al.* 1976), cows (Ford and Chenault 1981; Wise *et al.* 1982) and sows (Magness *et al.* 1983). Luteal regression is characterized by a decrease in P4 production (functional luteolysis), followed by a decrease in luteal size (structural luteolysis), which is largely achieved by apoptosis (Juengel *et al.* 1993; Rueda *et al.* 1995; Bacci *et al.* 1996; Rueda *et al.* 1997; McCracken *et al.* 1999). In ewes and cows, the decrease of blood flow during luteolysis occurs in parallel with the decrease of serum P4 level (Nett *et al.* 1976; Niswender *et al.* 1976; Ford and Chenault 1981; Wise *et al.* 1982). These findings suggest that the decrease of blood flow is related to functional luteolysis. The finding that vascular occlusion occurred following the sloughing of endothelial cells into the lumina of small blood vessels during

luteolysis suggests that vascular occlusion is the cause for the decrease of blood supply and resulting hypoxic conditions in the CL (Sawyer *et al.* 1990). However, the mechanisms how the decrease of blood flow induces luteolysis have remained unclear. We hypothesize that oxygen deficiency is related to luteolysis because the decreased blood flow during luteolysis is thought to be the cause for the decrease of $O_2$ supply. In cultured bovine luteal cells, P4 production decreased under hypoxic conditions (3% $O_2$) (Nishimura *et al.* 2006). The expression and activity of the enzyme P450scc (cytochrome P450 side-chain cleavage enzyme; CYP11A1), which converts pregnenolone into P4 by cleaving the side-chain, also decreased by hypoxia. Apoptosis, which is an essential phenomenon for structural luteolysis (Juengel *et al.* 1993), was also induced in cultured luteal cells under hypoxic conditions. Under hypoxic conditions, an effecter caspase in the apoptosis cascade, caspase-3, was induced in cultured luteal cells (Nishimura *et al.* 2008). BNIP3, which facilitates apoptosis under hypoxic conditions (Bruick 2000), is also induced by hypoxia. These findings suggest that the oxygen deficiency in the CL, which is induced by a decreased blood supply to the ovary, is one of the factors that accelerate luteolysis basically induced by uterine prostaglandin F2α and other luteolytic factors, such as cytokines, peptides and gases (McCracken *et al.* 1999). These ideas are illustrated Figure 2.

**Evaluation of mRNA expressions in ovarian cells and tissues under hypoxic conditions**

Hypoxia signaling induces erythropoiesis, angiogenesis, apoptosis and the expression of genes and proteins that are involved in other cellular responses (Wenger 2002). To evaluate hypoxia-induced signals, it is necessary to choose proper internal controls for the analyses of mRNA and protein expressions. Some of the better known internal controls include β-actin,

glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 18S ribosomal RNA, ubiquitin, β-2 microglobulin and ribosomal proteins (S9, S15a, L19, L29) (Hosseini *et al.* 2010; Frota *et al.* 2011; Varshney *et al.* 2012; Wisnieski *et al.* 2013). NADPH is not a suitable internal control for experiments analyzing the effects of hypoxia or the expressions of hypoxia-related genes or proteins because it is regulated by HIF1, and increases under hypoxic conditions (Wenger 2002). Hypoxia also increased the expression of *GAPDH* mRNA in bovine luteal cells but had no effect on 18S rRNA expression (Figure 3). In experiments with cell cultures, there is another requirement for properly evaluating hypoxia signaling: the experiments need to show evidence of hypoxic conditions. An excellent indicator of hypoxic conditions in cell cultures is an increase in the expression of HIF1α protein (not HIF1α mRNA) because hypoxia affects HIF1α expression mainly at the protein levels (Wang and Semenza 1995).

**Conclusions**

After the discovery of HIF1 (Wang and Semenza 1995), cellular responses to hypoxia and their importance have been found in the many fields, including oncogenesis, reproductive physiology, development (Jiang *et al.* 1996; Wenger 2002; Bruick 2003; Wiesener and Maxwell 2003; Lee *et al.* 2004; Hellwig-Burgel *et al.* 2005; Chen *et al.* 2009; Dunwoodie 2009). The expression of HIF has been found in a variety of organs and the function of some of these HIFs have been determined, which suggests that the cells in such organs have the ability to respond to hypoxic conditions. The response to hypoxia is important pathologically in the ischemic tissues, and is important physiologically in the process of embryo development in organ formation (Jiang *et al.* 1996; Wenger 2002; Bruick 2003; Wiesener and Maxwell 2003; Lee *et al.* 2004;

Hellwig-Burgel *et al.* 2005; Chen *et al.* 2009; Dunwoodie 2009). We have previously suggested that hypoxia plays roles in both life (luteal formation) (Nishimura and Okuda 2010) and death (luteal regression) (Nishimura *et al.* 2008) of CL. In the formation of CL, hypoxia induces angiogenesis (Nishimura and Okuda 2010), whereas it decreases P4 synthesis (Nishimura *et al.* 2006) and promotes apoptosis during regression of the CL (Nishimura *et al.* 2008). These ideas are summarized in Figure 4. It is still unclear how the fate of cells is determined under hypoxic conditions. When cells are exposed to hypoxic conditions, the cellular response to hypoxia tries to keep cells alive under hypoxia at first, while the response tries to kill cells under severe hypoxic conditions (anoxia) (Piret *et al.* 2002). Recently, in human granulosa cells (van den Driesche *et al.* 2008) and bovine developing luteal cells (Zhang *et al.* 2011), human chorionic gonadotropin (hCG) has been also reported to increase HIF1α expression under normoxia. Understanding the crosstalk in the HIF1 regulation between the signals affected by hormones and low oxygen conditions could help to clarify the roles of hypoxia in ovarian physiology.

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**Figure legends**

**Figure 1.**

Possible hypoxia-induced signaling before ovulation (upper panel) and after ovulation (lower panel). Before ovulation, decreasing ovarian blood flow induces moderate hypoxic conditions in dominant follicle. These hypoxic conditions generate HIF1-VEGF signaling, resulting in the induction of angiogenesis for follicular development. Steroidogenesis is also induced by hypoxia via stimulation of StAR expression. After ovulation, hypoxic conditions induced by the degraded vasculature of ovulated follicle and by decreased blood flow to the ovary, stimulate angiogenesis required for luteal formation by HIF-VEGF signaling. These hypoxia-induced signalings act with other factors related to follicular development, luteinization and luteal formation. *Abbreviations; hypoxia-inducible factor-1 (HIF1), vascular endothelial growth factor (VEGF), steroidogenic acute regulatory protein (StAR), progesterone (P4).

**Figure 2.**

Possible hypoxia-induced signaling during luteal regression. Before luteolysis (left panel), the oxygen supply to functional CL is enough to produce large amount of P4 via activation of steroidogenesis-related proteins, such as StAR, P450scc and 3β-HSD. During luteolysis (right panel), ovarian blood flow decreases, so that the microenvironment in CL is under hypoxic conditions. This hypoxia decreases P4 synthesis (functional luteolysis) by inactivation of P450scc, and induces apoptosis (structural luteolysis) by HIF1-BNIP3 signaling. These hypoxia-induced signalings act with the other luteolytic signalings for completing luteal regression. *Abbreviations; corpus luteum (CL), steroidogenic acute regulatory protein (StAR), cytochrome P450 side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase 3β-HSD, progesterone (P4), hypoxia-inducible factor-1 (HIF1), bcl-2/adenovirus E1B nineteen kilodalton interacting protein-3 (BNIP3).

**Figure 3.**

Time-dependent effects of reduced oxygen tension on the expression of *GAPDH* mRNA (**A**) and 18S ribosomal RNA (18S rRNA; **B**) in cultured bovine mid luteal cells (n=3). The cells were cultured under normoxia (20% $O_2$; open circle) or hypoxia (3% $O_2$; closed triangle) for the indicated times. Asterisks indicate significant differences between oxygen tensions within each time-period ($P<0.01$), determined by Student's *t*-test. *GAPDH* mRNA expression increased by hypoxia at over 8 h of culture, while 18S rRNA expression showed no significant difference between oxygen conditions at any culture periods.

**Figure 4.**

Possible signaling pathways induced by hypoxia leading to ovarian phenomena. Hypoxia generates multiple signals related to several ovarian phenomena. Before ovulation (Ov.), hypoxia assists follicular development by inducing angiogenesis via HIF1-VEGF signaling. Meanwhile, hypoxia also facilitates luteolysis by down-regulating P4 production (functional luteolysis) via P450scc inhibition, and by inducing luteal cell apoptosis (structural luteolysis) via HIF1-BNIP3 signaling. During peri-ovulatory period, moderate hypoxia supports luteinization by stimulating P4 production via StAR. After ovulation, hypoxia strongly induces angiogenesis via HIF1-VEGF signaling for luteal formation. *Abbreviations; ovulation (Ov.), steroidogenic acute regulatory protein (StAR), progesterone (P4), hypoxia-inducible factor-1 (HIF1), vascular endothelial growth factor (VEGF), cytochrome P450 side-chain cleavage enzyme (P450scc), bcl-2/adenovirus E1B nineteen kilodalton interacting protein-3 (BNIP3).