Effect of hypoxia on the release of vascular endothelial growth factor and testosterone in mouse TM3 Leydig cells

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1 Department of Physiology, School of Medicine, National Yang-Ming University, Taipei; 2 Department of Nursing, Chang Gung Institute of Technology, Kweisan, Taoyuan; 3 Department of Physiology, Chang Gung University, Kweisan, Taoyuan; and 4 Department of Medical Research and Education, Taipei City Hospital, Taipei, Taiwan

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Hwang G-S, Wang S-W, Tseng W-M, Yu C-H, Wang PS. Effect of hypoxia on the release of vascular endothelial growth factor and testosterone in mouse TM3 Leydig cells. Am J Physiol Endocrinol Metab 293: E1763–E1769, 2007. —Hypoxia has been shown to stimulate the expression of vascular endothelial growth factor (VEGF), which is a major mediator for angiogenesis and vasculogenesis. During hypoxia, VEGF promotes angiogenesis in the testis. However, the effect of VEGF on the steroidogenesis of testosterone and the cell proliferation in Leydig cells is unclear. To assess the effects and the action mechanisms of hypoxia, a mouse TM3 Leydig cell line was employed in the present study. The Leydig cells were incubated in an incubator chamber (95% N2-5% CO2) for 1–24 h. The cultured media were collected and assayed by testosteroneRIA and VEGF enzyme immunoassay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to detect the proliferation of Leydig cells. The present results showed that the proliferation of Leydig cells was enhanced significantly by hypoxia. The basal VEGF release was increased, and the response of VEGF production to human chorionic gonadotropin (hCG) was also enhanced in hypoxic condition. During hypoxia, administration of hCG or VEGF stimulated proliferation of Leydig cells, but the stimulatory effect was abolished by the administration of anti-VEGF antibody. Higher doses of VEGF stimulated testosterone release in a dose-dependent manner. Administration of anti-VEGF antibody abolished the stimulatory effect of VEGF on testosterone release. These data suggest that hypoxia stimulates cell proliferation and testosterone release in Leydig cells via an increase of VEGF production.

hypoxia; mouse TM3 Leydig cells; testosterone

OXYGEN IS ESSENTIAL FOR LIFE in humans and other mammals. Insufficient oxygen, hypoxia, in tissues or cells results in physiological and pathological responses such as ischemia, stroke, hemorrhage (6, 33, 39), and many cardiopulmonary injuries (6, 41). Cellular hypoxia causes an induction of hypoxia-response genes related to the angiogenesis, oxygen transport, and metabolism (1). It has been shown that hypoxia can increase the concentration of intracellular free calcium, nitric oxide (20), and inducible nitric oxide synthase (iNOS; see Refs. 28 and 38) and generation of polymorphonuclear neutrophils (38) and leukotriene B4 (27).

Recent studies demonstrated that treatment with iNOS inhibitor can reduce lipid peroxidation, apoptosome formation, and intracellular caspase-3 activity, maintain the ATP levels, and promote cell survival (27). Other studies have shown that intermittent hypoxia treatment for 14 days can protect the rats from oxidative injury because of by the presence of more bilirubin (5).

Vascular endothelial growth factor (VEGF), one of the genes induced by hypoxia, is a key regulator for angiogenesis and vascular formation in vascular endothelial cells. VEGF is a homodimeric glycopeptide and has been characterized as a potential growth factor in angiogenesis of endothelial cells (18). This growth factor is also called a vascular permeability factor because it is 5,000 times more permeable than histamine. Five human VEGF isoforms (VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206) have been characterized (21). VEGF121, VEGF145, and VEGF165 have been considered as secreted VEGF isoforms because they induce proliferation of endothelial cells mediated by VEGF receptor. Different types of VEGF receptors have also been identified. VEGFR-1 (Flk-1) is thought to have a negative role in angiogenesis (16), but VEGFR-2 (Flk-1/KDR) is the main mediator of the mitogenic and angiogenic effects of VEGF (15).

Luteinizing hormone (LH) and follicle-stimulating hormone (FSH), released from the anterior pituitary, are well known regulators of testicular functions in males. Apart from these endocrine effectors, autocrine and paracrine control of Leydig cells has been studied in both stimulatory and inhibitory roles of steroidogenesis. It has been shown that certain growth factors possess positive [e.g., insulin-like growth factor-I (IGF-I), inhibit, activin] or negative [e.g., transforming growth factor (TGF)-β, TGF-α/EGF, basic fibroblast growth factor (bFGF)] regulations of LH/hCG receptor numbers and mRNA and steroidogenic enzyme mRNA and activities, to alter the responsiveness to LH in the immature porcine Leydig cells (30).

We have previously shown that intermittent hypoxia (12% O2-88% N2) to male rats for 7–14 days caused an increase of plasma testosterone levels (7). Angiogenesis and vasodilation were observed in rat testicular tissues after intermittent hypoxia. It is possible to postulate that the increase of plasma testosterone levels might have resulted from certain angiogenesis-related growth factors that were triggered by hypoxia. In the present study, mouse Leydig cells were used to investigate the effects of hypoxia on 1) cell proliferation, 2) release of VEGF, and 3) the involvement of basic cellular regulatory pathways in regulation of VEGF secretion.

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Materials and Methods

Antibodies and reagents. Human chorionic gonadotropin (hCG) and mouse VEGF were purchased from Sigma (St. Louis, MO). PD-98059 was purchased from Tocris Cookson (Westwoods Business, Park Ellisville, MO). Anti-VEGF (1:200 dilution), anti-phospho (p)-extracellular signal-regulated kinases 1 and 2 (ERK1/2; 1:1,000 dilution), anti-β-actin (1:8,000 dilution), and anti-glycerolaldehyde-3-phosphate dehydrogenase (GAPDH; 1:500 dilution) were purchased from Santa Cruz (Watsonville, CA). The horseradish peroxidase-conjugated IgG, goat anti-rabbit IgG (1:6,000 dilution), and goat anti-mouse IgG (1:8,000 dilution) were purchased from ICN Pharmaceuticals (Aurora, OH).

Cell culture. TM3 Leydig cells, a nontumorogenic cell line derived from mouse testis (36), were obtained from the Culture Collection and Research Center (Food Industry Research and Development Institute, Taiwan, Republic of China). This cell line responds to LH by increasing testosterone production and secretion through mechanisms similar to those encountered in freshly isolated cells. Cells were cultured in 75-cm² flasks (Falcon, Franklin Lakes, NJ) in a 1:1 mixture of Ham’s F-12 and DMEM (Sigma) that contained 15 mM HEPES, 0.12% NaHCO₃, supplemented with 0.45% glucose, 5% horse serum, 2.5% FCS (Kibbutz Beit, Haemek, Israel), and 100 IU/ml potassium penicillin G + 100 µg/ml streptomycin sulfate (Sigma). Cells were cultured at 37°C in either a humidified atmosphere of normoxic conditions (95% air-5% CO₂) or in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) and flushed with hypoxic gas (95% N₂-5% CO₂).

Effect of hypoxia on basal and hCG-stimulated cell proliferation in TM3 Leydig cells. Mouse TM3 Leydig cells (2,000 cells in 200 µl) were preincubated for 48 h in 96-well plates and then incubated for 1–16 h with or without hCG at 1 IU/ml in normoxic or hypoxic conditions. To test the role of hypoxia on VEGF-stimulated cell proliferation in TM3 Leydig cells, the cells were incubated with VEGF (5–20 ng/ml) under hypoxic conditions.

To determine the proliferation in Leydig cells through VEGF receptor, TM3 cells were incubated with hCG (1 IU/ml) or VEGF (5 ng/ml) in the presence or absence of anti-VEGF antisera at 0.1 µg/ml. At the end of incubation, the cells were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell proliferation assessment.

Effect of hypoxia on basal and hCG-stimulated VEGF secretion in TM3 Leydig cells. After preincubation for 48 h in 12-well plates, the cells (10⁶ cells/ml) were incubated for 1–16 h with or without hCG at 1 IU/ml plus 50 µM PD-98059 [an inhibitor of mitogen/extracellular signal-regulated kinase (MEK)] in a normoxic or hypoxic condition. At the end of incubation, the cultured medium was collected and stored at −20°C until analyzed for VEGF by ELISA.

Effect of hypoxia on the expressions of ERK1/2. Mouse TM3 Leydig cells (10⁵ cells/10 ml) were seeded in 10-cm dishes and then incubated with or without hCG at 1 IU/ml for 1 or 16 h in a normoxic or hypoxic condition. At the end of incubation, cytoplasmic proteins were extracted from the cells and used to determine the expression of p-ERK1/2 through Western blot.

Effect of VEGF on testosterone release and ERK1/2 expression in TM3 Leydig cells. To ascertain the dose-dependent effect of VEGF, the cells (10⁵ cells/ml) were seeded in 12-well plates and then incubated with or without VEGF (5–20 ng/ml). The medium was collected and stored at −20°C until analysis for testosterone by RIA. Furthermore, the cells (10⁵ cells/10 ml) were seeded in 10-cm dishes and then incubated with or without hCG at 1 IU/ml and/or VEGF at 20 ng/ml in the presence or absence of PD-98059 at 50 µM. At the end of incubation, the cells were extracted for cytoplasmic protein to determine p-ERK1/2 expression by Western blot.

Cell proliferation assessment: MTT assay. We used the modified colorimetric MTT assay to quantify cell proliferation (9). Living cells reduced the yellow MTT to blue formazen, which was soluble in DMSO (Wako, Osaka, Japan). The culture medium, the intensity of blue staining was proportional to the number of cells alive at analysis. Described briefly, cells were incubated in 96-well microplates (Falcon) for 24 h. Cells were plated at 2,000 cells/200 µl per well with medium supplemented with 7.5% serum. The culture medium was removed and replaced by serum-free medium. After incubation for 24 h, the culture medium was replaced again by serum-free medium containing various drugs. After the treatments, the medium was removed and replaced by 50 µl MTT solution (1 mg/ml; Sigma). After a 4-h further incubation, the MTT solution was removed and replaced by 50 µl DMSO, and plates were shaken for 3 min. The optical density of each well was determined using a microplate reader (Dynatech Laboratories, Chantilly, VA) at a wavelength of 570 nm with a reference wavelength of 630 nm.

VEGF ELISA. At the end of incubation of Leydig cells under normoxic or hypoxic conditions, the supernatant was collected, and VEGF levels were determined using a Quantikine VEGF ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer’s instructions. This kit specifically measures rodent VEGF164 and VEGF120 variants; the limit of detection is 3 pg/ml. VEGF concentrations were normalized relative to cellular protein concentrations by Bradford protein assay.

Statistical analysis. The data were expressed as means ± SE. The treatment means were tested for homogeneity using ANOVA, and the difference between specific means was tested for significance using Duncan’s multiple-range test or Student’s t-test (47). A difference between two means was considered to be statistically significant when the P value was <0.05.

Results

Effect of hypoxia on basal and hCG-induced cell proliferation. To study the effect of hypoxia on TM3 cell proliferation, cell were exposed to normoxic or hypoxic conditions for 1–16 h. The proliferation status was assessed by MTT assay. As shown in Fig. 1, proliferation of TM3 cells was significantly increased by hCG treatment for 16 h in both normoxic and hypoxic conditions. As expected, hypoxia for, 16 h caused a greater induction in TM3 cell proliferation than normoxia did. Under hypoxic conditions, VEGF had significant stimulatory effect on cell proliferation compared with normoxic conditions in a dose-dependent manner (Fig. 2). To examine whether the VEGF receptor was involved in the induction of cell proliferation, we incubated the TM3 cell with anti-VEGF antibody. A similar inhibitory effect on cell proliferation was observed when the anti-VEGF antibody was added before stimulation of the cells with hCG, VEGF, and hypoxic treatment (Fig. 3).

Effect of hypoxia on the stimulation of VEGF release and expression by hCG. The effects of hypoxia on VEGF release by TM3 cells are shown in Fig. 4. After having been treated with hCG, the levels of medium VEGF were enhanced significantly after 4 h in normoxic conditions and at 2 h in hypoxic conditions. In addition to the stimulatory effect on cell proliferation, hypoxia also caused a significant elevation of VEGF release following treatment with or without hCG for 16 h. It has been shown that, in mouse Leydig cells, VEGF secretion could be mediated by an MEK1/2- and ERK1/2-dependent pathway (2). We therefore incubated the TM3 cells with the MEK inhibitor PD-98059 to block the phosphorylation of ERK1/2. Consequently, a significant enhancement in VEGF release in hypoxic conditions was observed after administration with PD-98059 (Fig. 4).

Activation of ERK1/2 by hCG and hypoxia in TM3 cells. It has been shown that hCG provokes a two- to threefold increase

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in the levels of phosphorylated ERK1/2 in MA-10 cells transiently transfected with a vector coding the wild-type human lutropin/choriogonadotropin receptor (53). Moreover, it has been demonstrated that, in human microvascular endothelial cells-1 (HMEC-1), ERK1/2 were activated during hypoxia (37). To verify whether the expression of ERK1/2 was affected by hypoxia in TM3 cells, we treated the cells with hCG in normoxic and hypoxic conditions. The results revealed that the expression of p-ERK1/2 was activated by hCG and hypoxia after 1 or 16 h (Fig. 5). The levels of p-ERK1/2 expression were judged by the staining of GAPDH, which showed consistent expression in the cells, as an internal control.

VEGF increased testosterone release in TM3 cells. It has been reported that steroids, including androgens, estrogens, and progestagens, are able to induce VEGF production in a variety of steroid-dependent cells. Both estrogen and androgens stimulate the expression of VEGF by increasing gene expression and mRNA stability (46). Our results indicated that the levels of VEGF were not altered by testosterone treatment (data not shown). However, VEGF significantly increased testosterone production in TM3 cells by a dose-dependent manner (Fig. 6). Administration of anti-VEGF antibody to block the signaling through VEGF receptor resulted in an inhibitory effect of testosterone release (Fig. 6). To further verify the mitogen-activated protein kinase (MAPK) signaling pathway associated with the stimulatory effect caused by VEGF on testosterone release, the expression of p-ERK1/2 was also detected. As shown in Fig. 7, VEGF induced an elevation of p-ERK1/2 expression that could be suppressed by the MEK inhibitor PD-98059.

DISCUSSION

In the present studies, we found that 1) hypoxia increased the proliferation of mouse TM3 Leydig cells and enhanced the stimulatory effect of hCG and VEGF on the release of testosterone, 2) hypoxia stimulated the formation and secretion of VEGF in Leydig cells and also enhanced the stimulatory effect of hCG on VEGF production, 3) VEGF stimulated the production of testosterone in Leydig cells, and 4) hypoxia, hCG, and
VEGF enhanced the phosphorylation of ERK1/2 in the MAPK pathway in Leydig cells. VEGF is an important factor in the regulation of angiogenesis. Recent studies demonstrated that hypoxia increased the secretion of VEGF by induction of the expression of veGF gene (1, 24). Hypoxia increased the proliferation of corticotrophs in the anterior pituitary gland and secretion of ACTH by the hypothalamus-pituitary-adrenal axis (17). Our studies demonstrated and confirmed that hypoxia increased the proliferation of Leydig cells. Hypoxia promoted the migration of epithelium in vessels and angiogenesis (26) because of a stimulation of VEGF121, VEGF145, and VEGF165 (42). hCG, similar to LH, increases the proliferation of Leydig cells. In our studies, hypoxia potentiated the proliferation of Leydig cells by VEGF and LH. Administration of anti-VEGF antibody decreased the proliferation caused by hypoxia, hCG, and VEGF. The secretion of VEGF induced by hCG indirectly increased the proliferation of Leydig cells.

Under hypoxic conditions, tissues increased the production and secretion of VEGF for angiogenesis (1). Recent studies demonstrated that the secretion of VEGF in mouse and rat Leydig cells following hCG treatment was via the protein kinase A (PKA) and MAPK pathway (2). VEGF receptors, including VEGFR-1 and VEGFR-2, have been found in human Leydig cells, and the secretion of VEGF in Leydig and Sertoli cells has also been reported (14). Our results demonstrated that hCG increased the secretion of VEGF in mouse TM3 Leydig cells and significantly enhanced the hypoxia effects on basal and hCG-treated Leydig cells. The activation of ERK1/2 by hCG was promoted by hypoxia. The phosphorylation of ERK1/2 was an important controlling factor in the release of VEGF via hCG stimulation. We used the MEK inhibitor PD-98059 to block the phosphorylation of ERK1/2. Administration of PD-98059 significantly inhibited the secretion of VEGF induced by hCG and insignificantly inhibited the basal secretion of VEGF during hypoxia. We therefore suggested that the secretion of VEGF by Leydig cells in hypoxia was partially activated by the MAPK pathway.

Recently, we have demonstrated that under intermittent hypoxic (88% N₂-12% O₂, 8 h/day) conditions for 7 or 14 days, male rats enhanced the plasma concentration of testosterone (7). We concluded that the mechanisms of testosterone release by intermittent hypoxia are the result of 1) activation of

Fig. 4. Effects of PD-98059 [a mitogen/extracellular signal-regulated kinase (MEK) inhibitor] on hCG-stimulated VEGF release in TM3 Leydig cells after treatment with or without hypoxia. *P < 0.05 and **P < 0.01 compared with vehicle group at indicated time. +++P < 0.01 compared with normoxic vehicle group at indicated time.

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the pathway of cAMP and cholesterol desmolase (cholesterol side-chain cleavage cytochrome P450, P450scc) activity, 2) increased intracellular calcium levels, and 3) increased expression of the steroidogenic acute regulatory (StAR) protein. Contrary to our postulation, hypoxia did not enhance testosterone secretion directly in Leydig cell, unless under hCG stimulation. It seems that hypoxia enhanced the testosterone in rats via an indirect effect to stimulate the Leydig cells. Recent studies demonstrated that growth factors (e.g., IGF-I, inhibin, activin, TGF-β/TGF-α, and bFGF) can modulate the number of LH receptors in Leydig cells and the secretion of the testosterone (30). Some studies demonstrated that prostate cells (32, 45), uterine cells (10, 23), and breast cancer cells (22) as well as androgens, estrogens, or progestagens can stimulate the release of VEGF. Our recent studies show that intermittent hypoxia (8 h/day) can produce the angiogenesis in the testes. In the present study, we have demonstrated that a high concentration of VEGF (10 ng/ml) significantly increased not only basal levels but also hCG-stimulated testosterone secretion by Leydig cells, and anti-VEGF antibody inhibited the secretion of testosterone caused by hCG and VEGF. The fact that hypoxia did not increase testosterone secretion directly might be because the secretion of VEGF by Leydig cells within 16 h was not long enough to enhance the production of testosterone.

Recent studies demonstrated that VEGF may act through VEGFR-1 and VEGFR-2 receptors on the endothelial cells to activate the phosphatidylinositol 3-kinase/protein kinase B and MAPK pathways to modulate its functions (13). Studies showed that the effects of LH and hCG on the gonad cells are through multiple signal transduction pathways, including cAMP, phospholipase C, modulation of intracellular calcium ion signaling, and MAPK (3, 31). These signal molecules stimulate the rapid translocation of cholesterol from the outer mitochondrial membrane into the inner mitochondrial membrane, in which it is converted into pregnenolone by the enzyme called cytochrome P450scc (8). It is now well established that StAR protein is a key regulatory protein in the rapid modulation of steroidogenesis in the gonadal cells (49, 51). The expression of the StAR protein is not only mainly modulated by cAMP-dependent mechanisms; other factors and signaling pathways, however, may play permissive roles (50). Studies indicated that protein kinase C (25) and several transcription factors, including the cAMP-response element (CRE)-binding protein/CRE modulator, the activator protein 1 family members Fos and Jun, DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region on the X chromosome, gene 1), and SR-B1 (scavenger receptor, class B, type-1) are involved in regulating StAR expression and steroidogenesis in gonadal and adrenal cells (11, 29, 34, 43, 44). In addition, the regulation of StAR expression and steroidogenesis by the MAPK/ERK signaling pathway has been demon-

Fig. 6. Effects of anti-VEGF antibody on VEGF-stimulated testosterone release in TM3 Leydig cells after treatment for 16 h. *P < 0.05 and **P < 0.01 compared with corresponding vehicle group. +P < 0.05 compared with vehicle group.

Fig. 7. Effects of PD-98059 (a MEK inhibitor) on basal, hCG-, and VEGF-stimulated p-ERK 1/2 expression in TM3 Leydig cells.
strated. Although some investigators have suggested that inhibition of the MEK/ERK signaling cascade is associated with enhanced ovarian steroid synthesis (19, 52), others have shown that increased steroid biosynthesis may be augmented by cAMP-induced activation of the MEK/ERK signaling cascade (4, 12). Excess androgen production is the classical endocrine disorder found in the reproductive-aged women with polycystic ovary syndrome (PCOS). Nelson-Degrafe et al. (40) found that, in PCOS theca cells, reduced levels of activated MEK1/2 and ERK1/2 are correlated with increased androgen production and CYP17 mRNA, which encodes the enzymes for the production of androgen. Our results showed that ERK1/2 was activated in the Leydig cells treated with VEGF. Administration of MEK inhibitor PD-98059 decreased the activity of ERK1/2. We therefore suggested that VEGF might modulate the function of Leydig cells by activation of ERK1/2. However, whether VEGF acting on the testosterone release from Leydig cells is through the regulation of StAR, P450ccc, or CYP17 enzymes remains to be explored.

Recent studies have shown that PKA plays a key role in the activation of steroidogenesis by gonadotropins (48). PKA increases the expression of the StAR protein. Gyles et al. (19) demonstrated that cAMP induced steroidogenesis by the activation and phosphorylation of ERK and that ERK activation could increase the phosphorylation of steroidogenic factor-1 and increase steroidogenesis through the transcription of StAR gene. Martinelle et al. (35) found that, in the Leydig cells activated by hCG, MAPKs may play a role in synthesis of StAR protein and the activity of the protein phosphatase 2A.

The present results have shown that hypoxia in vitro modulated the secretion of VEGF in Leydig cells and intermittent hypoxia in vivo increased the testicular angiogenesis and testosterone levels in rat plasma. In summary, we concluded that hypoxia increased the secretion of testosterone via a mechanism involving 1) enhanced production of VEGF in the testes, 2) proliferation of Leydig cells, and 3) increased activity of the ERK1/2 pathway in Leydig cells.

GRANTS
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