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Chronic intermittent hypoxia stimulates testosterone production in rat Leydig cells

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ABSTRACT

Aims: The hypoxia-stimulated response of the endocrine system depends on the kind and duration of hypoxia. Hypoxia has been reported to stimulate testosterone (T) production in rats, but the mechanisms remain to be investigated.

Materials and methods: Male rats were divided into two groups. The rats exposed to chronic intermittent hypoxia (CIH) at 8 h/day were housed in a hypoxic chamber (12% O_2) for 14 days. Normoxic rats were used as control animals. T was measured after challenging the rat Leydig cells (LCs) with different stimulators, including hCG (0.01 IU/ml), forskolin (10^{-5} M), 8-bromo-cAMP (10^{-4} M), A23187 (10^{-5} M), cyclopiazonic acid (10^{-4} M), and androstenedione (10^{-8} M). Meanwhile, the LCs were incubated with trilostane (10^{-5} M) and/or 25-OH-hydroxycholesterol (10^{-5} M); thereafter the media were collected for pregnenolone assay.

Key findings: In the CIH group, plasma T levels were increased, but the serum luteinizing hormone (LH) was decreased. Furthermore, at several time intervals after hCG injection, plasma T levels were higher in the CIH group. The evoked-release of T and pregnenolone were significantly increased in the CIH group. Compared with the normoxic group, the CIH group had higher mRNA and protein expression levels of the LH receptor and CYP11A1 but not StAR. The plasma and testicular microvasculature VEGF levels were increased in the CIH group. The testicular vessel distribution was more obvious in CIH rats.

Significance: CIH-induced T secretion might be partially mediated by mechanisms involving the induction of LH receptor expression, testicular angiogenesis, CYP11A1 activity, 17β-HSD activity, and calcium-related pathway.

1. Introduction

Oxygen is a metabolic substrate and a signaling mediator. Hypoxia is defined as deprivation of adequate oxygen supply at the tissue level of the whole body or a region of the body. It can result from conditions, such as low oxygen content in the inspired air, premature birth, and hematologic or cardiopulmonary disease, and can lead to physiologic or pathologic response [1–3]. Hypoxia can be further distinguished as normobaric or hypobaric, the latter mimics a high-altitude environment. It initiates angiogenesis and metabolic reprogramming through activating the hypoxia signaling pathway, which is predominantly regulated by hypoxia-inducible factor (HIF) [4]. Moreover, hypoxia affects the hormone biosynthesis in different endocrine glands, such as the pituitary [5], adrenal gland [6,7], thyroid [5], and gonads [8]. The

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hypoxia-stimulated response of the endocrine systems depends on the variety and duration of hypoxia.

Hypoxia mediated the physiologic response in hormone regulation. In rat adrenal cortical cells, the production of aldosterone and ACTH-evoked corticosterone in the zona glomerulosa and zona fasciculata/reticularis cells, respectively, were reduced after 1 h of hypoxia. In addition, the hypoxia-suppressed productions of ACTH-stimulated corticosterone, aldosterone and cAMP, is dependent on the oxygen concentration [9]. Moreover, exposure of male rats to 28 days of hypoxia under a barometric pressure of 380 mmHg, which is equivalent to an altitude of 5500 m, had been reported to increase the proliferation of corticotrophs in the anterior pituitary and the ACTH level [6] but it decreased the number of thyrotrophs and thyroid weight [5]. In rat adrenal cortex, a decrease in oxygen inhibits CYP11A1 and CYP11B2 activities [9].

Steroidogenesis in Leydig cells (LCs) requires sequential actions that convert cholesterol into testosterone (T) [10,11], which is stimulated by pulses secretions of the luteinizing hormone (LH) through cAMP-PKA pathway. The first and the most critical step on steroidogenesis is the conversion of cholesterol to pregnenolone by cytochromes CYP11A1 (P450scc) and the steroidogenic acute regulatory (StAR) protein [12]. Thereafter, pregnenolone is converted to T by 3β-hydroxysteroid dehydrogenase isomerase (3β-HSD), 17α-hydevrepoxylase/C17-20 lyase (P450c17) and 17β-HSD in the smooth endoplasmic reticulum [13].

Hypoxia also affects T levels. T and LH levels were reported to decrease in male rats housed in a hypoxic hypobaric chamber (428 Torr, pO_2:89.6 mmHg, 8 h/day, 5 days/week) for 30 days [8]. Moreover, sperm number was decreased after 3 weeks of hypoxia at an altitude of 5000 m [14]. In contrast, the plasma T level was increased after 48 h of hypoxia at a high-altitude (4300 m) [15]. In apnea-related hypoxia, which is found in patients with obstructive sleep apnea, the LH and T levels were reduced [16], and this effect was associated with increased oxidative stress and sexual dysfunction [17]. With intermittent exercise, plasma T was showed to have a tendency to increase during normoxia, but it was suppressed during hypobaric hypoxia [18]. In contrast, T release in rat LCs was stimulated by four-day of intermittent hypoxia, partially through induction of the activities of adenylyl cyclase, cAMP, L-type calcium channel, and 17β-HSD [19]. Interesting, the bi-phasic effect of hypoxia on steroidogenesis was also found in granulosa cells [20]. Therefore, hypoxia-regulated T release had been a controversial issue.

Hypoxia has been shown to alter the activities of steroidogenesis-related enzymes. The direct effects and mechanisms of chronic intermittent hypoxia (CIH) on T production remain to be determined. Therefore, we used an animal model and purified LCs, in order to 1) investigate the effects of CIH on T secretion capacity; 2) determine the effects of CIH on the cAMP-, Ca^{2+}-, and 17β-HSD-evoked T production; 3) investigate the effects of CIH on the expressions of the LH receptor, CYP11A1, and StAR, and 4) determine the effects of CIH on testicular angiogenesis.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 300–350 g were maintained under controlled temperature (22 ± 2 °C) and light (06:00–20:00) conditions, with food and water *ad libitum*. The use of the animals was approved by the Institutional Animal Care and Use Committee of National Yang-Ming University. All animals received human care in compliance with the Principles of Laboratory Animal Care and the Guide for Care and Use of Laboratory Animals (National Science Council, Taiwan).

2.2. CIH exposure

Forty male rats were divided randomly into two groups: normoxia (n = 20) and CIH (n = 20). The CIH rats were housed in a 12% O_2-hypoxic chamber maintaining by a hypoxic generator (HYP123, Hypoxico Inc., New York, NY, USA), 8 h daily for 14 days. Normoxic rats were used as control animals.

2.3. Blood sample collection

After 14-d CIH exposure, blood samples were collected directly after decapitation. The plasma samples were collected to determine the concentrations of plasma T, serum LH, and serum VEGF. Moreover, the blood samples from testicular vein were also collected for serum VEGF determination.

2.4. Preparation of rat LCs

After hypoxic exposure, rat testes were removed after decapitation and digested by collagenase. The digestion was then filtered through the nylon mesh and applied to a continuous percoll gradient. After centrifugation, LCs was found in 10% height of the tube from the bottom. The purity of LCs was approximately 85% [21].

2.5. The functional capacity to secrete T in vitro and in vivo

In the *in vitro* study of functional capacity to secrete T, LCs were challenged with human chorionic gonadotropin (hCG, 0.05 IU/ml) at 34 °C for 1 h. After centrifugation, the supernatant was collected for T radioimmunoassay (RIA).

In the *in vivo* study, rats were anesthetized then catheterized *via* the right jugular vein [22]. Twenty hours later, the conscious rats were challenged with hCG (5 IU/kg). Blood samples (0.5 ml at each time point) were collected before and 15, 30, 60, 90 and 120 min after the hCG-administration in order to determine plasma T levels.

2.6. Effects of CIH on cAMP-, Ca^{2+}-, and 17β-HSD-evoked T secretion

To determine the effects of CIH on cAMP-, Ca^{2+}-, and 17β-HSD-related T secretion, LCs were incubated with different stimulators including forskolin (an adenylyl cyclase activator, 10^{-5} M), 8-bromo-adenosine 3′,5′-cyclic monophosphate (8-Br-cAMP, 10^{-4} M), cyclopiazonic acid (CPA, 10^{-4} M), A23187 (10^{-5} M) and androstenedione (10^{-8} M) at 34 °C for 1 h. After centrifuging, the supernatant was collected for T RIA.

2.7. Determination of the CYP11A1 activity in LCs

LCs were incubated with trilostane (an inhibitor of 3β-hydroxysteroid dehydrogenase, 10^{-5} M), and/or 25-hydroxycholesterol (25-OH-C, 10^{-5} M) at 34 °C for 1 h. After centrifuging, the supernatant was collected for pregnenolone EIA.

2.8. Western blot

LCs were extracted with lysis buffer and 20 μg of protein extracts were separated by 10% SDS-PAGE and transferred to PVDF membrane [21,23]. An enhanced chemiluminescence kit (Amersham, Piscataway, NJ, USA) was used for immunodetection. The protein bands were quantified by the Image J software (NIH, Bethesda, MD, USA). The following antibodies were used: anti-CYP11A1 and anti-β-actin antibodies were purchased from Millipore (Chemicon, Temecula, CA, USA) and anti-LH receptor antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-StAR antibody was kindly provided by Dr. D. M. Stocco (Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX,

USA).

2.9. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

The mRNA expressions of LH receptor, CYP11A1, and StAR were investigated [21,24]. Total RNA was extracted with TRlzol (Invitrogen, Carlsbad, CA, USA). RT reactions and PCR reactions were used by the SuperScript™ III Reverse Transcriptase (Invitrogen) and the Taq DNA polymerase master mix (Ampliqon, Copenhagen, Denmark). The applied L19, CYP11A1, StAR, and LH receptor RT-PCR primers and the expected PCR products lengths described elsewhere [24,25]. The PCR products were further size-fractionated on 2% agarose gels, and then stained with ethidium bromide. The signals were measured by Image J software.

2.10. T RIA

The T concentrations of plasma and media were measured by RIA [26,27]. The sensitivity of the T RIA was 2 pg/tube. The intra- and inter-assay coefficients of variation were 4.1% (n = 6) and 4.7% (n = 10), respectively.

2.11. Pregnenolone EIA

The concentration of medium pregnenolone was determined by EIA [22,28] with anti-pregnenolone serum purchased from Biogenesis Inc. (Sandown, NH, USA). 3,30,5,50-tetramethylbenzidine (TMB) was purchased from Sigma and used as substrate. The absorbance values were measured at wave length of 450 nm.

2.12. Serum LH levels

Serum LH levels were analyzed by rat pituitary kit of MILLIPLEX™ MAP by the Luminex® xMAP® technology (Millipore Corp., St. Louis, MO, USA). The LH sensitivity of the bioassay was 4.9 pg/ml. The intra- and inter-assay coefficients of variation were lower than 15% (n = 12) and 9.5% (n = 5), respectively.

2.13. Serum VEGF levels of peripheral and testicular vein

The concentration of serum of VEGF was examined by using kit (R& D Systems, Minneapolis, MN, USA). This kit specifically measures rodent VEGF 164 and VEGF120 variants; the limit of detection is 3 pg/ml.

2.14. Statistical analysis

All values are presented as mean ± SEM of at least three independent experiments. Data were processed by Student's *t* test [29]. Statistically significant was considered at $p < 0.05$. SigmaStat (Systat Software Inc., Chicago, IL, USA) was used for data analysis.

3. Results

3.1. Plasma T and serum LH

CIH increased the plasma T level but it decreased the serum LH level. The plasma T of the CIH group showed a 2.4-fold increase, compared with that of the normoxic group (1.61 ± 0.13 ng/ml vs. 0.68 ± 0.09 ng/ml, Fig. 1a). The serum LH level was approximately 63% lower in the CIH group than in the normoxic group (0.13 ± 0.02 ng/ml vs. 0.35 ± 0.09 ng/ml, Fig. 1b).

3.2. Functional capacity to secrete T in vivo and in vitro

The T production capacity was evaluated *in vivo*. The plasma T before hCG (5 IU/kg) administration was significantly higher in the CIH group than in the normoxic group (Fig. 2a). Further, the plasma T levels in the CIH group increased by 2.3-, 2.7-, and 2.1-fold at 60, 90, and 120 min (Fig. 2a). The area under the curve (AUC) for hCG-evoked plasma T was 2.3-fold greater in the CIH group than in the normoxic group (Fig. 2b). In an *in vitro* study, compared with the normoxic group, the CIH group showed a 1.7-fold increase in the hCG-evoked T secretion and a 2.2-fold increase in the vehicle T secretion (Fig. 2c). Therefore, CIH significantly induced the functional capacity to release T, both *in vitro* and *in vivo*.

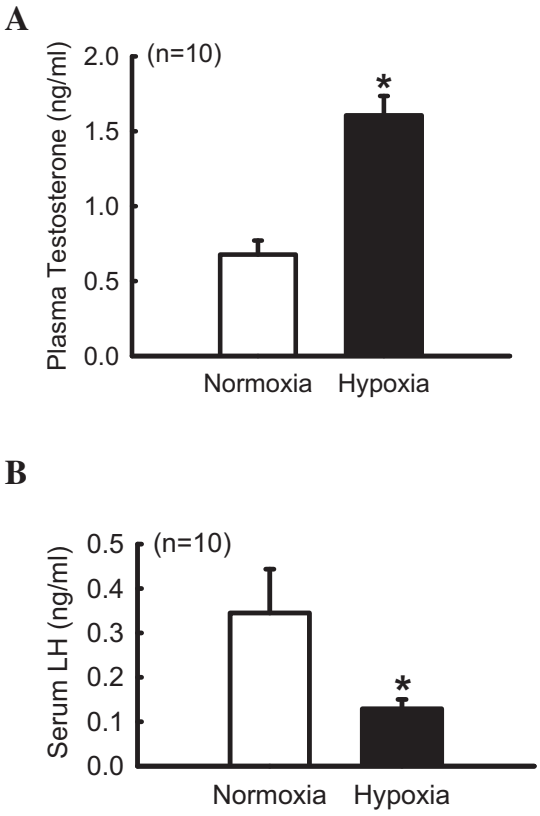

Fig. 1. CIH (A) increased the plasma T concentration but (B) decreased the LH level. Values are expressed as mean ± SEM. *$p < 0.05$, *vs.* the normoxic group.

3.3. Expression level of the mRNA and protein of the LH receptor in LCs

The mRNA and protein expressions of the LH receptor in LCs were determined by RT-PCR (Fig. 3a–b) and Western blot (Fig. 3c–d), respectively. Compared with the normoxic group, the CIH group showed significant increase in the LH receptor mRNA and protein levels by 1.5-fold and 1.7-fold, respectively.

3.4. CIH induced the cAMP-related T release in LCs

Compared with normoxic group, the CIH had increased T secretion in response to 8-Br-cAMP and forskolin (Fig. 4a) by 2-fold and 2.2-fold, respectively. Therefore, CIH induced T release partially through the cAMP-related pathway.

3.5. Calcium-related T release in LCs

Compared with the normoxic group, the CIH group had significantly increased CPA- and A23187-stimulated T release by 1.8-fold and 1.9-fold, respectively (Fig. 4b). Therefore, CIH induced T release partially through the Ca^{2+}-related pathway.

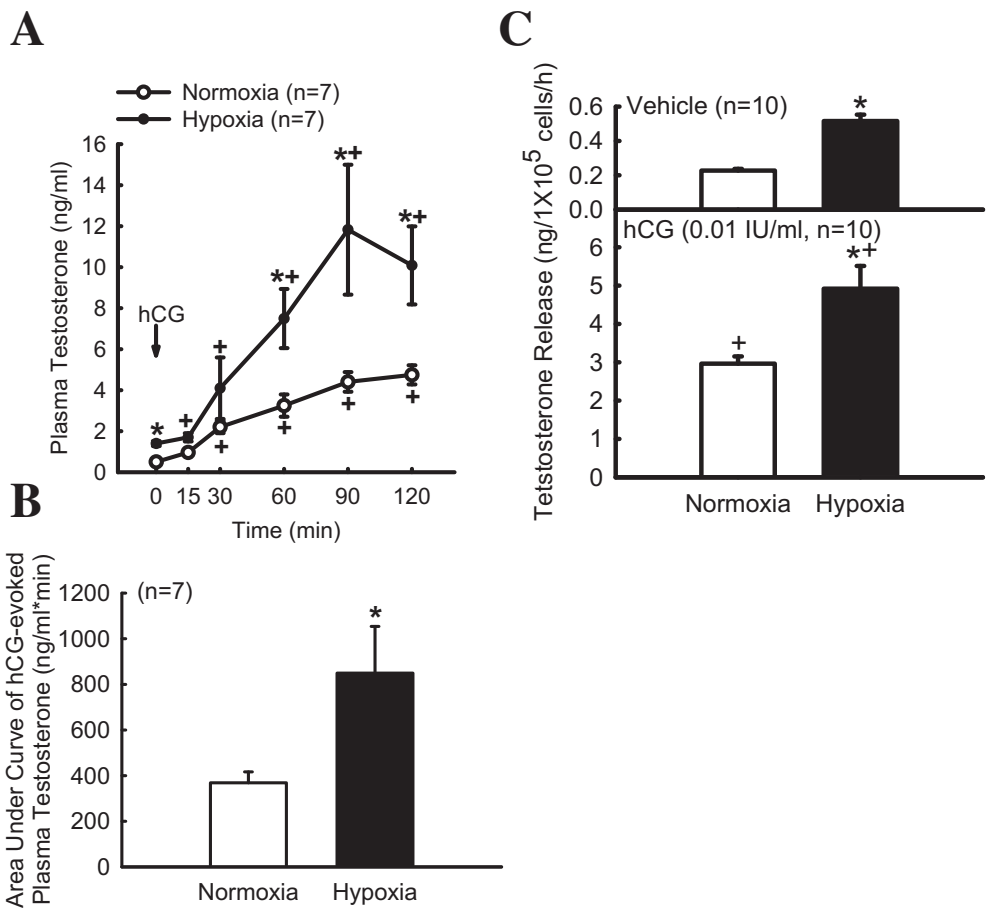

Fig. 2. Effects of CIH on T secretion both *in vivo* and *in vitro*. (A) The rats are given a single intravenous injection of hCG (5 IU/kg) *via* a right jugular catheter and are bled for T determination at different time intervals after injection. (B) The AUC of hCG-evoked plasma T. (C) Effects of CIH on the release of T in LCs incubated with hCG (0.01 IU/ml). Values are expressed as mean ± SEM. *$p < 0.05$, *vs.* the normoxic group. + $p < 0.05$, vs. time 0 min (baseline) or the vehicle group.

3.6. 17β-HSD activity

Androstenedione is an immediate precursor of T biosynthesis. Compared with the normoxic group, the CIH group had significantly increased androstenedione-stimulated T release by 3-fold (Fig. 4c).

3.7. Protein and mRNA expressions of CYP11A1 and StAR

StAR and CYP11A1 proteins are rate-limiting steps in steroidogenesis. The mRNA and protein expressions of StAR and CYP11A1 in LCs were investigated by RT-PCR (Fig. 5a–b) and Western blot (Fig. 5c–d), respectively. The mRNA and protein levels of CYP11A1 were significantly increased by 6.3-fold and 1.5-fold, respectively following CIH. However, both the mRNA and protein expressions of StAR were not different between the 2 groups.

3.8. CYP11A1 activity

To further determine the CYP11A1 activity, the supernatant was collected for pregnenolone measurement after trilostane treatment. CIH increased the trilostane-stimulated pregnenolone secretion (Fig. 5e). However, there was no difference in the 25-OH-C-evoked pregnenolone production between the 2 groups. Therefore, CIH induced the CYP11A1 activity in LCs.

3.9. Serum VEGF levels and testicular vessel distribution

The serum VEGF levels in both peripheral and testicular veins were significantly increased after exposure to CIH. Compared with the normoxic group, the CIH group showed increase in the serum VEGF by 1.4-fold in the peripheral vein (20.38 ± 2.36 pg/ml vs. 14.42 ± 0.74 pg/ml) and by 2.0-fold in the testicular vein (72.98 ± 9.93 pg/ml vs. 36.24 ± 4.20 pg/ml) (Fig. 6a). The vessel distribution in rat testes was more apparent after CIH exposure (Fig. 6b).

4. Discussion

The results from this present *in vivo* study suggested that the increase in the plasma T after 14 days of intermittent hypoxia might be partially medicated by mechanisms involving the enhancement of LH receptor expression; the activities of the CYP11A1 and 17β-HSD; and the calcium-, and cAMP-related pathways. Moreover, CIH significantly increased testicular angiogenesis and the serum VEGF levels in the peripheral and testicular vein (Fig. 7).

A previous study has shown that the plasma total T and free T levels in men significantly increased to the highest value after 48 h of hypoxia at a high-altitude of 4300 m, then gradually declined to the baseline levels on day 19 [15]. On the other hands, plasma LH and T levels were reported to decrease after 30 days of hypobaric hypoxia exposure [8]. Additionally, serum T and PaO_2 were reported to have a significant positive correlation [30]. In our animal study, 14-day of intermittent hypoxia significantly increased plasma T and decreased serum LH. This result was consistent with that of previous reports, which demonstrated stimulation of T release after 4 days of intermittent hypoxia [19]. Taken together, acute hypoxia may induce T production but long-term continuous hypoxia may decrease the T level. We speculated that the plasma T level may represent an adaptive response to prolonged hypoxic exposure, which contributes to the decline in T levels over time. The hypoxia-induced increase in plasma T level seemed to be maintained by repeated hypoxia-reoxygenation such as in intermittent hypoxia.

The T production of LCs depends on the pulsatile secretion of LH by

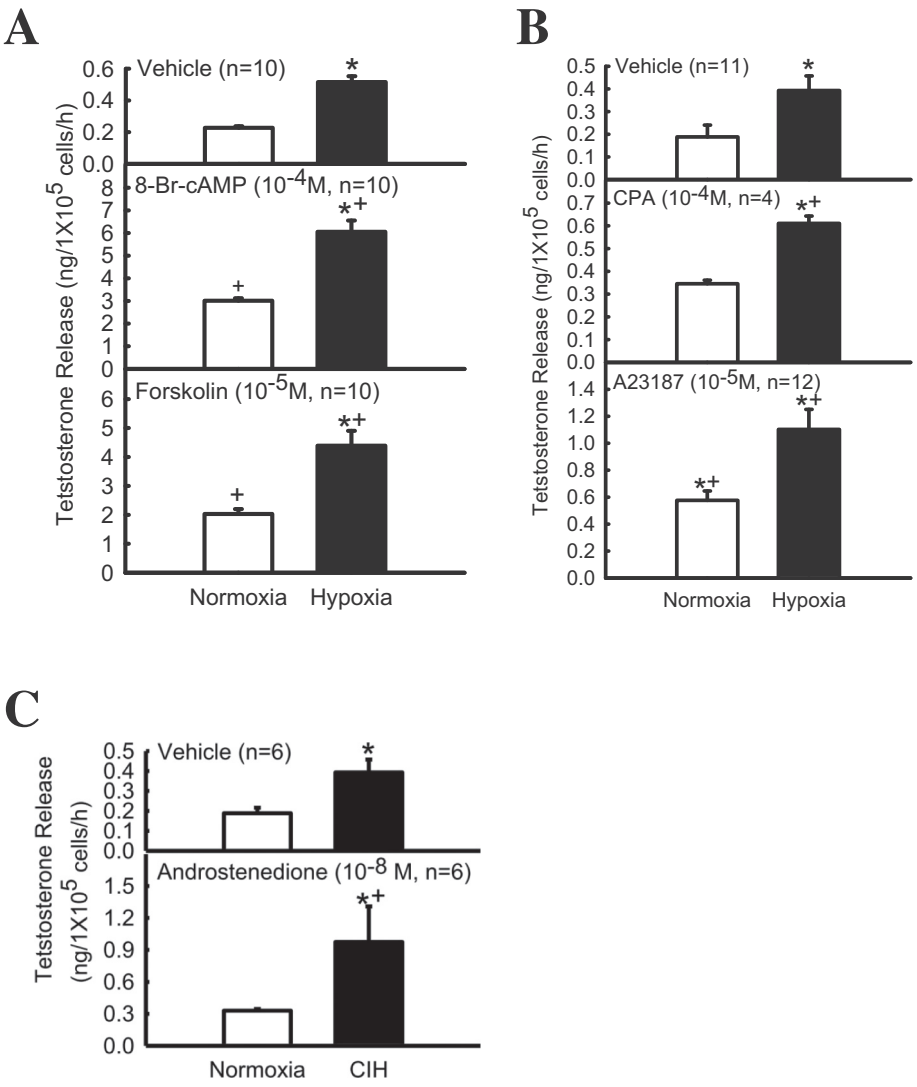

Fig. 3. Molecular effects of CIH on the T release in LCs. After incubating, the rat LCs with 8-Br-cAMP, forskolin, cyclopiazonic acid (CPA), A23187, or androstenedione for 1 h, the (A) cAMP-, (B) Ca^{2+}-, and (C) 17β-HSD-stimulated T release are increase in the CIH group compared with the normoxic group. Values are expressed as mean ± SEM. *$p < 0.05$, *vs. the* normoxic group. +$p < 0.05$, vs. the vehicle group.

the pituitary gland. Hypobaric hypoxia decreases androgen production by inhibiting the hypothalamic-pituitary function [31]. Moreover, urine T was found to decrease after hCG stimulation under hypoxia [32]. In the present *in vivo* study, the decreased serum LH level may have been due to feedback regulation from the increased plasma T. This mechanism may amplify the inhibition of serum LH secretion and lead to increased LH sensitivity by upregulation of the LH receptor in LCs.

The bioactivities of LH and hCG were similar. Binding of the LH receptor with hCG activates adenylyl cyclase and increases the production of intracellular cAMP, followed by enhancing the T production [33]. Therefore, the T production capacity in response to hCG was evaluated in our current study. Indeed, we found that hCG-evoked T production significantly increased after CIH exposure, both *in vivo* and *in vitro*. These results were confirmed by the decreased serum LH level and overexpression of LHR. Moreover, hypoxia was shown for decrease cAMP production in the urine [34]. Our results showed that CIH potentiated the stimulatory effects of both 8-Br-cAMP and forskolin on T production. The activity, as well as the mRNA and protein levels, of CYP11A1 was enhanced in the CIH group. Apparently, the resulting hypersecretion of T response to intermittent hypoxia seems to have been partially due to an LH-independent mechanism. A previous study revealed that relatively low O_2 concentration increased in hCG-stimulated progesterone synthesis and maintained T production in cultured LCs [35]. Hypoxia might increase T production by increasing the proliferation of LCs [36]. Therefore, increased T secretion capacity after CIH exposure might be directly mediated by the LH-cAMP pathway.

Intracellular calcium ions are involved in T secretion. The results of the present study indicated that A23187 and CPA increase T secretion. This result was similar with that of our previous study, which showed that calcium channel blockers and calcium-signaling messengers enhanced T production after exposure to hypoxia [19]. The present study further confirmed that hypoxia-induced T secretion was associated with the calcium-related pathway. Notably, other pathways may also regulate T biosynthesis [37].

StAR and CYP11A1 are the well-known proteins of rate-limiting steps in T biosynthesis. The StAR protein increases the delivery of cholesterol to the inner mitochondrial membrane, where cholesterol is then transformed to pregnenolone by the CYP11A1 enzyme [38]. The present study found that CIH exposure significantly increased the mRNA and protein expressions of CYP11A1 but not the expressions of StAR in LCs. Therefore; we used trilostane to detect pregnenolone accumulation, in order to investigate the enzyme activity of CYP11A1. Pregnenolone synthesis is related with the oxygen concentration in the

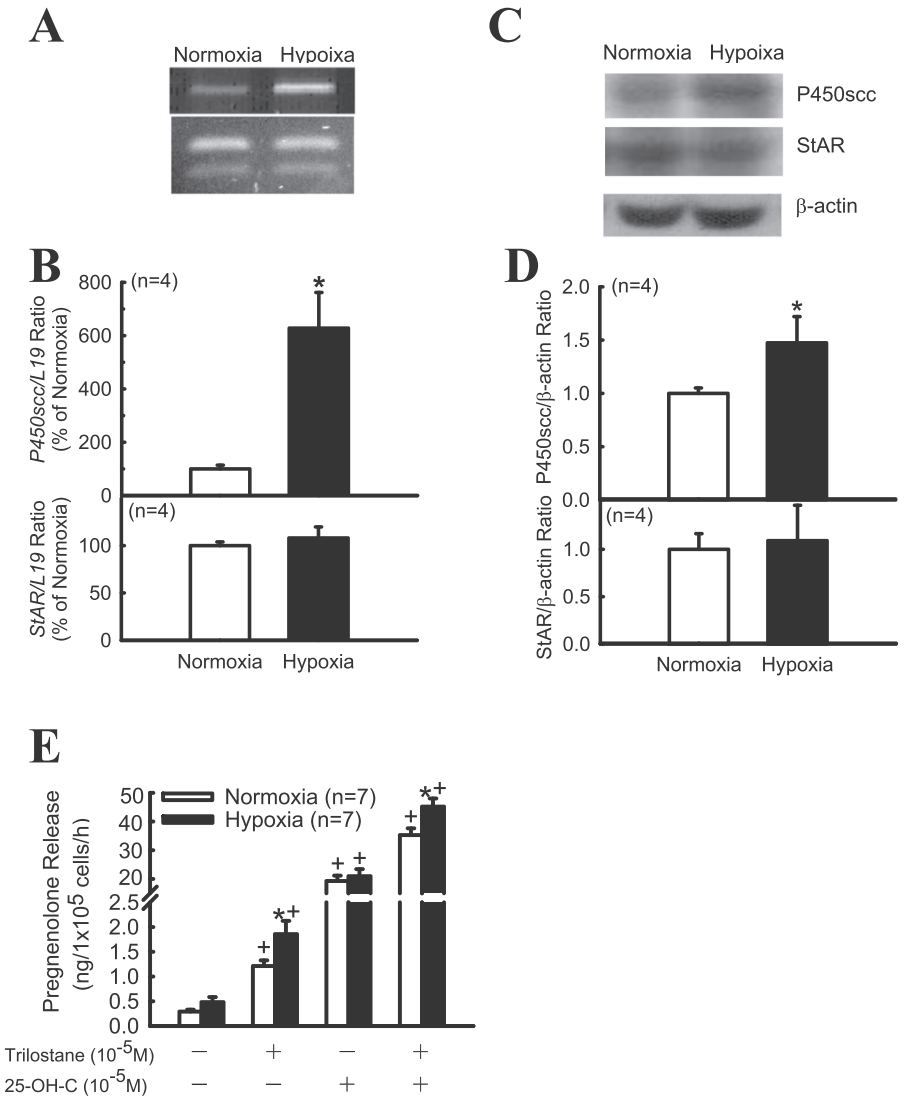

Fig. 4. CIH increased the mRNA and protein expressions of CYP11A1 (P450scc) but not StAR. (A) A representative gel pattern shows the expressions of CYP11A1 and StAR mRNA. (B) Quantitative comparisons of CYP11A1 and StAR mRNA are carried out by RT-PCR. L19 is used as an internal control. (C) A representative gel pattern shows the protein expressions of CYP11A1 and StAR. (D) Quantitative comparisons of the CYP11A1 and StAR protein are detected by Western blot. β-Actin is used as an internal control. (E) CIH induced the CYP11A1 activity in rat LCs. Cells incubated with trilostane and/or 25-OH-C increase pregnenolone production. Compared with the normoxic group, trilostane-induced pregnenolone production is further elevated in the CIH group. Values are expressed as mean ± SEM. $*p < 0.05$, vs. the normoxic group. $+p < 0.05$, vs. the vehicle group.

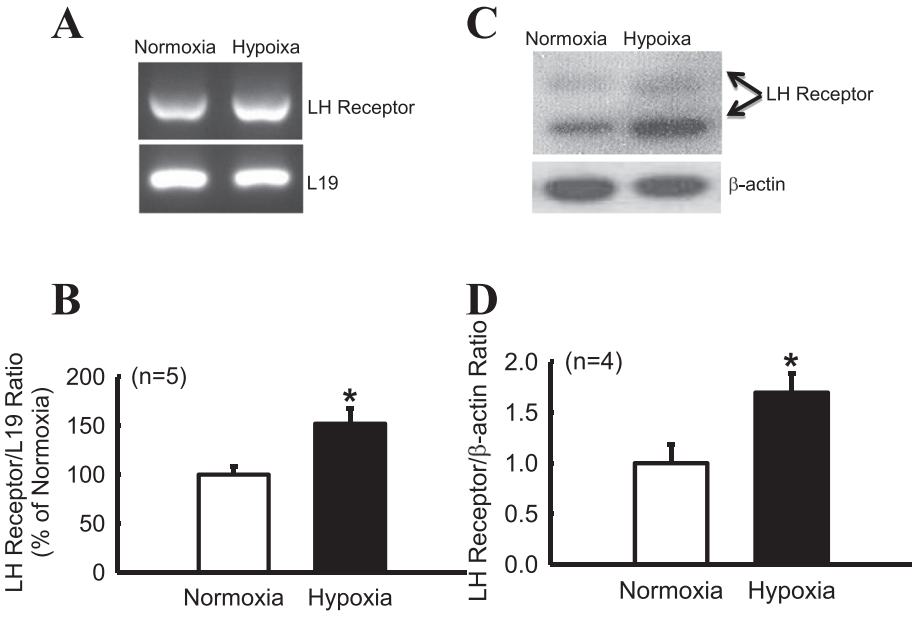

Fig. 5. CIH increased the mRNA and protein expressions of the LH receptor. (A) A representative gel pattern shows the expression of the LH receptor mRNA. (B) Quantitative comparisons of the LH receptor mRNA is carried out by RT-PCR. L19 is used as an internal control. (C) A representative gel pattern shows the expressions of the LH receptor. (D) Quantitative comparisons of the LH receptor protein are carried out by Western blot, which shows the LH receptor (two bands) at 60–85 kDa and β-actin at 43 kDa. β-Actin is used as an internal control. Values are expressed as mean ± SEM. $*p < 0.05$, *vs.* the normoxic group.

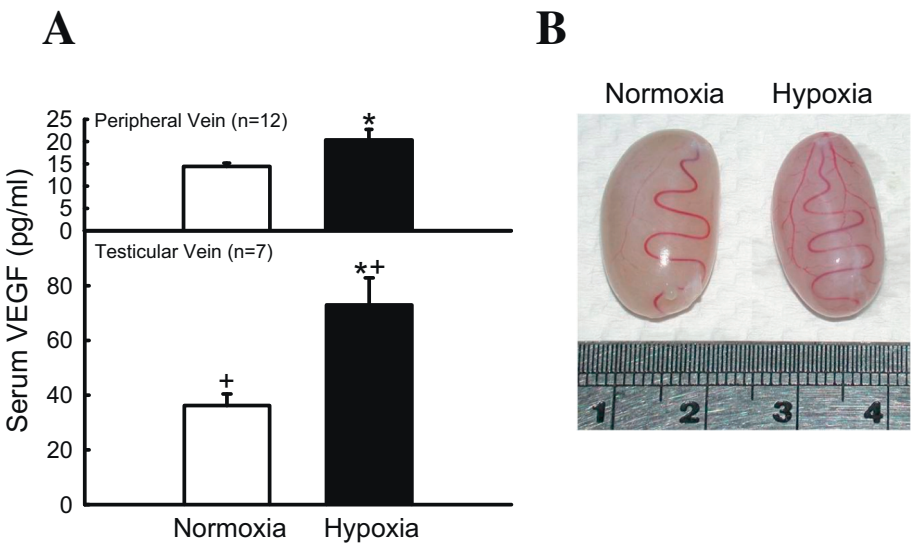

Fig. 6. CIH increased the VEGF levels and vessel distribution. (A) Serum VEGF concentration is higher in the CIH group then in the vehicle group. (B) The vessel distribution in rat testes is more apparent after 14 days of intermittent hypoxia. Values are expressed as mean ± SEM. *$p < 0.05$, *vs.* the normoxic group. +$p < 0.05$, vs. the peripheral serum level.

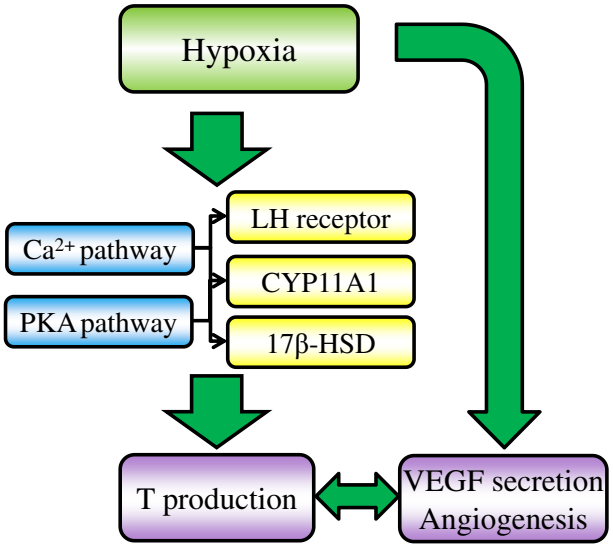

Fig. 7. Scheme of the proposed mechanism of CIH on T release in the testes. Hypoxia activates Ca^{2+}- and the PKA-signaling pathway. Subsequently, the translation/activation of the LH receptors, CYP11A1 and 17β-HSD increase, thereby stimulating T production. LH, luteinizing hormone; 17β-HSD, 17β-hydroxysteroid dehydrogenase; T, testosterone.

adrenal cortex [39]. Our result implied that the increased T secretion capacity after CIH exposure may have been directly mediated by the increase in pregnenolone. CIH significant increases the expression and activity of CYP11A1 in LCs. Moreover, incubation of LCs with the T precursor, androstenedione, can stimulate the levels of T following CIH. CIH seemed to activate 17β-HSD during T biosynthesis.

Hypoxia has been shown to induce endothelial cell migration by accumulation of HIF and increased VEGF level. Both HIF and VEGF play important roles in angiogenesis and vasodilation in response to hypoxia [40]. Moreover, VEGF and its receptor have been reported to be up-regulated by androgen [41,42]. In addition, the release of erythropoietin from the kidneys is T-dependent [43]. In the present study, CIH significantly increased T production in LCs. Therefore, the CIH-induced testicular angiogenesis might be related with the hypoxic-induced T production and the increased serum VEGF in testicular vein.

We further explored the effects of T on VEGF expression. A previous study demonstrated that T increased the mRNA and protein expressions of VEGF in ovarian granulosa cells [44]. In castrated rats with diabetes, a reduced capillary VEGF level was reversed after T administration [45]. In contrast, T was shown to downregulate the gene expression of VEGF in rat pancreatic tissue [46] and bone marrow mesenchymal stem cells [47]. These results demonstrated that the T-mediated regulation VEGF production varied among different tissues.

In summary, CIH-induced T production and testicular angiogenesis might be partially mediated by increased testicular VEGF. Compared with long-term hypobaric hypoxia exposure at a high altitude, which inhibits the hypothalamic-pituitary-testis axis and leads to sterility, our CIH intervention seemed to be effective in avoiding the reproduction problems. CIH-induced endogenous T secretion might be a good choice to avoid doping crisis in athletes who need to increase the muscle mass and enhance training efficacy. We hope that the concepts presented in this present study would provide non-invasive and non-hormonal preparation methods for patients who need androgen supplementation.

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Author contributions

Study concepts, quality control of data and algorithms: Shyi-Wu Wang; Paulus S. Wang

Study design, data acquisition, data analysis and interpretation, statistical analysis: Yu-Min Cho

Manuscript preparation: Yu-Min Cho, Jou-Chun Chou, Kai-Lee Wang

Manuscript editing: Kai-Lee Wang

Manuscript review: Shyi-Wu Wang; Paulus S. Wang, Chia-Mei Fang, Sindy Hu

Declaration of Competing Interest

No conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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