Effects of hypoxia on testosterone release in rat Leydig cells

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Hwang GS, Chen ST, Chen TJ, Wang SW. Effects of hypoxia on testosterone release in rat Leydig cells. Am J Physiol Endocrinol Metab 297:E1039–E1045, 2009. First published August 18, 2009; doi:10.1152/ajpendo.00010.2009.—The aim of this study was to explore the effect and action mechanisms of intermittent hypoxia on the production of testosterone both in vivo and in vitro. Male rats were housed in a hypoxic chamber (12% O2 + 88% N2, 1.5 l/min) 8 h/day for 4 days. Normoxic rats were used as control. In an in vivo experiment, hypoxic and normoxic rats were euthanized and the blood samples collected. In the in vitro experiment, the enzymatically dispersed rat Leydig cells were prepared and challenged with forskolin (an adenylyl cyclase activator, 10−4 M), 8-Br-cAMP (a membrane-permeable analog of cAMP, 10−4 M), hCG (0.05 IU), the precursors of the biosynthesis testosterone, including 25-OH-Cholesterol (10−6M), pregnenolone (10−6M), and androstenedione (10−7M), and NAADP (calcium-signaling messenger causing release of calcium from intracellular stores, 10−5M). The concentrations of testosterone in plasma and medium were measured by radioimmunoassay. The level of plasma testosterone in hypoxic rats was higher than that in normoxic rats. Enhanced testosterone production was observed in rat Leydig cells treated with hCG, 8-Br-cAMP, or forskolin in both normoxic and hypoxic conditions. Intermittent hypoxia resulted in a further increase of testosterone production in response to the testosterone precursors. The activity of 17β-hydroxysteroid dehydrogenase was stimulated by the treatment of intermittent hypoxia in vitro. The intermittent hypoxia-induced higher production of testosterone was accompanied with the influx of calcium via L-type calcium channel and the increase of intracellular calcium via the mechanism of calcium mobilization. These results suggested that the intermittent hypoxia stimulated the secretion of testosterone at least in part via stimulatory actions on the activities of adenylyl cyclase, cAMP, L-type calcium channel, and steroidogenic enzymes.

In the respiratory system, hypoxia increases the breath rate and depth and enhances the oxygen pressure (45). In the cardiovascular system, hypoxia induces the migration of endothelial cells, the production of hypoxia-inducible factor (HIF), and the vessels angiogenesis and dilation (30). In the endocrine system, hypoxia affects the function of the hypothalamic-pituitary-thyroid axis and reduces the thyrotroph number and thyroid weight (19). Also, hypoxia also acts on the hypothalamus-pituitary-adrenal axis and increases the corticotroph number and the levels of plasma adrenocorticotropic hormone (ACTH) (20). The aged hypoxic rats displayed a prolonged plasma corticosterone stress response and had higher adrenal weight than controls (52). Hypoxia stimulates the expression of the steroidogenic acute regulatory protein and enhances the secretion of glucocorticoids but inhibits the aldosterone synthesis and increases the volume of urea (56, 57). In addition, Bruder et al. (8) have shown that hypoxia decreases aldosterone synthesis via a decrease of cAMP production in rats and found that P-450 side-chain cleavage enzyme (P450scs) activity is decreased during hypoxia. Moreover, we have demonstrated that hypoxia and testosterone stimulated the secretion of erythropoietin (70).

It has been shown that the basal levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are significantly lower in hypoxic patients (61). Wang (68) demonstrated that there are great disturbances of hypothalamic-pituitary-gonadal function in male patients with acute attack of chronic cor pulmonale. Earlier studies showed that lower serum testosterone levels are accompanied in patients following hypoxia administration (60). Recently, it has been shown that hypoxia (40 mmHg O2) significantly inhibits cAMP and ACTH-stimulated productions of aldosterone, cortisol, and dehydroepiandrosterone (55). Farias et al. (16) have demonstrated that the FSH and LH levels decrease in rats exposed to chronic hypobaric hypoxia. Also, dysfunction of the pituitary-gonadal axis has been observed in the patients with obstructive sleep apnea (OSA) (37), which is characterized by recurrent upper airway collapse during sleep (53). In contrast, Coste et al. (12) have shown no significant effects of hypoxia on the circadian profile of the gonadal axis hormones. Moreover, increased testosterone secretion from TM3 Leydig cells by hypoxia treatment has been observed in our laboratory (29).

Multiple studies have demonstrated that chronic hypoxia induced the reproduction dysfunction (1, 15, 64). Moreover, earlier studies showed that plasma insulin, glucagon, and LH did not significantly change in graded exercise in normoxic or acute hypoxic conditions. Plasma Δ4-androstenedione and testosterone increased in a similar manner in both conditions (5). However, chronic hypoxia and intermittent hypoxia are exhibited in different conditions. Also, chronic hypoxia and inter-

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OXYGEN IS ESSENTIAL FOR LIFE IN HUMAN BEINGS and other mammals. Insufficient oxygen or hypoxia in tissues or cells results in physiological changes and pathological responses such as ischemia, stroke, hemorrhage (9, 42, 46), and many cardiopulmonary injuries (10, 50). It has been shown that hypoxia increases the concentration of intracellular free calcium, nitric oxide (NO) (25), and the activity-inducible NO synthase (31, 32) as well as generation of polymorphonuclear neutrophils (47, 48) and leukotriene B4 (32). Moreover, cellular hypoxia causes an induction of hypoxia response genes that is related to angiogenesis, oxygen transport, and metabolism (23).
tient hypoxia exert different physiological effects on neurological, cardiovascular (17), and respiratory systems (43). In addition, OSA has been related to intermittent hypoxia, not chronic hypoxia (11). However, intermittent hypoxia in the reproduction system is still unclear. In the present study, we have tested the hypothesis that acute intermittent hypoxia acts through the pathways of the actions of gonadotropin and cAMP, calcium channel, and P450scc to affect the secretion of testosterone by rat Leydig cells.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), N-2-hydroxyethylpipеразине-N'-2-ethanesulfonic acid (HEPES), Hanks’ balanced salt solution (HBSS), medium 199, sodium bicarbonate, penicillin G, streptomycin, heparine, collagenase, human chorionic gonadotropin (hCG), forskolin, 8-bromo-cAMP (8-Br-cAMP), 25-hydroxycholesterol, pregnenolone, progesterone, androstenedione, nifedipine, nimodipine, tetradrine, and nicotinic acid adenine dinucleotide phosphate (NAADP) were purchased from Sigma Chemical (St. Louis, MO). [3H]testosterone was obtained from Amersham International (Bucks, UK). The doses of drugs were expressed in their final mol concentrations in the flask.

Animals. Male Sprague-Dawley rats weighing 300–350 g were housed in a temperature-controlled room (22 ± 1°C) with 14 h/day of artificial illumination (6:00 AM to 8:00 PM) and given food and water ad libitum.

The use of the animals was approved by the Institutional Animal Care and Use Committee of the National Yang-Ming University. All animals received human care in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals published by the National Science Council, Taiwan.

Treatment of hypoxia. Eight male rats were divided into normoxia and intermittent hypoxia groups. Rats in the intermittent hypoxia group were housed in an environment aerated with 12% O2 and intermittent hypoxia groups. Rats in the intermittent hypoxia Laboratory Animals published by the National Science Council, ad libitum.

The method for preparation of rat Leydig cells. The method for preparation of rat Leydig cells has been described elsewhere (21). Rat testicular interstitial cells, dispersed using collagenase as described by Lin et al. (36), were gently applied to the upper layer of a continuous Percoll gradient (20 ml/tube; composed of 9 parts of Percoll and 11 parts of 1.8-fold medium 199) generated by centrifugation at 20,000 g for 60 min at 4°C (3). The added testicular interstitial cells in continuous Percoll gradient were then centrifuged at 800 g for 20 min at 4°C. After two further washes, the cell pellet was resuspended in a final volume of 10 ml in incubation medium consisting of medium 199 containing 1% BSA, 25 mM HEPES, 2.2 g/l sodium bicarbonate, 100 IU/l penicillin G, 100 mg/l streptomycin sulfate, and 2,550 USP U/l heparin, pH 7.3, aerated with 95% O2 and 5% CO2. The cell concentration (1 × 10^5 cells/ml) and viability (>98%) were determined using a hemocytometer, and the Trypan blue method was awake (n = 4).

Experimental design. Cell suspensions (1 × 10^5 cells/ml) of normoxia and acute intermittent hypoxia groups were preincubated for 1 h and then incubated for 1 h in the forskolin (an adenyllyl cyclase activator, 10^{-4} M), 8-Br-cAMP (a membrane-permeable analog of cAMP, 10^{-4} M), hCG (0.05 IU), and the precursors of the biosynthesis testosterone, 25-OH-cholesterol (10^{-5} M), pregnenolone (10^{-7} M), progesterone (10^{-7} M), and 17-OH-progesterone (10^{-7} M). The androstenediones (10^{-7}-10^{-5} M) nifedipine (L-type Ca^{2+} channel blocker, 10^{-5}-10^{-4} M), nimodipine (L-type calcium channel blocker, 10^{-5} M), tetradrine (L-type calcium channel blocker, 10^{-5} M), and NAADP (calcium-signaling messenger causing release of calcium from intracellular stores, 10^{-6}-10^{-4} M) are in the presence or absence of treatment with normoxia or hypoxia. At the end of incubation, 0.5 ml of ice-cold gelatin phosphate-buffered saline was added and immediately followed by centrifugation at 100 g for 10 min at 4°C. The supernatant fluid was stored at −20°C until analyzed for testosterone by RIA.

RIA of testosterone. The concentrations of testosterone in plasma and medium samples were determined by RIA, as described elsewhere (35, 69). With anti-testosterone serum no. W8, the sensitivity of testosterone RIA was 2 pg/assay tube. The intra- and interassay coefficients of variation were 10.6 (n = 5) and 6.2% (n = 7), respectively.

Statistical analysis. All data were given as means ± SE. In some cases, the means of treatment were tested for homogeneity by analysis of variance, and the difference between specific means was tested for significance by Duncan’s multiple range test (65). In other cases, Student’s t-test was employed. A difference between two means was considered statistically significant when P < 0.05.

RESULTS

Plasma testosterone concentration. Male rats were treated with or without intermittent hypoxia 8 h/day for 4 days. The plasma testosterone was determined by RIA. The level of plasma testosterone in hypoxic rats was significantly higher than that in normoxic rats (P < 0.01; Fig. 1).

Testosterone secretion in response to 8-Br-cAMP, forskolin, and hCG in vitro. It is well known that 8-Br-cAMP is a permeable analog of cAMP. Forskolin is an activator of adenylcyclase to increase the intracellular levels of cAMP. Incubation of rat Leydig cells with hCG, 8-Br-cAMP, or forskolin enhanced the production of testosterone in both normoxic and hypoxic rats. Hypoxia resulted in a further increase of testosterone production in response to the above stimulants by Leydig cells compared with normoxia rats. (P < 0.01; Fig. 2).

Testosterone release in response to steriodogenic precursors in vitro. Administration of the precursors of the biosynthesis of testosterone, 25-OH-cholesterol (10^{-5} M), pregnenolone (10^{-7} M), progesterone (10^{-7} M), or 17-OH-progesterone (10^{-7} M) were added to the cultured Leydig cells. The effects of intermittent hypoxia on testosterone secretion are shown in Figs. 1 and 2.
stimulated the production of testosterone in vitro from rat Leydig cells five- to 10-fold (Fig. 3). Intermittent hypoxia resulted in a further increase of testosterone production in response to the above precursors ($P < 0.01; \text{Fig. 3}$).

Activity of 17β-hydroxysteroid dehydrogenase. Androstenedione is an immediate precursor of the biosynthesis of testosterone. Administration of androstenedione ($10^{-7} - 10^{-5}$ M) enhanced the production of testosterone in both normoxic and hypoxic groups ($P < 0.01; \text{Fig. 4}$). The levels of testosterone release in response to androstenedione at $10^{-7}$ and $10^{-6}$ M were higher in the hypoxia group than in the normoxia group ($P < 0.01; \text{Fig. 4}$). When the high dose of androstenedione was employed, the response of testosterone release was the same between the hypoxia and normoxia groups (Fig. 4).

Testosterone release in response to calcium channel blockers in vitro. Nifedipine, nimodipine, and tetrandrine are L-type Ca$^{2+}$ channel blockers. Administration of nifedipine ($10^{-6} - 10^{-4}$ M) decreased the production of testosterone in both normoxic and hypoxic groups (Fig. 5). The levels of testosterone release in response to nifedipine were lower in the hypoxia group than in the normoxia group ($P < 0.01; \text{Fig. 5}$). When the high dose of nifedipine was employed, the decreased response of testosterone release was the same between the hypoxia and normoxia groups (Fig. 5). Also, administration of nimodipine ($10^{-5}$ M) or tetrandrine ($10^{-5}$ M) decreased the production of testosterone in both the normoxic and hypoxic groups (Fig. 6).

Testosterone release in response to NAADP in vitro. NAADP is one of the most potent calcium-signaling messengers, causing release of calcium from intracellular stores. Administration of NAADP ($10^{-5} - 10^{-4}$ M) stimulated the production of testosterone in both the normoxic and hypoxic groups ($P < 0.01; \text{Fig. 7}$). The levels of testosterone release in response to NAADP ($10^{-6} - 10^{-5}$ M) were higher in the hypoxia group than in the normoxia group ($P < 0.01; \text{Fig. 7}$). The response of testosterone was dose dependent with NAADP in both the normoxia and hypoxia groups.

DISCUSSION

In the present study, we have demonstrated that 1) intermittent hypoxia increased the secretion of testosterone and enhanced the stimulatory effects of hCG, 8-Br-cAMP, and forskolin on the release of testosterone, 2) intermittent hypoxia stimulated the production of testosterone in Leydig cells in the presence of steroidogenic precursors, 3) intermittent hypoxia

Fig. 2. Effects of intermittent hypoxia for 4 days on 8-bromo-cAMP (8-Br-cAMP), forskolin, and human chorionic gonadotropin (hCG)-induced testosterone secretion by rat Leydig cells in vitro. **$P < 0.01$ compared with normoxia group. Each value represents means ± SE.

Fig. 3. Effects of intermittent hypoxia for 4 days on 25-OH-cholesterol-, pregnenolone-, progesterone-, and 17-OH-progesterone-induced testosterone secretion by rat Leydig cells in vitro. **$P < 0.01$ compared with normoxia group. Each value represents means ± SE.
resulted in more reduction of testosterone production in response to L-type calcium blockers compared with the normoxia group, and a calcium-signaling messenger enhanced more testosterone production in the intermittent hypoxia group than in the normoxia group.

The studies of interaction between hypoxia and reproduction systems are few and not clear. Recent studies have shown that hypoxia influences the nervous, circulatory, respiratory, and endocrine systems in human beings (30, 44, 56). In the endocrine system, hypoxia suppresses the function of the hypothalamic-pituitary-thyroid axis, decreases the number of thyrotrophs, and reduces the function of thyroid gland (21). Hypoxia also enhances the function of the hypothalamus-pituitary-adrenal axis and increases the corticotroph numbers and ACTH secretion (19, 20).

Hu et al. (28) have shown that continuous exercise induces a decrease of testosterone under either normoxia or hypobaric hypoxia. They also showed that testosterone secretion could be suppressed in the case of both continuous and intermittent exercise under hypoxic conditions. They suggested that the hypoxic exercise resulted in a suppression of Leydig cell testosterone biosynthesis, not pituitary dysfunction. It has been demonstrated that the acute intermittent prenatal hypoxia attenuates the postnatal testosterone surge in rats. Postpartum plasma corticosterone levels were also suppressed by hypoxia in rats (24). In addition, the plasma LH and testosterone levels decreased in Wistar rats exposed to chronic hypoxia (16). In contrast, Barnholt et al. (2) have shown that testosterone increased acutely in all subjects within the first 48 h at 4,300 meters.

Our studies were designed to explore the mechanisms of the testosterone production in Leydig cells after intermittent hypoxia in vivo and in vitro. In the present studies, we have shown that those testosterone levels in rat plasma and rat Leydig cell culture medium are increased following intermittent hypoxia. These results are different from those in the recent studies.

![Fig. 4](image4.png)

**Fig. 4.** Effects of intermittent hypoxia on androstenedione-induced (10^{-7}–10^{-5} M) testosterone secretion by rat Leydig cells in vitro. **P < 0.01 compared with normoxia; +++P < 0.01 compared with androstenedione-free group. Each value represents means ± SE.

![Fig. 5](image5.png)

**Fig. 5.** Effects of intermittent hypoxia for 4 days on testosterone secretion in vitro in response to nifedipine (10^{-6}–10^{-4} M). **P < 0.01 compared with normoxia group; +++P < 0.05; +++P < 0.01 compared with nifedipine-free group. Each value represents means ± SE.

![Fig. 6](image6.png)

**Fig. 6.** Effects of intermittent hypoxia on testosterone secretion in vitro in response to nimodipine (10^{-5} M) and tetrandrine (10^{-5} M). **P < 0.01 compared with normoxia group; +++P < 0.01 compared with vehicle group. Each value represents means ± SE.

![Fig. 7](image7.png)

**Fig. 7.** Effects of nicotinic acid adenine dinucleotide phosphate (NAADP; 10^{-6}–10^{-4} M) on testosterone secretion by rat Leydig cells following treatment with normoxia or intermittent hypoxia for 4 days. **P < 0.01 compared with normoxia group; +++P < 0.01 compared with vehicle group. Each value represents means ± SE.
Previous studies have shown that androstenedione biosynthesis and the weight of the testes were reduced in hypoxia, and the testosterone synthesis was not changed (41). Another study has demonstrated that testosterone and its precursors decrease in the testes of old men (54). These authors assumed that these age-dependent changes are caused by an impaired oxygen supply of the ageing testes. In the present study, we found that incubation of the precursors of testosterone (25-OH-C, pregnenolone, progesterone, 17-OH-progesterone, and androstenedione) can stimulate the levels of testosterone in intermittent hypoxia (Figs. 3 and 4). Hypoxia seemed to activate the enzymes of testosterone biosynthesis.

Recent results have shown that calcium entry blockers can maintain regional cerebral cortical blood flow and may have a significant role in cerebral resuscitation following cardiac arrest (71). Other results have demonstrated that hypoxia inhibits high K+-induced catecholamine release and that this inhibition is a result of the inhibition of high K+-induced increases in Ca2+ subsequent to the inhibition of Ca2+ influx via voltage-dependent Ca2+ channels (34). High doses of 17β-estradiol protect the neurons from ischemia by inhibiting the release of calcium ion from the intracellular Ca2+ stores (9). Gupte et al. (22) have shown that the dehydroepiandrosterone metabolite epiandrosterone may act as an L-type Ca2+ channel antagonist with properties similar to those of 1,4-dihydropyridine. Other studies have shown that testosterone is a vasodilator in both coronary and pulmonary circulation. The beneficial effects of testosterone were immunomodulation, altering expression of cytokines, and an antithrombotic action. (63). These results indirectly showed the interaction of hypoxia and testosterone, and they did not show that the acute hypoxia influences the production of testosterone via calcium channel. Our results have shown that the production of testosterone was increased by enhancing the intracellular Ca2+ and calcium-signaling messenger in the condition of intermittent hypoxia.

In the steroidogenesis, the cholesterol is converted to pregnenolone within the inner mitochondrial membrane by cytochrome P450scce (CYP11A1). This conversion is caused by the cholesterol side-chain cleavage reaction and is the rate-limiting step in the synthesis of steroids. pregnenolone is subsequently converted to the various steroids produced by the different steroidogenic tissues by gland-specific pathways. In the case of the placenta, pregnenolone is converted to progesterone by type I 3β-HSD (3, 49, 66). ACTH increases cholesterol binding to cytochrome P450scce by increasing the enzyme’s affinity for its substrate or availability of cholesterol and promotes turnover of the enzyme (67). A low-oxygen condition decreases progesterone synthesis by attenuating P450scce production and activity in bovine luteal cells (51). Sustained hypoxia activates adrenal steroidogenesis in the older fetal sheep. The resultant increase in cortisol synthesis is associated with decreased expression of adrenal IGFI-II mRNA (6).

A recent study has shown that HIF-1α is constitutively present in the Leydig cells of the murine testis and potentially regulates HSD-3b1 transcription and male reproduction function (40). In 1998, Braems et al. (6) showed that, in the older sheep fetuses, hypoxemia resulted in significantly increased levels of mRNAs encoding P450scce, P450C21, and 3β-HSD (6). These results are similar to ours. Rudolfsson and Bergh (58) showed that treatment of castrated rats with testosterone resulted in an increase of HIF-1α, VEGF, and carbonicanydrase 9 levels in ventral prostate epithelial cells. In our study, indirect evidence from testosterone precursor 25-OH-C administration suggests that increased testosterone secretion induced by intermittent hypoxia could be related to the activity of P450scce. HIF-1α may influence the enzymes of testosterone production. More studies are needed to clarify this point.

OSA is characterized by recurrent upper airway collapse and repetitive oscillations in oxyhemoglobin saturation during sleep (11, 72). OSA results in a chronic exposure to intermittent hypoxia (17). Although studies investigating the pathophysiological effects of hypoxia are concentrated on the chronic hypoxia (e.g., high-altitude training or exercise) (14, 27), recent research has focused mainly on the intermittent hypoxia, which is thought to be related to OSA. OSA is associated with heart diseases such as hypertension, coronary artery disease, pulmonary hypertension, stroke, and heart failure (17). Also, patients with OSA have endocrine disorders, including acromegaly, hypothyroidism, diabetes mellitus, and Cushing’s syndrome (4). Moreover, sexual dysfunction has been found in OSA patients (11). Decreased plasma testosterone concentrations were found frequently in the patients with OSA (18, 26, 37–39, 59, 73), in hypoxia-treated people (62), and in rats (28). The mechanism for the decreased plasma testosterone in the patients with OSA is not yet known. However, obesity is thought to be the main risk factor for OSA. In addition, aged males have much greater chance to have OSA (13). Moreover, the decline in plasma testosterone concentrations may be due to obesity and advanced age (18, 37, 38), not OSA or sleep fragmentation. In contrast, there were no significant effects of hypoxia on the circadian profile of plasma testosterone in young healthy males after 8 h spent in a hypobaric chamber, equivalent to 8,000 or 12,000 feet above sea level (12). In our study, young and healthy male rats were used. Increased testosterone was observed after intermittent hypoxia treatment. More studies are needed to clarify whether obesity and age are involved in the decreased plasma testosterone observed in the patients with OSA.

In conclusion, our results demonstrate that intermittent hypoxia stimulates the testosterone secretion through the activation of adenylyl cyclase, enhanced activities of P450scce, 3β-HSD, and 17β-HSD, increased levels of calcium ion, and influx into the Leydig cells.

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GRANTS

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REFERENCES


