Diversities in hepatic HIF-1, IGF-I/IGFBP-1, LDH/ICD, and their mRNA expressions induced by CoCl₂ in Qinghai-Tibetan plateau mammals and sea level mice

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Chen, Xue-Qun, Shi-Jun Wang, Ji-Zeng Du, and Xiao-Cheng Chen. Diversities in hepatic HIF-1, IGF-I/IGFBP-1, LDH/ICD, and their mRNA expressions induced by CoCl₂ in Qinghai-Tibetan plateau mammals and sea level mice. Am J Physiol Regul Integr Comp Physiol 292: R516–R526, 2007. First published September 21, 2006; doi:10.1152/ajpregu.00397.2006.—Ochotona curzoniae and Microtus oeconomus are the native mammals living on the Qinghai-Tibetan Plateau of China. The molecular mechanisms of their acclimatization to the Plateau-hypoxia remain unclear. Expressions of hepatic hypoxia-inducible factor (HIF)-1α, insulin-like growth factor-I (IGF-I)/IGF binding protein (BP)-1 (IGFBP-1; including genes), and key metabolic enzymatic genes [lactate dehydrogenase (LDH)-A/isoform dehydrogenase (ICD)] are compared in Qinghai-Tibetan-Plateau mammals and sea-level mice after injection of CoCl₂ (20, 40, or 60 mg/kg) and normobaric hypoxia markedly increased HIF-1α only in mice, 2) hepatic and circulatory IGF-I in M. oeconomus, 3) hepatic IGFBP-1 in mice and O. curzoniae, and 4) LDH-A but reduced ICD mRNA in mice (CoCl₂ 20 mg/kg) but were unchanged in the Tibetan mammals. Normobaric hypoxia markedly increased HIF-1α and LDH-A mRNA in mice and M. oeconomus (8.0% O₂) not in O. curzoniae, and 2) reduced ICD mRNA in mice and M. oeconomus (8.0% O₂) not in O. curzoniae. Results suggest that 1) HIF-1α responsiveness to hypoxia is distinct in lowland mice and plateau mammals, reflecting a diverse tolerance of the three species to hypoxia; 2) CoCl₂ induces diversities in HIF-1, IGF/I, IGFBP-1 protein or genes in mice, M. oeconomus, and O. curzoniae. In contrast, HIF-1 mediates IGFBP-1 transcription only in mice and in M. oeconomus (subjected to severe hypoxia); 3) differences in IGF/I, IGFBP-1 expressions induced by CoCl₂ reflect significant diversities in hormone regulation and cell protection from damage; and 4) activation of anaerobic glycolysis and reduction of Krebs cycle represents strategies of lowland-animals vs. the stable metabolic homeostasis of plateau-acclimatized mammals.

hypoxia-inducible factor; insulin-like growth factor; lactate dehydrogenase-A; Tibetan mammal; cobalt chloride; hypoxia

HOW ANIMALS LIVING AT THE Qinghai-Tibetan plateau acclimatize to the hypoxic environment has long been a topic of interest and study. Over the past decades, an increasing number of studies have reported on the physiological and ecological adaptive responses at the intact animal, organ, or tissue level of the plateau animals under acute or chronic hypoxia, but the underlying mechanisms, in particular, the cellular and molecular mechanisms, have not been well understood.

Although high-altitude native animals show indications of protective adjustment that are lacking in the sea level animals, the nature and essential mechanisms of such adjustment are still being investigated. One of these protective factors is insulin-like growth factor I (IGF-I), synthesized primarily in the liver, which participates in growth and functions in almost every organ in the body (1, 38). IGF-I is also an extremely potent mitogen, and cellular growth requires O₂ consumption, suppressing cell growth and proliferation (10). Endogenous IGF-I also plays a significant role in recovery of tissues from damage (12, 48). This protective effect is mediated through a receptor IGF-IR and its binding proteins (IGFBPs), which play an “integrator” role in endocrine growth and IGF-I distribution and bioavailability (9, 19). Among all of the IGFBPs, IGFBP-1 is unique: its expression is dramatically altered by changes in the metabolic state (24, 23). IGFBPs can both enhance and reduce IGF action depending on the tissues, differentiation status of embryonal development, and other factors during hypoxia (4, 5, 11, 17, 44). IGFBP-1 can also protect against liver injury by reducing the level of proapoptotic signals (25).

In studies of gene transcription, the hypoxia-inducible factor (HIF)-1, a dimer consisting of HIF-1α and HIF-1B subunits, stimulates transcription of several hypoxia-activated target genes such as oxygen-dependent target genes encoding glucose transporters and glycolytic enzymes (lactate dehydrogenase-A). HIF-1 is a critical component of the cellular and systemic response to hypoxia in mammals (40) and fish (33). HIF-1α is unique in being tightly controlled by the cellular oxygen tension: it is continuously degraded under normoxia by the ubiquitin-proteosome system, but it is stabilized by hypoxia (6, 40). Therefore, increased HIF-1α in tissues suggests an increase in hypoxia-triggered target genes.

The small mammals Ochotona curzoniae (Hodgson, 1857) and Microtus oeconomus (Pallas, 1776) are the predominant species of native mammals on the China Qinghai-Tibetan plateau alpine meadow; they live at an altitude of about 3 km and are completely acclimatized to the hypoxic environment (7, 46). O curzoniae is a unique model of hypoxic tolerance. We previously reported that acute and chronic simulated altitude hypoxia of 5 km did not cause any significant biochemical alteration in the permeability of lysosomal membrane and protease activity of hepatic cells in these native high-altitude mammals. In contrast, in lowland rats, mice, and guinea pigs, there was hepatic cell damage shown by extensive cytolysis (8,
26, 27), We hypothesize that the hepatic IGF-I and IGFBP-1, as survival factors, may be crucially involved in rapid adaptive shifts in cell survival and/or protection during hypoxia exposure.

The present experiments compare the changes between lowland mice and plateau native mammals in hepatic HIF-1α, IGF-I, and IGFBP-1 (see Fig. 1), as well as in the key enzyme genes in anaerobic and aerobic energy metabolism pathway: lactate dehydrogenase (LDH)-A, isocitrate dehydrogenase (ICD), and succinate dehydrogenase (SDH) mRNA induced experimentally by CoCl2 injection. Dramatic differences in these genes in native mammals are expected when compared with laboratory mice under normoxic and CoCl2 mimic hypoxic conditions. The results of this study provide novel evidence and possible mechanisms (a "multimode" response) of adaptation under hypoxia in lowland mice and native plateau mammals. In addition, the adaptive response to hypoxic damage may serve as another example of plastic adaptive response to hypoxia.

**MATERIALS AND METHODS**

**Animals.** Adult male ICR mice (30 ± 5 g) in healthy, clean grade (certification No. 2001001) were purchased from the Laboratory Animal Center of Zhejiang Province, China. Adult male *O. curzoniae* Hodgson (150 ± 10 g) and *M. oeconomus* Pallas (30 ± 5 g) mice were collected around the Haibei Alpine Meadow Ecosystem Station of Chinese Academy of Sciences, China (37° 40' N, 101° 23' E, altitude: 3.2 km, air pressure: 508 mmHg) (Fig. 2). All animals were maintained at 12:12-h light-dark cycle (lights on 0600–1800), at room temperature 20 ± 2°C, and with free access to food and water, according to National Institutes of Health guidelines for laboratory animal care. The study’s project was approved by the National Science Foundation of China, Laboratory Animals Center of China, and the local animal administration authority. Mice were housed in a group of six, whereas each native animal was housed separately. All animals were adapted to the conditions for 1 wk before the experimental manipulation.

**Mimic hypoxia.** Co2+ has been shown to be a substrate for ferrochelatase and therefore to be incorporated into the porphyrin moiety, resulting in Co2+-protoporphyrin IX (CoPP), which cannot bind O2, and CoCl2 injection reduces key enzyme of heme synthesis, as well as mimics venous PO2 (reducing concentration of PO2 in the blood) (20). CoCl2 has been used by many researchers for mimic hypoxia, so we used it in our present study.

Mimic cellular hypoxia was performed by an intraperitoneal injection of CoCl2 (the hypoxia-mimic compound: cobalt chloride of 20, 40, and 60 mg/kg). Animals (mice and plateau mammals) were randomized into four groups of 6–8 animals: *group 1*: 0.9% NaCl ip (control), *group 2*: 20 mg/kg CoCl2 ip, *group 3*: 40 mg/kg CoCl2 ip; and *group 4*: 60 mg/kg CoCl2 ip. All groups of animals were killed at 6 h after the CoCl2 injection. Additionally, in group 3, *O. curzoniae* were injected with 40 mg/kg of CoCl2 and killed at 3, 6, and 12 h, respectively, after the injection.

Mimic normobaric hypoxia stress was performed in a chamber of 70 × 70 × 50 cm ventilated with a mixed gas (O2/N2% of 16.0% O2, 10.8% O2, and 8.0% O2, respectively, and controlled by oxygen sensor (Innovative Instruments, Wake Forest, NC). The flow rate of the mixed gas was kept at 3.5 l/min for 6 h. All animals were placed individually in a separate compartment and divided randomly into four groups of 6–8 mice and 3 groups of 6–8 Tibetan mammals. In mice, four groups were set: *group 1*: 20.9% O2 (sea level, control), *group 2*: 16.0% O2 (~2.3 km altitude), *group 3*: 10.8% O2 (~7 km), and *group 4*: 8.0% O2 (~7 km). In Tibetan mammals, three groups were set: *group 1*: 16.0% O2; *group 2*: 10.8% O2; and *group 3*: 8.0% O2.

Overall hypoxia operations were started at 10:00 AM. The normobaric and CoCl2 mimics hypoxia experiments for the Tibetan mammals were carried out at 2.3 km altitude in the Xining Laboratory (The
Northwest Plateau Institute of Biology, The Chinese Academy of Sciences), and for the lab mice, these experiments were carried out at about sea level in the Zhejiang University Laboratory.

**Tissue preparation.** Animals were rapidly decapitated, trunk blood was collected, and centrifuged at 3,000 g for 30 min, and plasma was collected and stored at −80 °C until assayed. The livers were quickly removed from the animals, frozen immediately in liquid nitrogen, and stored at −80 °C in a freezer. Serial 20-μm liver sections were cut with a cryostat microtome (model HM505E; Microm, Waldorf, Germany). The sections were thaw mounted on gelatin-coated slides, desiccated under vacuum overnight, and stored at −80 °C until use.

**Immunohistochemistry.** Immunohistochemical studies were performed on tissue sections as previously described (36). Sections were fixed in fresh, chilled 4% paraformaldehyde in PBS (pH 7.4) for 30 min and washed three times with PBS (pH 7.4). Then sections were incubated in Triton X-100 (0.4%) for 15 min and washed again three times with PBS (pH 7.4). Endogenous peroxidase activity was destroyed with 0.3% hydrogen peroxide (H2O2). Nonspecific binding was blocked by 10% normal goat serum for HIF-1α and IGFBP-1 or horse serum for IGF-I (Zhongshan Biotechnology, Beijing, China) at 37°C for 30 min. Then all sections were incubated overnight in humidified chambers at 4°C with the following primary antibodies: monoclonal anti-HIF-1α antibody (~120 kDa, 1:200; cat no: BA0912; Boster Biotechnology, Wuhan, China). Polyclonal anti-IGFBP-1 antibody (1:200; donated by Dr. Cuming Duan, University of Michigan), monoclonal anti-IGF-I antibody (1:500, donated by Dr. Cuming Duan, University of Michigan). All of the primary antibodies were diluted in PBS (pH 7.4) containing 0.4% Triton X-100 and 1% BSA. Sections were washed three times in PBS (pH 7.4) and then detected by use of a streptavidin-biotin-peroxidase conjugate IgG (Santa Cruz, CA; 1:10,000) for 1 h. After the membrane was washed with Tris-buffered saline-Tween (TBS-T) followed by incubation with appropriate horseradish peroxidase-conjugated IgG (Santa Cruz, CA; 1:10,000) for 1 h. After the membrane was washed with TBS-T, the signals were detected by enhanced chemiluminescence (Santa Cruz, CA). Positive and negative controls for antibody of HIF-1α were shown in Fig. 3.

Plasma IGFBP-1 concentration was measured by Western ligand blot, as previously described (14). Briefly, nonreduced samples of plasma were electrophoresed on 12.5% SDS acrylamide gels under nonreducing conditions and eleetrotransferred onto nitrocellulose. The blotted proteins were incubated with 1,000,000 cpm 125I-labeled IGF-I overnight at 4°C. Washed, air-dried, and exposed to radiographic film. The optical density of IGFBP-1 bands (30 kDa) were analyzed by Image Master VDS Software (Hoefer Pharmacia Biotech, Piscataway, NJ). Goat plasma was used as a control, and plasma...
IGFBP-1 content was calculated and expressed as a percentage of goat plasma control.

ELISA. Plasma IGF-I concentration was measured by ELISA following the instructions of the manufacturer (Boster Biotechnology). IGF-I concentration was measured with a maximum absorbance of 0.450 nm. Plasma IGF-I content was calculated and expressed as IGF-1 ng/ml plasma.

Semiquantitative RT-PCR. RT-PCR was performed according to a previously described protocol (37). Total RNA from livers was isolated with RNA Isolation Kit (Bio Basic, Markham, ON, Canada) following the instructions of the manufacturer. The amount of total RNA was determined by spectrophotometry (Lambda Bio 4.0; Perkin Elmer) at 260 nm. The reverse transcription was carried out on 2 μg of total RNA by M-MLV reverse transcriptase (Promega, Madison, WI) using an oligo(dT) primer in a solution (25 μl) containing 0.8 mM of all four dNTP, 5 μl × 5X buffer (250 mM Tris·HCl, pH 8.3, 375 mM KCl, 15 mM MgCl2, 50 mM DTT), 1 unit/μl ribonuclease. The reverse transcription was carried out at 37°C for 2 h, heated for 5 min at 90°C, and then cooled to 4°C to synthesize the first cDNA strand. The synthesized cDNA (2 μl) was used as a template, amplified by PCR in a reaction mixture (25 μl) containing 200 IU Taq DNA polymerase (Sangon, Shanghai, China), 10× buffer [20 mM MgSO4, 100 mM KCl, 80 mM (NH4)2SO4, 100 mM Tris·HCl, pH 9.0, 0.5% NP40], 0.2 μM each of dNTP, and 0.25 mM of the following primers: IGFBP-1: forward: 5'-CACAGGGTCTGACGTGCCGGCG-3', reverse: 5'-GGCGTTCCACAGGATGGGCTG-3' (29), IGF-I: forward: 5'-TCGTCTTCATCATCTTCTACC-3', reverse: 5'-CTCTCTACATTCTTGAGTCTTT-3' (NM_178866), LDH-A: forward: 5'-ACAGTGGTGGGGTGGTG-3', reverse: 5'-CCGCATTCCCTTCCTG-3' (22), SDH: forward: 5'-AACCTACGAGGCAACAC-3', reverse: 5'-ACCTGTCCGTTCGCCACCAACGAC-3' (35), ICD: forward: 5'-AAATTTGCTCTTCCTGCTTAC-3', reverse: 5'-CCTGACAGCGATGGTGC-3'(21), β-actin: forward: 5'-GCAGCTTTGACATCCTGAAG-3', reverse: 5'-ACAGTGAGGCCTAGGAGAG-3' (49).

All of the primers were synthesized in Sangon (Shanghai, China).

In general, PCR was performed by a cycle of denaturing at 94°C for 45 s, annealing at appropriate temperature for suitable seconds and extension at 72°C for an appropriate time for suitable cycles. The annealing time and temperature were set as follows: 45 s at 57°C for ICD and SDH, 45 s at 59°C for LDH-A, 60 s at 55°C for ICD. The extension time was set as follows: 60 s for IGF-I, LDH-A, and ICD, 45 s for SDH, and 120 s for IGFBP-1.

The amplified products were separated on 1% agarose gel and visualized by ethidium bromide staining. Semiquantitative analysis was performed during the exponential phase of the PCR reaction, at which time the PCR products were deemed proportional to the number of cDNA templates, and the suitable cycles were 26 cycles for IGF-I, 33 cycles for IGF-I, 22 cycles for LDH, ICD, and SDH. The negative control contained all reagents, except that 2 μl H2O was substituted for the RT reaction product. Products of RT-PCR reactions were photographed and analyzed by Image Master VDS Software (Hoefer Pharmacia Biotech), and mRNA contents were expressed as cDNA relative densitometric units (ratio of cDNA/β-actin).

Quantitative analysis. The immunoreactive densities of HIF-1α, IGF-I, and IGFBP-1 in livers were determined using Optimas 6.5 Software (Argis-Schoen Vision Systems, Alexandria, VA). In brief, sections were placed under a microscope (Nikon TE2000; Tokyo, Japan), and the images were transferred via a digital camera (Nikon CoolPix 950) to a computer. The mean density of the positive area was measured. Five separate positive regions in each section were selected. Six sections were examined from each animal. The results were expressed as the percentage of relative optical density units (in arbitrary units) compared with the control group. Control groups were given the value of 100%: optical densities obtained in six sections per animal were averaged and used to calculate group means after being subtracted from a mean of controls.

Statistical analysis. The data are presented as the means ± SD. The significance of differences was assessed with Student’s t-test and one-way ANOVA with Duncan’s test. The SPSS statistical package (Version 11.0) was used for the analysis, and statistical significance was accepted at P < 0.05.

RESULTS

Effects of CoCl2 mimic hypoxia on hepatic HIF-1 protein expression in mice, M. oeconomus, and O. curzoniae.

The changes of hepatic HIF-1α, IGF-I, and IGFBP-1 protein expression in response to injections of 20, 40, and 60 mg/kg CoCl2 were examined. The expression levels of different hepatic markers (HIF-1α, IGF-I, IGFBP-1, LDH, and ICD) were measured in mice, M. oeconomus, and O. curzoniae after 14 days of CoCl2 treatment.

Table 1. Changes in protein and/or mRNA levels for five different markers: HIF-1α, IGF-I, IGFBP-1, LDH, and ICD during 20 mg/kg CoCl2 challenge

<table>
<thead>
<tr>
<th>Marker</th>
<th>Mice</th>
<th>M. oeconomus</th>
<th>O. curzoniae</th>
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<tr>
<td>HIF-1α</td>
<td>↑↑</td>
<td>↑↑</td>
<td>—</td>
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<tr>
<td>IGF-I</td>
<td>↑</td>
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<td>↑</td>
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<tr>
<td>IGFBP-1</td>
<td>↑</td>
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<td>↑</td>
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<tr>
<td>LDH mRNA</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ICD mRNA</td>
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<tr>
<td>Plasma IGF-I</td>
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<tr>
<td>Plasma IGFBP-1</td>
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ICD, isocitrate dehydrogenase. ↑, significantly upregulated, P < 0.05; ↑↑, P < 0.01; —, no significance difference compared with the control; ↓, significantly downregulated, P < 0.05.
are summarized in Table 1 and illustrated in Figs. 4, 6, and 7. The HIF-1α protein expression was increased markedly and proportionately to dose by 51.1% (1.5 ± 0.2, P < 0.01), 35.1% (1.4 ± 0.3, P < 0.05), and 21.4% (1.2 ± 0.2, P > 0.05) in mice compared with unchanged controls. The HIF-1α protein expression was unchanged in O. curzoniae and M. oeconomus compared with untreated high-altitude controls (Tables 1 and 2, Figs. 1, A–G, and 4, on HIF-1α by immunohistochemistry). The data (HIF-1α in mice) measured by immunohistochemistry (Fig. 4A) are confirmed by Western blot analysis (Fig. 4B).

Effects of normobaric hypoxia on hepatic HIF-1α protein expression in mice, M. oeconomus, and O. curzoniae. The changes of hepatic HIF-1α protein in response to normobaric hypoxic stress of 16.0%, 10.8%, and 8.0% O2 of sea level are summarized in Table 2 and illustrated in Fig. 5. During exposure to the normobaric hypoxia for 6 h, the HIF-1α protein expression markedly increased by 44.9% (1.4 ± 0.3, P < 0.01), 119.2% (2.2 ± 0.6, P < 0.001), and 54.3% (1.5 ± 0.6, P < 0.05), respectively, in mice compared with untreated control. The HIF-1α protein was significantly increased by 53.7% (1.5 ± 0.3, P < 0.05) in the liver of M. oeconomus only when exposed to 8.0% O2 (the lowest O2 concentration we tested) but unchanged in O. curzoniae compared with untreated control.

Effects of CoCl2 mimic hypoxia on hepatic IGF-I and IGFBP-1 mRNA expression in mice, M. oeconomus, and O. curzoniae. Hepatic IGF-I protein expression was significantly increased depending on the dose of CoCl2 (20, 40, and 60 mg/kg) by 42.1% (1.4 ± 0.1, P < 0.01), 84.4% (1.8 ± 0.2, P < 0.01), and 94.3% (1.9 ± 0.3, P < 0.01) vs. control, respectively, in M. oeconomus and unchanged in O. curzoniae and mice (Table 1, Figs. 1, A–G, middle, and 6).

Hepatic IGFBP-1 protein expression was significantly enhanced after CoCl2 injection of 20, 40, and 60 mg/kg CoCl2 by 102.6% (2.0 ± 0.1, P < 0.01), 12.9% (1.1 ± 0.2, P > 0.05), and 3.1% (1.0 ± 0.2, P > 0.05) in mice and by 112.7% (2.2 ± 0.2 P < 0.01), 93.5% (1.9 ± 0.2 P < 0.01), and 112.3% (2.1 ± 0.1, P < 0.01) in O. curzoniae, respectively, compared with controls, while unchanged in M. oeconomus (Table 1, Figs. 10, A–F, and G on IGFBP-1 and Fig. 7). The data (IGF-I in M. oeconomus and IGFBP-1 in mice) measured by immunohistochemistry (Fig. 6A and Fig. 7A) are confirmed by Western blot analysis (Fig. 6B and 7B).

Effects of CoCl2 on hepatic IGF-I mRNA and IGFBP-1 mRNA expressions. Hepatic IGF-I mRNA expression was markedly increased at 6 h by 93.5% (1.9 ± 0.2, P < 0.01 vs. control) and 12 h by 1.3% (2.3 ± 0.2, P < 0.01 vs. control) after administration (Fig. 8). The data (IGFBP-1) measured by immunohistochemistry (Fig. 8A) are confirmed by Western blot analysis (Fig. 8B).

Effects of CoCl2 on plasma IGF-I and IGFBP-1 protein level. Plasma IGF-I protein levels (by ELISA analysis) were significantly increased in M. oeconomus and unchanged in mice and O. curzoniae after injection of CoCl2 (40 mg/kg) (Fig. 9C). Hepatic IGF-I mRNA expression was unchanged by CoCl2 injection in mice (Fig. 9A) and O. curzoniae but increased only in M. oeconomus after the CoCl2 injection (60 mg/kg) (Fig. 9B).

Effects of CoCl2 on plasma LDH-A, ICD, and SDH mRNA expressions. Levels of hepatic LDH-A mRNA (Table 1, Fig. 11A) and ICD mRNA (Table 1, Fig. 11B) were significantly increased by 102.6% (2.0 ± 0.1, P < 0.01), 12.9% (1.1 ± 0.2, P > 0.05), and 3.1% (1.0 ± 0.2, P > 0.05) in mice and by 112.7% (2.2 ± 0.2 P < 0.01), 93.5% (1.9 ± 0.2 P < 0.01), and 112.3% (2.1 ± 0.1, P < 0.01) in O. curzoniae, respectively, compared with controls, while unchanged in M. oeconomus (Table 1, Figs. 10, A–F, and G on ICD and Fig. 7). The data (IGF-I in M. oeconomus and IGFBP-1 in mice) measured by immunohistochemistry (Fig. 6A and Fig. 7A) are confirmed by Western blot analysis (Fig. 6B and 7B).

Table 2. Comparison of the expression level of HIF-1α treated with two protocols of hypoxic stresses in Tibetan mammals M. oeconomus and O. curzoniae and laboratory mice

<table>
<thead>
<tr>
<th>16% O2</th>
<th>10.8% O2</th>
<th>8.0% O2</th>
<th>20 mg/kg</th>
<th>40 mg/kg</th>
<th>60 mg/kg</th>
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<tbody>
<tr>
<td>Mice</td>
<td>↑ ↑ ↑</td>
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<tr>
<td>M. oeconomus</td>
<td>↑</td>
<td></td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>O. curzoniae</td>
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The oxygen concentration of 16% O2 is equal to 2.3 km high altitude, 10.8% O2 is equal to 5 km high altitude, and 8.0% O2 equates to 7 km high altitude. ↑, Significantly upregulated; ↑ P < 0.05; ↑↑ P < 0.01, ↑↑↑ P < 0.001. - , No significant difference compared with the untreated control.
enhanced (1.3 ± 0.2, P < 0.05) or reduced (0.8 ± 0.2, P < 0.05), respectively, in mice by an injection of CoCl2 (20 mg/kg) but unchanged in M. oeconomus and O. curzoniae (Table 1, Fig. 11, A and B); hepatic SDH mRNA was unchanged in the three species (Table 1, Fig. 11C).

Effects of normobaric hypoxia on hepatic LDH-A, ICD, and SDH mRNA expressions. Levels of hepatic LDH-A mRNA and ICD mRNA were significantly enhanced (P < 0.05 and P < 0.001) and reduced in mice during 10.8% and 8.0% O2 stress; SDH mRNA was significantly enhanced only during 8.0% O2 (P < 0.05), while unchanged in O. curzoniae (Fig. 12, A and B); in addition, hepatic SDH mRNA was unchanged in the three species (not shown here).

DISCUSSION

Species-dependent HIF-1α expression. HIF-1α, a basic-helex-loop-helix-PAS transcription factor, is a key regulator of O2 homeostasis. HIF-1α activates transcription of genes whose protein products increase O2 availability or promote metabolic adaptation to hypoxia. Adjustments of functional genes to hypoxia, including upregulation of the erythropoietin, of VEGF, of the glucose transporters (GLUT), and of the glycolytic enzymes that upregulate adequate ATP in the absence of oxidative phosphorylation (40). A number of known HIF-1α-responsive genes in mammals, such as the glycolytic genes, heme oxygenase-1 and IGFBP-1 in fish (e.g., Illichthys mirabilis) and HepG2 cells and others, have been reported during hypoxia (11, 42, 45). These relevant genes by HIF-1α transcript are beneficial for homeostasis and survival, potentially through hormone regulation, metabolic shift, and cell cycle control, as well as cellular damage protection against hypoxia. HIF-1α activation and HIF-1α transcriptional regulation to hypoxic targeting genes are marks of hypoxic stress response and are significant to an organism’s survival. In addition to hypoxia, the divalent cation such as cobalt chloride (CoCl2), an iron chelator, may mimic hypoxia and can also induce HIF-1 activity (48). As Co2+, a substrate for ferrochelatase, incorporates into the porphyrin moiety, it forms CoPP, which is unable to bind O2; consequently, Co2+ leads to a reduced tissue and cellular O2 supply (mimics hypoxia) (20). CoCl2 may also produce an inhibition of HIF-α-hydroxylases that is widely regarded as the main mechanism of cobalt action that could influence the stabilities of HIF-1α. In the present study, a significant increase in hepatic HIF-1α proteins occurred in sea level laboratory mice but not in plateau mammals after an injection of 20 mg/kg CoCl2 (Fig. 4). At the same time, we operated normobaric hypoxia (low Po2 in mixed O2/N2) and found that 16% (light), 10.8% (middle), and 8.0% O2 (severe hypoxia) also markedly caused an increase of HIF-1α protein in the liver of mice and did also only by 8.0% O2 in M. oeconomus, but did not in O. curzoniae (Fig. 5). This increased HIF-1α emphasizes the high sensitivity of sea-level mice, in contrast to the greater resistance of high-altitude plateau ani-

Fig. 5. Western blot analysis of HIF-1α expression in the liver of mice, M. oeconomus, and O. curzoniae at various O2 concentrations (16%, 10.8%, and 8.0% O2). In lanes 1–4 are presented mice control, 16% O2, 10.8% O2, and 8.0% O2; in lanes 5–7 are presented M. oeconomus control, 10.8% O2, and 8.0% O2; in lanes 8–10 are presented O. curzoniae control, 10.8% O2, and 8.0% O2. Values are given as the means ± SD; n = 6 or 7; *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the controls.

Fig. 6. Hepatic insulin-like growth factor-I (IGF-I) protein expression induced by various CoCl2 doses (20, 40, 60 mg/kg) in mice, M. oeconomus, and O. curzoniae. A: ratio of IGF-I OD relative to control values. Values are given as the means ± SD; n = 6 or 7; *P < 0.05, **P < 0.01 compared with the control. *P < 0.05, compared between 20 mg/kg CoCl2 and 60 mg/kg CoCl2. B: Western blot analysis of IGF-I. Results shown are representative of three independent experiments, **P < 0.01 compared with the control.
mals to both hypoxia models. These data suggest that 1) both CoCl2 mimic hypoxia and normobaric hypoxia can induce HIF-1α expression through a low PO2 supply in tissues and cells, although the hypoxia patterns are different; 2) there are diverse responses of the HIF-1α to hypoxia among the three species not only between sea level mice and plateau mammals but between the plateau mammals; 3) the response of HIF-1α to normobaric hypoxia is conditioned in M. oeconomicus, e.g., a severe hypoxia (8.0% O2) is required, compared with marked increased response in mice and no response in O. curzoniae to all of the doses of normobaric hypoxia (Fig. 5). The mice response to hypoxia in our present study conforms to that induced by hypoxic ischemia and preconditioning (3, 16). In our data, these observations in different animal species suggest that different responsiveness of HIF-1α represent a diverse mechanism of hypoxia adaptation and a diversely acclimatized evolutionary model.

Diversities of IGF-I and IGFBP-1 genes expression. Both IGF-I and IGFBP-1, the members of IGF families, are the hypoxia-targeting gene, which are regulated by hormones and IGF family receptors.

IGF-I, as an anabolic and survival factor, is upregulated by ischemia (45) and strongly antiapoptotic (32, 34, 50), and it has a critical role in protecting CNS against various types of injury (30). A treatment with IGF-I significantly ameliorated brain injury caused by hypoxia/ischemia (2, 28, 48). IGF-I and induction of IGF-I receptors within the damaged brain regions suggest a possible role for this hormone in brain recovery (12). In addition, the robust induction of endogenous IGF-I within damaged regions of the liver might suggest a therapeutic potential for the IGF-I system in preventing liver failure (31, 39). HIF-1α is one of the transcriptional factors for the important hypoxia-targeting genes. We found that hepatic IGF-I significantly enhanced in M. oeconomicus but not in mice and in O. curzoniae after CoCl2 administration (Fig. 6); thus this increase may not be directly correlated with HIF-1α, as HIF-1α keeps silent during CoCl2 challenge (Fig. 4); however, in the normobaric hypoxia model, an increased HIF-1α was noted only under 8.0% O2 in M. oeconomicus (Fig. 5), suggesting that this increased HIF-1α may work for the IGF-I transcript only during severe hypoxia stress. Kajimura et al. (17, 18) reports that CoCl2-activated HIF-1α is responsible for the increase of hypoxia-targeting IGF-BP-1 expression in mice through the IGF-BP-1 gene transcription in zebrafish embryo model. Recently, Guan et al. (12) also observed an increased IGFBP-1 in hypoxia ischemia-damaged region of brain, suggesting IGFBP-1 may also be a potential factor for prevention hypoxia-induced injury. Similarly, in the present study, we found that hepatic IGFBP-1 levels were markedly elevated in mice and O. curzoniae 6 h after injection of 20 mg/kg CoCl2, and the responses of IGFBP-1 to CoCl2 in O. curzoniae were stronger than in mice. IGFBP-1 mRNA and protein are also induced in HepG2 cells by hypoxia (PO2 = 2%) (42). In contrast to the mice, the IGFBP-1 increase is not correlated with HIF-1α, as no response of hepatic HIF-1α occurred at the same time in O. curzoniae (Figs. 4, 7, and 8); it is possible that different transcriptional mechanisms operate for both IGF-I and IGFBP-1 activations, not only between mice and plateau mammals but also between the two types of native plateau animals.
Diversities of LDH-A and ICD gene expression. Both LDH-A and ICD genes are the hypoxia-targeting genes. We found in this study that LDH-A genes encoding LDH-A enzymes (modulating anaerobic metabolism) were markedly upregulated as shown by the increased LDH-A mRNA, while ICD genes encoding ICD enzymes (modulating aerobic metabolism, TCA) via an unknown modulator were downregulated by 20 mg/kg CoCl$_2$ and by normobaric hypoxia (all doses of PO$_2$), as shown by the decreased ICD mRNA in the liver of mice (Figs. 11 and 12, A and B). In contrast to the mice, in the Tibetan native mammals, _O. curzoniae_ and _M. oeconomus_, 20 mg/kg of CoCl$_2$ or normobaric hypoxia produced a lack of change in LDH-A and ICD mRNA expressions (Table 1, Figs. 11, Fig. 12, A and B). In the case above, therefore, the hypoxia-activated HIF-1/ATF may acutely take part in the LDH-A mRNA transcript in mice and _M. oeconomus_ (under 8.0% O$_2$), not in _O. curzoniae_. Under an acute hypoxia, the enhancement of LDH-A mRNA and the reduction of ICD mRNA in mice

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**Fig. 9.** RT-PCR amplifications of IGF-I and/or IGFBP-1 in livers during 20 mg/kg CoCl$_2$ challenge in mice (A), _M. oeconomus_ (B), and _O. curzoniae_ (C). Results shown are representative of three independent experiments, *P < 0.05, compared with the control, respectively.

**Fig. 10.** Plasma IGF-I (A) and IGFBP-1 (B) levels after intraperitoneal CoCl$_2$ in mice, _M. oeconomus_, and _O. curzoniae_. A: plasma IGF-I was detected by ELISA, and the content was calculated and expressed as IGF-I ng/ml plasma. B: plasma IGFBP-1 was detected by Western ligand blot. OD of IGFBP-1 bands (30 kD) were analyzed by Image Master VDS Software (Hoefer Pharmacia Biotech). Goat plasma was used as a control, and plasma IGFBP-1 content was calculated and expressed as percentage to goat plasma control. Lanes 1–7 show the mice control, the mice with intraperitoneal 40 mg/kg CoCl$_2$, the control of _M. oeconomus_, the _M. oeconomus_ with intraperitoneal 40 mg/kg CoCl$_2$, the control of _O. curzoniae_, the _O. Curzoniae_ with intraperitoneal 40 mg/kg CoCl$_2$, and the goat plasma (control), respectively. _M, M. oeconomus; O, O. curzoniae_. Values are given as the means ± SD; n = 6; *P < 0.05 compared with the control.
and *M. oeconomus* (under 8.0% O₂) would benefit them by increasing anaerobic metabolism and decreasing the aerobic metabolism and reactive oxygen, which protect against cell injury. The lack of a response of LDH-A mRNA and ICD mRNA in the high-altitude plateau mammals to mimic hypoxia indicates that the plateau mammals have evolved their metabolism to sufficiently adapt and survive a plateau environment. This evolutionary adaptation of metabolism at a natural Tibetan Plateau environment is supported by our previous study showing that *O. curzoniae* had a high ratio of oxygen utilization under plateau hypoxic stress (7). The underlying precise mechanism remains unknown.

It has been thought that LDH leakage is associated with cell damages, and there is evidence that shows that IGF-I decreases LDH leakage from the cell (13, 41). It is well established that during hypoxia, LDH is activated through HIF-1α, resulting in lactic acid accumulation and glycogen decrease (40). Similar results are presented in this study in mice liver by two types of hypoxia. Previous reports showed that the damage of liver was induced only in rats but not in *O. curzoniae* under acute hypoxia (8, 27). Therefore, the acute upregulated LDH-A/ICD mRNA in mice may provide more ATP via glycolytic pathway and benefit cell survival, as well as cell protection via activated IGF-I/IGFBP-1, which acts as a hypoxia response element in the first intron of the hIGFBP-1 gene (43) and antiapoptosis via suppression of the activation of specific proapoptotic factors (25).

In conclusion, CoCl₂ mimic hypoxia and normobaric hypoxia: 1) upregulates hepatic HIF-1α, IGF-I/IGFBP-1, and LDH-A mRNA and downregulates ICD mRNA in mice, and 2) enhances hepatic IGF-I in *M. oeconomus* and IGF/FB-1 in *O. curzoniae*, respectively. Additionally, both hepatic LDH-A and ICD mRNA are unchanged in plateau animals. These data suggest that those genes and proteins are locally activated by a protective effect in liver cells against hypoxia. CoCl₂ mimic and normobaric hypoxia-activated HIF-1α may be involved in the upregulation of hepatic IGF/FB-1 and LDH-A mRNA in mice during hypoxia, but the upregulation of hepatic IGF/FB-1 in plateau animals may be correlated with other transcription factors other than HIF-1α. The adaptive strategies of highland mammals to hypoxia seem to differ genetically from those of...
lowland mammals by a long evolutionarily acclimatization to the hypoxic environment.

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