ORIGINAL ARTICLE

Intermittent hypoxia causes a suppressed pituitary growth hormone through somatostatin

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Abstract

OBJECTIVES: The aim of this study was to investigate the response of the growth hormone (GH) in rat anterior pituitary to intermittent hypoxia (IH) and its modulation by hypothalamic somatostatin (SS).
SETTING AND DESIGN: To observe the hypoxic response, rats were exposed to simulated altitude hypoxia (2km or 5km) in a hypobaric chamber for various days (4h/d); to clarify SS-involvement, rats were pretreated with SS antagonist (cysteamine, CSH, 200mg/kg/d, s.c.) then exposed to IH (5km) for 2d. The GH mRNA and immunostaining GH in pituitary as well as immunostaining SS in median eminence (ME) of hypothalamus were assayed by RT-PCR and immunohistochemistry, respectively.
RESULTS: IH of 5 km altitude (IH5) markedly suppressed the body weight gain (BWG) of rats from 1d to 10d, and it was returned to control level henceforth, while no significant influence was showed in the group of IH of 2 km altitude (IH2). IH5 for 2 and 5d significantly decreased GH mRNA expression in the pituitary. The pituitary immunostaining GH was remarkably increased in groups of IH2 for 5, 10, and 15 d, and in groups of IH5 for 2, 5, and 10d. Immunostaining SS in ME was significantly reduced in group of IH2 for 5d, and in groups of IH5 for 2d and 5d. Pretreatments (s.c.) with SS antagonist (CSH) significantly reversed IH5-caused increase of immunostaining GH and reduction of mRNA level in pituitary.
CONCLUSIONS: IH may cause a short-term and recoverable suppression of GH release, and reduce GH mRNA expression in anterior pituitary, which may depend on the intensity and duration of the hypoxia. This suppression may be due to a modulation of hypoxia-activated SS.

Abbreviations:

BWG	body weight gain
CNS	central nervous system
CRF	corticotropin-releasing factor
CSH	cysteamine
GH	growth hormone
GHRH	GH-releasing hormone
IH	intermittent hypoxia
ME	median eminence
OD	optical density
PVN	paraventricular nucleus
PeN	periventricular nucleus
SS	somatostatin

Introduction

Somatic growth is genetically a programmed sequence of events, which requires the maintenance of homeostasis proceeding. It has been clinically observed that lower body weight of the newborn occurs in residents at high altitude such as Tibetan Plateau. Hypoxia, one of the environmental factors at plateau, is due to a lower oxygen pressure, which leads to oxygen deficiency of the whole organism, even in tissues as well as cells, consequently disturbs physiological activities and harms the health. It is noteworthy that suppressed body growth seems to be associated with hypoxia in both human beings and animals [1–5]. We have already found that continual hypoxia acutely and chronically induced suppression in the body growth and development of rats, which may in part be correlated with an inhibition of the GH release and/or biosynthesis [5].

It is well known that the growth hormone (GH) plays an important role in body's growth, neural, and reproductive physiology, and performs an important control over the function of bone and muscle cells [6–8]. The release of GH is regulated by two hypothalamic neuropeptides from the median eminence (ME). They are stimulatory GH-releasing hormone (GHRH) and inhibitory somatostatin (SS), which modulate somatotroph in the anterior pituitary [9, 10]. In male rat, SS and GHRH are released in reciprocal 3 to 4 hours' cycles (about 180° out of phase) from the ME into the hypophyseal portal blood, and act upon the pituitary somatotrophs to generate the ultradian rhythm of GH secretion observed in the peripheral blood [11]. SS plays a key role in inhibiting GH release. Chen et al. has reported that the hypoxia makes SS mRNA expression increasing in the periventricular nucleus (PeN) of the hypothalamus in rats [12].

Cysteamine (CSH) (2-mercaptoethylamine; NH_2-CH_2-CH_2-SH) has been found to provoke a marked specific decrease of endogenous brain SS [13]. CSH induces a loss of both immunological and biological SS by acting on the disulphide bond-containing segment of the SS molecule [14]. In order to provide evidence to support the view that SS exerts a modulation on GH under hypoxia, we used CSH to decay endogenous hypothalamic SS and examined its effect on the protein and mRNA levels of GH in rat pituitary. The purpose of this study was to clarify whether intermittent hypoxia (IH) would also alter pituitary GH expression in rats, and whether the alterations would be due to the hypoxia-altered hypothalamic SS.

Materials and Methods

Animals: Adult male Sprague-Dawley rats (150±20g) in healthy, clean grade (certification No.2001001) were purchased from the Experimental Animal Center of Zhejiang Province, China, and maintained at 12:12 h light/dark cycle (light on 06:00–18:00 hr), at room temperature (20±2°), and with free access to food and water. Animals were housed in a group of six and adapted for 1 week prior to the experimental manipulation. The principles of NIH laboratory animal care were followed. Body weights were recorded daily. The BWG was determined by subtracting the body weight on 1st day of IH from the body weight on subsequent days.

Intermittent hypoxia simulation: The rats were placed in a hypobaric chamber. The altitude was simulated at two levels of hypoxia, 2 km (16.0% O_2, 79.97 kPa) and 5 km (10.8% O_2, 54.02 kPa) for 1, 2, 5, 10, and 15 d, setting 4 h per day (from 07:00 to 11:00 h AM). Control groups were set at sea level (20.9% O_2, 100.08 kPa).

Cysteamine (CSH) administration: Rats were divided into 3 groups: (1) Control group: rats were set at sea level and given subcutaneous injection of saline (2ml/kg) daily. (2) Hypoxia (5 km) group: rats were given subcutaneous injection of saline (2ml/kg), then exposed to hypoxia of 5km for 2 days, 4h per day (from 07:00 to 11:00 h AM). (3) CSH group: rats were given subcutaneous injection of CSH-HCl (200mg/kg, Sigma, USA), then exposed to hypoxia as same as hypoxia group. CSH-HCl was dissolved in 0.9% NaCl (saline) with the concentration of 100mg/ml and pH was adjusted to 7.2 with 1N NaOH [15].

RNA preparation: Animals were decapitated immediately during 11:00 to 11:30 h to minimize circadian rhythm effects. Rat pituitary sample was homogenized, and total RNA was performed using TRIZOL Reagent (SNBC China). RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water, and the concentrations were determined spectrophotometrically at 260 nm.

RT-PCR: RT-PCR protocol used was largely adapted from the technique described by Yoshizato et al [16]. The RT reaction was carried out at 37° for 2 h in 25 μl of the reaction mixture containing 1 μg total RNA, 0.5 μg oligo(dT) primer (Sangon, China), 10 mM deoxy (d)-NTPs, 25 U RNase inhibitor (Bio Basic Inc, Canada), and 200 U Moloney murine leukemia virus reverse transcriptase (Promega). Two microliter of the reaction mixture was amplified by 20 cycles of PCR on a thermal cycler (Eppendorf, Mastercycler personal, Germany) in the reaction buffer containing 20 mM $MgSO_4$, 100 mM KCl, 80mM $(NH_4)_2SO_4$, 100 mM Tris-HCl (pH 9.0), 0.5% NP-40, 2.5 mM dNTPs, 0.4 μM of sense and antisense primer pairs for GH or β-actin, and 5 U Taq plus DNA polymerase (SNBC, China) in the final volume of 25 μl. The hot start method was

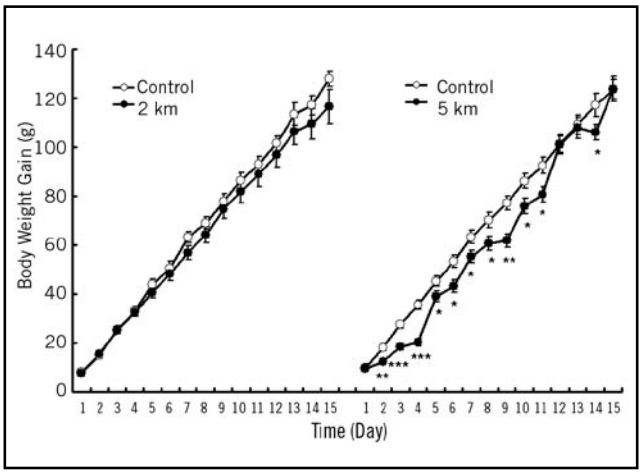

Figure 1. IH2 did not markedly affect rat BWG, compared to control. The BWG was suppressed before 10 d of IH5, and then gradually recovered. Values are given as the mean ± SEM; n=6; * P<0.05, ** P<0.01 compared with the control.

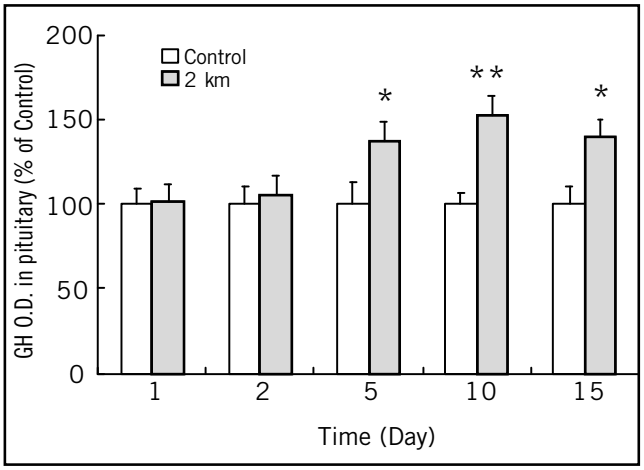

Figure 2. IH2 caused an increased immunostaining GH in the pituitary of rats during 1-15d, indicating its secretion was inhibited. Values are given as the mean ± SEM; n=6; * P<0.05, ** P<0.01 compared with the control.

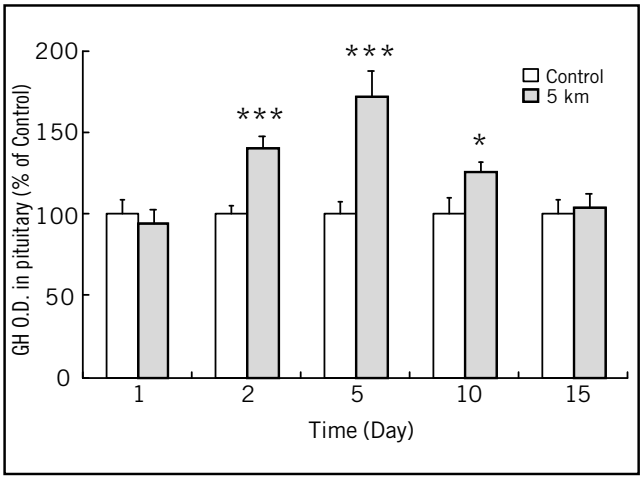

Figure 3. IH5 caused an increased immunostaining GH in the pituitary of rats during 1-15d, indicating its secretion was inhibited. Values are given as the mean ± SEM; n=6; * P<0.05, *** P<0.001 compared with the control.

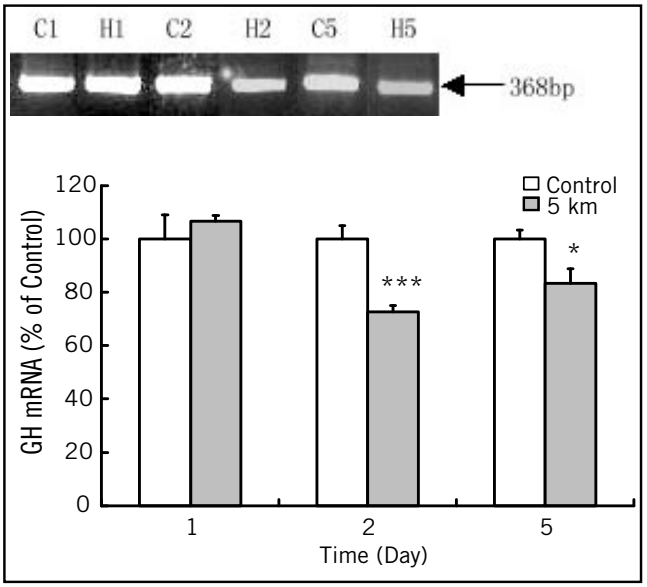

Figure 4. IH5 suppressed GH mRNA levels in rat pituitary during exposure for 1-5d. GH and β-actin mRNA levels in the pituitary were detected by RT-PCR. Values are given as the mean ± SEM; n=6; * P<0.05, *** P<0.001 compared with the control.

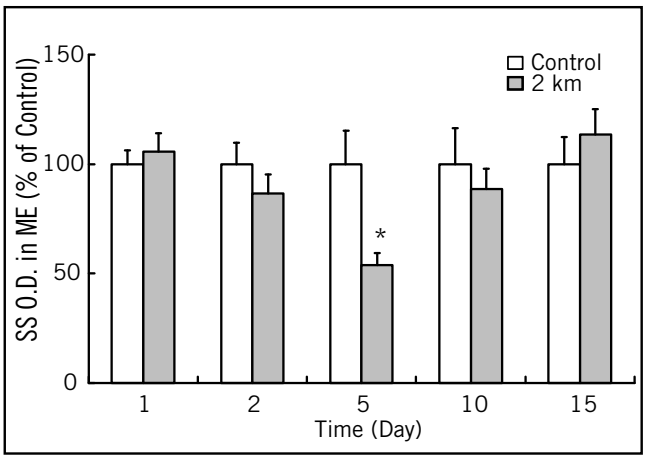

Figure 5. IH2 decreases immunostaining SS levels in the ME of rats for 1-15d, indicating its release was increased. Values are given as the mean ± SEM; n=6; * P<0.05 compared with the control.

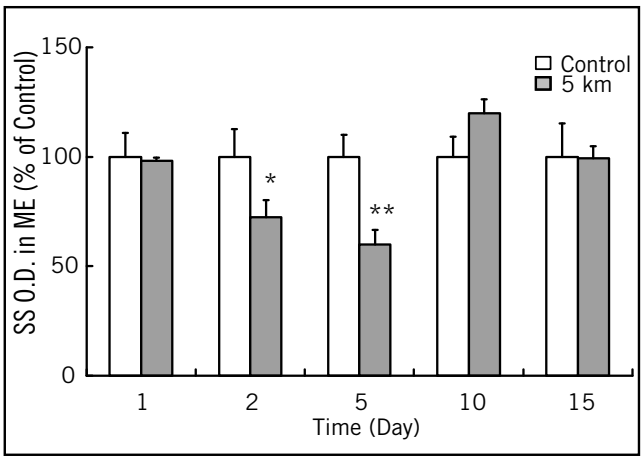

Figure 6. IH5 reduced immunostaining SS in the ME of rats during exposure for 1-15d, indicating its secretion was increased. Values are given as the mean ± SEM; n=6; * P<0.05, ** P<0.01 compared with the control.

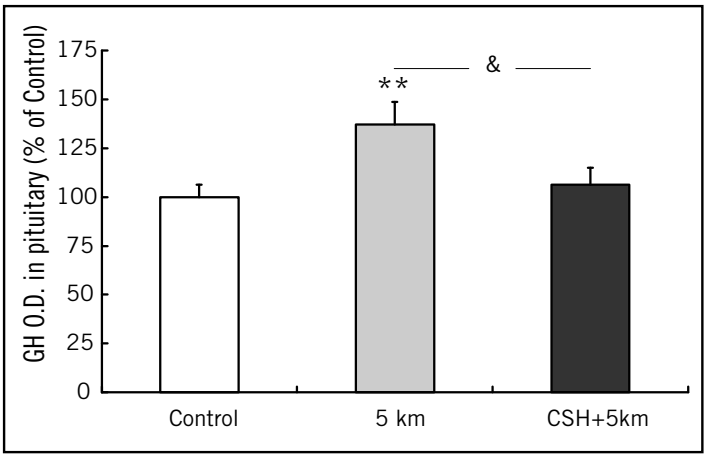

Figure 7. CSH injection (s.c.) reversed IH5-caused immunostaining GH levels increases in anterior pituitaries of rats, indicating inhibitory secretion was blocked. Values are given as the mean ± SEM; n=6;
** P<0.01 compared with the control;
& P<0.05 CSH group compared with hypoxic one.

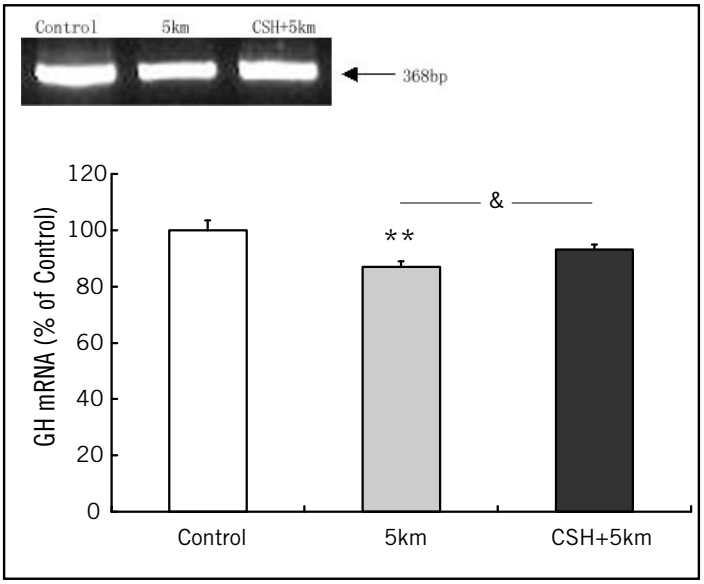

Figure 8. CSH injection (s.c.) reversed IH5-caused GH mRNA decline in anterior pituitaries of rats, indicating inhibitory GH biosynthesis was reversed. Values are given as the mean ± SEM; n=6;
** P<0.01 compared with the control;
& P<0.05 CSH group compared with hypoxia one.

employed for this PCR, and each cycle consisted of denaturing at 94° for 30 sec, annealing at 55° for 30 sec, and extension at 72° for 45 sec. The PCR reaction was completed by addition of the extension reaction at 72° for 8 min before termination. The PCR for β-actin mRNA was performed for 26 cycles. The primers used for RT-PCR of GH mRNA were 5'-TGACACCTACAAAGAGTTCGAGCG-3' (primer 1 for the 5'-end) and 5'-TGTTGGCGTCAAACTTGTCATAGG-3' (primer 2 for the 3'-end). The predicted length of amplified fragments is 368 bp. For β-actin mRNA, the primers 5'-TGACGTTGACATCCGTAAAG-3' (for the 5'-end) and 5'-ACAGTGAGGCCAGGATAGAG-3' (for the 3'-end) were used. The predicted length of the amplified fragments is 194 bp. The amplified products were separated on 2% agarose gel and visualized by ethidium bromide staining. Products of RT-PCR reactions were photographed and analyzed by densitometry.

Tissue preparation for immunohistochemistry: The anterior pituitaries were immediately removed from the rats and immersed in fresh chilled paraformaldehyde (0.4% in 0.01M phosphate buffer, pH 7.4). Fixation was carried out for 48 h at 4°. Serial 20 μm pituitary sections from all rats were cut with a cryostat microtome (HM505-E, Germany). The brains were quickly removed from the animals, frozen immediately and stored at –80°. Using the atlas of The Rat Brain in Stereotaxic Coordinates [17] as an anatomical guide, 20 μm thick section of brain was cut with the cryostat microtome. For each rat, two sections were selected from the portion of 2.3 mm to the bregma. Two sections were collected from the region of 2.8 mm to the bregma. The sections were thaw-mounted on gelatin-coated slides, desiccated under vacuum overnight, and stored at –80° until use.

Immunohistochemistry: Immunohistochemical staining was performed as described by Bhatnagar [18]. Brain sections were fixed in fresh chilled paraformaldehyde for 30 min and washed with PBS (pH 7.4). After incubated in Triton X-100 (0.4%) for 15 min and washed with PBS (pH 7.4), all sections were incubated in 10% normal goat serum for 10 min and then incubated overnight in either GH antisera (NIDDK-anti-rGH-IC-1, AFP411S, diluted 1:20,000 in PBS pH 7.4 containing 0.4% Triton X-100, 1% BSA) or rabbit anti-somatostatin serum (1:600, Phoenix Pharmaceuticals, INC.USA), in humidified chambers at 4°. These sections were rinsed in PBS and were incubated in biotinylated goat anti-human IgG (1:300, Beijing Zhongshan biotechnology CO., LTD.) or goat anti-rabbit IgG (1:200, Beijing Zhongshan biotechnology CO.,

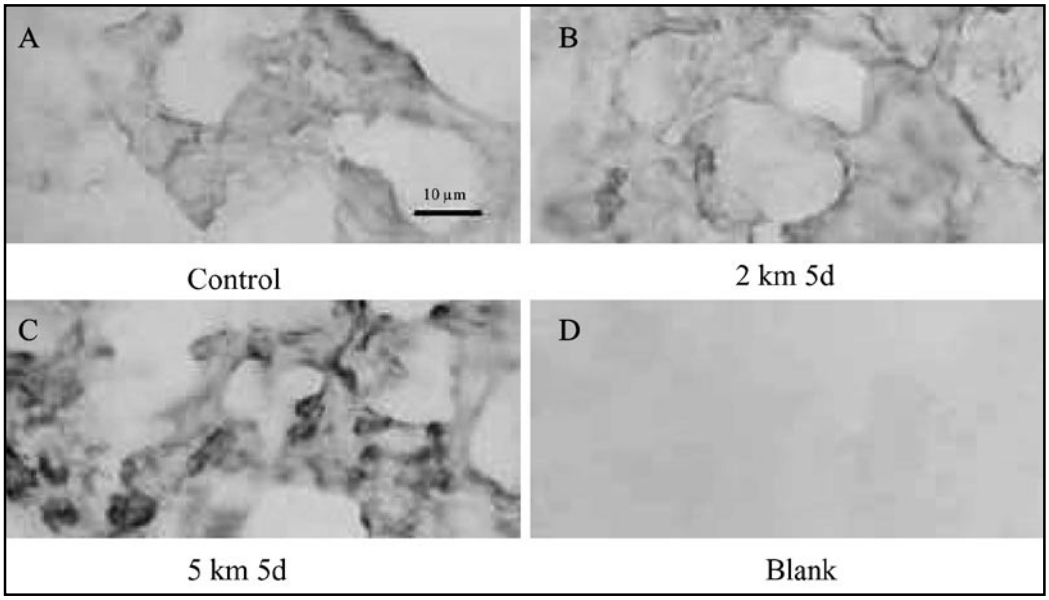

Figure 9.
The immunostaining GH in the anterior pituitaries of rats during IH were detected by immunohistochemistry. The augmentations of the immunostaining GH were found in the pituitaries of rats exposed to IH 2km (B) and 5km (C) for 5d vs. control group (A). The immunostaining signal was undetectable in blank (D) incubated in diluent instead of GH antisera. The scale bar is 10 μm.

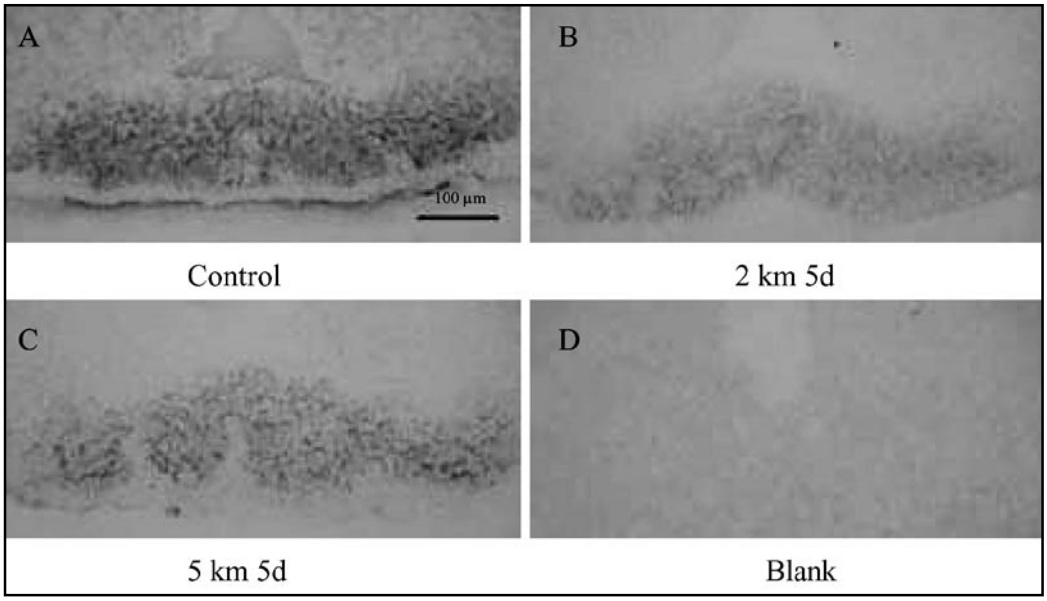

Figure 10.
The immunostaining SS in the ME of rats during IH were detected by immunohistochemistry. Noted the less immunostaining SS at IH 2 km (B) and 5 km (C) for 5d compared to the more immunostaining SS in the ME of control rats (A). The immunostaining signal was undetectable in blank (D) incubated in diluent instead of SS antisera. The scale bar is 100 μm.

LTD.) diluted in PBS (pH 7.4, 1% BSA) in humidified chambers for 1 h at 37°. Sections were rinsed again in PBS pH 7.4 and were incubated in avidin biotin peroxidase complex (ABC, Beijing Zhongshan biotechnology CO., LTD.) diluted 1:300 in PBS (pH 7.4) for 1 h in humidified chambers at 37°. Sections were rinsed again in PBS, pH 7.4 and then immunoreactivity was visualized by incubating sections for 3 min in chromogen 3, 3'-diaminobenzidine hydrochloride (DAB) in presence of 0.01% hydrogen peroxide (H_2O_2). Sections were rinsed in PBS (pH 7.4), dehydrated through a graded series of ethanol solutions, cleared in xylene, and observed under an optical microscope (Olympus BX 40, Japan). The reactions for specificity of GH and SS immunoreactivity were also carried out by omission of primary antibodies in the incubating medium and no immunoreactivity was observed in these sections.

Quantitative analysis: The immunostaining density of GH in pituitary or SS in ME was quantitatively assessed with the Optimas 6.5. In brief, sections were placed under a microscope and the image was transferred via a digital camera (Nikon CoolPix 950, Japan) to a computer. The five separate positive areas in each section of the immunostaining GH in the anterior pituitary and the immunostaining SS in the ME were selected, and the integrated optical densities (OD) were measured. The results were derived by subtracting the background and expressed as the percentage of optical density units compared to the control groups. Control groups were given the value of 100%. Optical densities obtained in five areas were averaged and used to calculate the group means.

Statistics: The data were presented as the mean ± SEM, and Student's t-test and one-way ANOVA were performed for the data analysis. $P<0.05$ was considered significant.

Results

Compared to control (Fig. 1), IH of 2 km (IH2) through 1–15d did not significantly influence the BWG. IH of 5km (IH5) for 1–15d markedly resulted

in a decrease of BWG. The BWG was lowered by 31.2% (from 18.2±1.4 g of control to 12.5±1.1 g, P<0.01) for 2 d, and 11.8% (from 86.2±3.0 g of control to 76.0±3.2 g, P<0.05) for 10d, respectively. The BWG returned to the control level after 15d.

IH2 for 5, 10 and 15d enhanced the immunostaining GH in the anterior pituitary: by 37.0% (137.0±11.6, P<0.05, Fig. 9B), 52.0% (152.0±11.6, P<0.01), and 38.9% (138.9±11.0, P<0.05), respectively (Fig. 2). The IH5 for 2, 5 and 10d increased the immunostaining GH by 39.7% (139.7±7.8, P<0.001), 71.7% (171.7±15.3, P<0.001, Fig. 9C), and 25.5% (125.5±6.2, P<0.05), respectively (Fig. 3).

When rats were exposed to IH5, the GH mRNA expressions in pituitaries were not changed for 1d, but were remarkably suppressed for 2d (p<0.001) and 5d (p<0.05, Fig. 4).

IH2 caused a progressed reduction and return of the immunostaining SS in ME of hypothalamus. The marked decrease (the lowest point) by 46.3% (53.7±5.7, P<0.05, Fig.5 and 10B) occurred on day 5. IH5 also caused a progressed reduction and return of the immunostaining SS, but the significant decreases of 27.7% (72.3±7.9, P<0.05) and 40.0% (60.0±6.7, P<0.01, Fig. 10C) took place on day 2 and day 5, respectively (Fig.6).

CSH injection significantly reversed IH5 2d-induced increase of immunostaining GH in pituitary from increase by 37.1% (IH5 vs. control, P<0.01) to decrease by 22.3% (CSH+IH5 vs. IH5, P<0.05) (Fig.7), and this injection markedly reversed the IH5 2d-caused GH mRNA expression reduction in pituitary from reduction by 13.0%, (IH5 vs. control, P<0.01) to increase by 7.2%, (IH5+CSH vs. IH5, P<0.05) (Fig.8).

Discussion

We have previously reported that chronic continual hypoxia produced suppressed growth and BWG in not only young but also neonatal male rats [3–5]. In this study, the suppressed growth and BWG in adult rats were also remarkably induced by IH5 rather than by IH2, which indicated that this suppression was dependent on hypoxia intense and time course.

The GH mRNA in the pituitary was significantly decreased for 2 and 5 d under IH5. Although a direct relationship between the amount of mRNA and the quantity or rate of synthesis of translated product cannot be assumed, mRNA availability is a prime factor in protein biosynthesis [19]. The reduction of pituitary GH mRNA suggested that intermittent hypoxia suppressed biosynthesis of GH in the anterior pituitary. Meanwhile, the immunostaining GH in the pituitary was enhanced significantly for 5, 10 and 15d under IH2, and for 2, 5 and 10d under IH5. The increase of GH level observed here could theoretically result from either increased synthesis or decreased release of GH but the result of RT-PCR showed that GH biosynthesis was suppressed. The evidence above suggested that the enhanced immunostaining GH in the pituitary might be due to reducing the GH secretion into circulation. We have previously demonstrated that acute hypoxia for 0.5, 2 and 24 h and continual hypoxic exposure as long as to 25d inhibited GH release from pituitary into circulation [5]. Based on the fact mentioned above, IH5 not only enhancing pituitary immunostaining GH but also suppressed GH mRNA levels, suggesting that the IH played an inhibitory role in GH release and biosynthesis in the anterior pituitary. It is well known that the GH plays an important role in body's growth [6]. The suppression of GH biosynthesis and release might be at least in part correlated with the suppressed BWG.

The hypothalamic SS is the governor of GH release from the pituitary. It has been documented that the SS levels in the hypothalamic regions were changed by multi-stressors, for instance, cold exposure [20], food deprivation [21, 22], sleep deprivation [23], depression [24] and immobilization [25]. Our previous studies demonstrated that hypoxia, as one of the stressors, at different degrees of altitude, acutely and chronically produced variable responses of SS, for instance, increased or decreased SS release from ME of hypothalamus in rats, being dependent on the hypoxia intensity and time course [26]. The continual hypoxia increased SS mRNA expression in PeN of hypothalamus, from which a synthesized SS was transported to terminals of SS neurons in the ME where the SS released into pituitary portal system [12]. In this study, the immunostaining SS in the ME was significantly decreased during the IH exposure (2 km for 5d, 5 km for 2d and 5d). Presumably, the decreased immunostaining SS might be due to an increased release from the ME.

There are evidences that SS receptors are located on GH cells that serve to regulate the GH activity [9]. The response of GH to stress has known to be mediated at least in part by the release of SS from the hypothalamus [27]. CSH induces a loss of both immunological and biological SS via acting on the disulphide bond-containing segment of the SS molecule [14] and provokes a marked specific decrease of endogenous brain SS. Therefore in this study, CSH was used to decay endogenous hypothalamic SS, and its modulation on GH was examined during IH. The results showed the injection of CSH markedly reversed IH5 5d-induced pituitary GH mRNA reduction and pituitary immunostaining GH enhancement, suggesting that hypothalamic SS was involved in such a suppression of GH through inhibitory release and biosynthesis of GH during IH5 exposure.

We have already demonstrated that the continual hypoxia stimulated SS release from the ME and SS mRNA expression in the PeN, which were in part regulated by increased circulating corticosterone [12, 26] that might contribute to lower GH release from pituitary. There is evidence showing functional and structural linkages between corticotropin-releasing factor (CRF) and SS neuron within the hypothalamus, which were involved in the secretion of corticotropin (ACTH) and GH from the pituitary [28]. We have found that CRF release from the paraventricular nucleus (PVN) and ME in adult rats was activated

by acute hypoxia, and the CRF mRNA expression in PVN of hypothalamus in rats was remarkably increased by chronic continual hypoxia of 10% O_2 [29]. The continual hypoxia activated CRF may be presumed as a potential factor to stimulate SS release from ME. In adrenalectomized rats with preinjection of CRF antagonist, alpha-helical CRF (9–41), immunostaining GH declined in pituitary after acute hypoxia, suggesting hypoxia-activated CRF played an inhibitory role in GH release, which might presumably act via SS mediation in the hypothalamus (our unpublished data).

In a comparison of the data based on the BWG and GH changes from chronic continual [5] and intermittent hypoxia study (present study) at same altitude (5km), we suggest one should be careful when athletes are training at high altitude in an attempt to produce some features (stimulating an increase in erythropoietin, leading to increased red cell mass as well as building muscles) of altitude acclimatization, with the ultimate goal to improve sea-level athletic performance, because IH5 produces inhibitory GH mRNA expression and GH release in pituitary, which may cause suppression of BWG and immune activity as well as hurt health.

In summary, IH5 produces hypoxia-intensity and time course dependent inhibition of GH release, and suppression of GH mRNA expressions in pituitary, which are correlated with hypoxia-triggered hypothalamic SS secretion. This inhibitory role may partly be responsible for decreasing BWG.

Acknowledgements

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