Polycythemic responses to hypoxia: molecular and genetic mechanisms of chronic mountain sickness


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Ou, L. C., S. Salceda, S. J. Schuster, L. M. Dunnack, T. Brink-Johnsen, J. Chen, and J. C. Leiter. Polycythemic responses to hypoxia: molecular and genetic mechanisms of chronic mountain sickness. J. Appl. Physiol. 84(4): 1242–1251, 1998.—We examined erythropoietin (EPO) gene expression and EPO production during hypoxia in two Sprague-Dawley rat strains with divergent polycythemic responses to hypoxia. Hilltop (H) rats develop severe polycythemia, severe hypoxemia, and pulmonary artery hypertension. The H rats often die from a syndrome indistinguishable from chronic mountain sickness (CMS) in humans. Madison (M) rats develop polycythemia and pulmonary artery hypertension that is modest and suffer no excess mortality. We tested the hypothesis that these rat strains have different stimulus-response characteristics governing EPO production. Rats of each strain were exposed to hypoxia (0.5 atm, 73 Torr inspired Po2), and renal tissue EPO mRNA and EPO levels, plasma EPO, ventilation, arterial and renal venous blood gases, and indexes of renal function were measured at fixed times during a 30-day hypoxic exposure. During extended hypoxic exposure, H rats had significantly elevated renal EPO mRNA, renal EPO, and plasma EPO levels compared with M rats. Ventilatory responses and indexes of renal function were similar in the strains during the hypoxic exposure. H rats had greater arterial hypoxemia from the onset of hypoxia and more severe renal tissue hypoxemia and greater polycythemia after 14 days of hypoxic exposure. When EPO responses were expressed as functions of renal venous Po2, the two strains appeared to lie on the same dose-response curves, but the responses of H rats were shifted along the curve toward more hypoxic values. We conclude that H rats have significantly greater polycythemia secondary to poorer renal tissue oxygenation, but the stimulus-response characteristics governing EPO gene expression and EPO production do not seem to differ between M and H rats. Finally, the regulation of EPO levels during hypoxia occurs primarily at the transcriptional level.

erythropoietin; gene expression; renal oxygenation; renal work; tissue hypoxia; high altitude

POLYCYTHEMIA is a compensatory mechanism to sustain O2 delivery during life at high altitude, but excessive polycythemia is associated with chronic mountain sickness (CMS). There is, on average, an increase in hematocrit (Hct) after altitude exposure, but there is considerable individual variation, and only a small number of high-altitude residents develop CMS. The origin of the variation among individuals is unknown, but variation in erythropoietin (EPO) responses to equivalent hypoxic stress is a tenable hypothesis. EPO, a glycoprotein growth factor, regulates the rate of red blood cell production by stimulating the proliferation and differentiation of erythroid precursor cells (12). EPO synthesis in adult mammals occurs primarily in the kidney (19), and hypoxia in the kidney from a variety of causes (e.g., high-altitude exposure and anemia) stimulates the synthesis and release of EPO, which are followed by increased production of red blood cells. Recent molecular studies in vivo (2, 32–34) and in vitro (17) showed that hypoxia or cobalt treatment led to rapid accumulation of EPO mRNA in the kidney or in cultured hepatoma cell lines, and detection of EPO mRNA preceded the appearance of EPO in plasma or in cultured medium. Hence, accelerated EPO gene expression is the first step in the polycythemic responses to hypoxic or cobalt treatment. The location(s) of the hypoxic sensing mechanism is controversial. The adequacy of renal tissue oxygenation at the EPO-producing sites is thought to be the immediate signal regulating EPO production (22), but a more potent extrarenal sensing mechanism has also been postulated (31). Renal tissue oxygenation depends on the relationship between renal tissue O2 delivery, a function of arterial O2 content [Ca O2, which depends on arterial Po2 (Pa O2) and Hct] and renal blood flow, and renal tissue O2 consumption (V O2), a function of the excretory activity of the kidney. Therefore, there may be a link between renal excretory function and the regulation of EPO production (10, 11, 13). Polycythemia following EPO release ought to improve renal tissue oxygenation and reduce EPO synthesis. However, changes in EPO levels, renal tissue oxygenation, and Hct are not well correlated in time, and the classic concept of a negative-feedback control of EPO production by a polycythemic response remains debatable (3, 9, 28).

We have studied two Sprague-Dawley rat strains with marked differences in the propensity to develop CMS after chronic exposure to hypoxia: Hilltop (H) rats develop excessive polycythemia, accentuated hypoxemia, and severe pulmonary hypertension associated with a high mortality rate; Madison (M) rats develop only moderate polycythemia and pulmonary hypertension and no significant mortality (30). Excessive polycythemia apparent in H rats after 3 wk of exposure to a simulated altitude of 5,500 m was associated with persistent elevation of plasma and renal EPO (28). Metabolic degradation of EPO did not differ in the two rat strains, and the persistent elevation of EPO probably resulted from sustained EPO gene expression. In past experiments, high blood viscosity associated with excessive polycythemia in the H rats (Hct often >70%) may have compromised renal oxygenation and thereby
enhanced EPO production (13, 28). In addition, there was a positive correlation between the extent of polycythemia and EPO production, which was not compatible with a negative-feedback control mechanism regulating EPO production. The purposes of the present study were to use the H and M rat strains 1) to characterize EPO gene expression and EPO production during chronic hypoxia, 2) to examine the relationships among the levels of EPO gene expression and EPO production and renal tissue oxygenation, and 3) to examine the notion of a negative-feedback control mechanism of EPO production.

**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley rats weighing 270–320 g were obtained from Hilltop (H), Scottsdale, PA (altitude-sensitive strain) and Madison, WI (M) breeding laboratories. There were two separate series of experiments: in one series of experiments, levels of EPO mRNA and EPO were measured; in the other, ventilatory and blood-gas responses, renal function, and renal oxygenation were measured. In each set of experiments, five groups of five to six rats from each strain were exposed to a simulated altitude of 0.5 atm (5,500 m, 73% O2) for various durations (0, 6, and 24 h and 1, 3, 7, 14, 21, 28, and 30 days). Supplementary experiments were performed to test the effects of cobalt or combined cobalt and hypoxia treatment on EPO and EPO mRNA production. Two methods were used to measure EPO mRNA: Northern and slot-blot analyses using polyadenylated RNA [poly(A)⁺ RNA] were used in early experiments, and a highly sensitive RNase protection assay was employed later in the study. EPO gene probes for Northern blot studies were provided by Dr. E. Goldwasser (University of Chicago). The probes were 1.0- and 1.2-kb Pst I restriction fragments of the cloned mouse EPO gene. The 1.2-kb fragment contained all of exons 2 and 3 and part of exon 4. The 1.0-kb fragment contained the remainder of exon 4 and part of exon 5. The probes were labeled with [32P]CTP (3,000 Ci/mmol; ICN, Costa Mesa, CA) to high specific activities (2 × 10⁷ dpm/µg) using a nick translation kit (BRL, Bethesda, MD) according to the manufacturer’s instructions. Probes were denatured by heating at 100°C for 10 min and quenched on ice immediately before use. RNAs were isolated from tissue homogenates by extraction with 4 M guanidine isothiocyanate and sedimentation of RNA through 5.7 M cesium chloride (4). Poly(A)⁺ RNA was isolated by chromatography on oligo dT-cellulose. For electrophoresis, 6–30 µg of poly(A)⁺ RNA per lane were loaded on formaldehyde-1.0% agarose gels. After electrophoresis, the gels were blotted onto nitrocellulose filters and dried under vacuum at 80°C for 2 h. Northern and slot blots were hybridized with 32P-labeled probe at 42°C for 18 h and washed using standard methods. The blots were exposed to Kodak XAR-5 X-ray film (Eastman Kodak, Rochester, NY) with an intensifying screen at −70°C. The EPO mRNA was quantitated by scanning densitometry. Results from Northern blots were expressed as densitometric units per microgram of RNA loaded. As an internal control for RNA loading, Northern and slot blots were probed with a mouse β-actin probe. Spleenic poly(A)⁺ RNA was used as a negative control.

Northern blot analysis was not sensitive enough to detect renal EPO mRNA in rats at sea level or rats exposed to a simulated altitude of 5,500 m (10.5% O2 or 73 Torr inspired PO2). For this reason, an RNase protection assay was employed to follow the activity of EPO gene expression during the development of CMS in rats exposed to a simulated altitude of 5,500 m. In RNase protection assays, total RNA was probed for EPO mRNA without separation of the poly(A)⁺ RNA. The details of the RNase protection assay for EPO mRNA have been described previously (33). Briefly, total tissue RNA samples prepared by the acid guanidinium thiocyanate-phenol-chloroform method (4) were analyzed using an RNase protection assay (16). The template for production of the complementariry strand and RNA probe was a polymerase chain reaction-derived rat EPO cDNA fragment cloned into the plasmid vector pCR1000 (Invitrogen, San Diego, CA). The labeled RNA probe was generated by in vitro transcription by T7 RNA polymerase (Life Technologies, Gaithersburg, MD) using [α-32P]UTP (29.6 TBq/mM; Amersham, Arlington Heights, IL). The protected fragments were separated on a denaturing 8% agarose gel electrophoresis to verify quantification and integrity of samples. RNA samples were hybridized overnight in 30 µl of hybridization buffer at 45°C with 0.5 × 10⁹ cpm of labeled probe. RNase digestion was performed at 30°C for 1 h using RNase T1 (Boehringer-Mannheim, Indianapolis, IN). The protected segments were separated on a denaturing 8% acrylamide-7 M urea gel and analyzed by autoradiography. The autoradiograms were quantitated by scanning with a BIO-Imaging analyzer (Fuji Medical System). RNase protection assays using antisera against human recombinant EPO (Incstar, Stillwater, MN), as previously described (28).

Measurements of ventilation, renal function, and renal tissue oxygenation in fully awake and chronically instrumented rats. Ventilation was measured in a whole body plethysmograph, as previously described (28). The following renal variables were measured at each designated time: urine output, sodium and potassium excretions, plasma sodium and potassium concentrations, and sodium reabsorption. Potassium and sodium concentrations were measured with a flame photometer (model FLM3, Radiometer America, Cleveland, OH). The glomerular filtration rate (GFR) was measured using polyfructose (15), and renal plasma flow was measured using p-aminohippuric acid by the method of Bratton and Marshall as modified by Smith et al. (35). Urine flow and clearance rates are expressed per 100 g of body weight. Protein was determined by a micro-Kjeldahl method. Arterial and venous blood gas samples were obtained anaerobically by withdrawing 300 µl from the rat; only the last 160 µl were used for analysis. The residue was returned to the rat. pH, PO2, and PCO2 were measured with microelectrodes at 37°C (model BMS 3 MK2, Radiometer America). Hypoxic exposure did not affect the shape of the oxyhemoglobin dissociation curve in the H or M rats, and the O2 content in arterial and venous blood was estimated from a rat oxyhemoglobin dissociation curve after correction for the blood pH measured in vivo (21). Renal blood flow was calculated from renal plasma flow and the Hct. The renal coefficient of O2 delivery (COD) was calculated by multiplying renal blood flow by CaO2. Renal VO2 was calculated by multiplying renal blood flow by the renal arteriovenous O2 content difference.

Surgical preparation. Four to 5 days before an experiment, catheters were implanted in each animal in the urinary bladder, femoral artery, and left renal and right external jugular veins under anesthesia using a combination of ketamine (60 mg/kg body wt im) and pentobarbital sodium (20 mg/kg body wt ip), as previously described (29). After surgery, each rat was treated with penicillin (100,000 U daily im for 5 days) and returned to the presurgical altitude immediately after recovery from anesthesia. All rats were allowed free access to water and laboratory rat chow. The animals were acclimated to a plastic restraining cage for 2 days before the surgery.
renal function studies. The animals recovered from surgery uneventfully, and the majority gained weight by the time of measurements.

Experimental procedures. During the measurements, each rat was housed in a restraining cage. A Plexiglas hood was fitted over the front of the restraining cage so that the desired gas mixture could be flushed through the hood. Renal hemodynamic values were measured (29), and the exposed end of the bladder catheter was extended with a short length of polyethylene tubing to allow collection of urine under the restraining cage. Urine volume was measured by weight. After 30–40 min of equilibration, two to three samples of urine and arterial and renal venous blood were obtained from each animal under appropriate PO2 conditions. To avoid possible adverse effects of repeated blood sampling that might change the Hct, each rat was studied only once.

RESULTS

Effect of extreme hypoxia and cobalt on EPO gene expression and EPO levels in H and M rats. Northern blot analysis failed to detect EPO mRNA in the kidney in either rat strain during exposure to a simulated altitude of 5,500 m, even though poly(A)+ RNA was used. To test the sensitivity of the Northern blot analysis, extreme stimuli, severe hypoxia alone (7,300 m simulated altitude or 65 Torr inspired O2) or combined severe hypoxia (7,300 m) and cobalt chloride (60 mg/kg sc) treatment, were used. The results are presented in Fig. 1. Under sea-level conditions, no EPO mRNA was detected in the kidney (Fig. 1 B, lanes 1 and 2), and no strain difference in plasma EPO levels was detected (Fig. 1 A, lanes 1 and 2). The EPO mRNA increased markedly in both rat strains during severe hypoxia (data not shown) and during combined hypoxia and cobalt injection (Fig. 1 B, lanes 1 and 2), but there were no strain differences under these conditions. The combined stimuli elevated the plasma EPO levels from control values of 59.5 ± 12.7 to 2,234.4 ± 632.5 mU/ml in the H rats and from 56.0 ± 6.1 to 2,705.7 ± 451.3 mU/ml in the M rats. These findings indicate that the Northern blot analysis could detect EPO mRNA but was insensitive to the EPO mRNA changes during exposure to 10.5% inspired O2 (5,500 m). Therefore, a more sensitive RNase assay was used in subsequent experiments.

Effect of 10.5% inspired O2 on EPO gene expression and EPO levels in H and M rats. The results of the EPO mRNA RNase protection analysis of total RNA from the kidney are summarized in Fig. 2 A, and the quantitative relationships of renal EPO mRNA as estimated by phosphor image analysis are shown in Fig. 2 B for each rat strain. At sea level, EPO mRNA levels were equivalent in the two rat strains. After 6 h of hypoxic exposure, EPO mRNA markedly increased in both rat strains, but the increase was at least 100% higher in the H than in the M rats. The EPO mRNA fell precipitously after 24 h of hypoxic exposure but remained significantly elevated above the control values in the H rats throughout the entire period of exposure. In contrast, EPO mRNA levels in the M rats fell toward the sea-level control values after 24 h of hypoxia. The results demonstrate exaggerated expression of the EPO gene in kidneys of H rats during hypoxic exposure. EPO mRNA was undetectable in the spleen, heart, or liver in H and M rats under control and hypoxic conditions. In contrast to the response to 10.5% inspired O2, cobalt injection (60 mg/kg sc) elicited equivalent accumulation of EPO mRNA in the kidney 6 h after treatment in both rat strains (Fig. 3). Thus RNase and Northern blot assays demonstrate no strain difference in EPO gene expression after cobalt treatment.

The time courses of changes in circulating and renal tissue EPO levels during chronic hypoxia in the two rat strains are portrayed in Fig. 4. There were no strain differences in plasma or renal tissue EPO levels under sea-level control conditions. EPO levels rose markedly at 12 h after exposure to hypoxia in both rat strains. The mean value of this increase was higher in the H than in the M rats, but the difference was not statistically significant. The EPO levels declined at 24 h in the plasma and the kidney in both rat strains, but the decrease was more profound in the H than in the M rats. Thereafter, the circulating and renal tissue EPO levels remained low or continued to decline toward the
sea-level control levels in the M rats, whereas those in the H rats tended to rise. As a result, the circulating and renal tissue EPO levels in the chronically hypoxic H rats were significantly higher than the sea-level control values and higher than in the hypoxic M rats at each time studied. The patterns of changes in circulating and renal tissue EPO levels during hypoxic exposure parallel those of EPO mRNA.

Effect of 10.5% inspired O$_2$ on renal function in conscious H and M rats. We examined a variety of aspects of renal function in H and M rats under the hypoxic conditions. These results are summarized in Table 1. There were no strain differences in urine output, plasma sodium, sodium excretion, or GFR at sea level or at any point during hypoxic exposure. Neither the plasma potassium (range 3.7–4.5 meq/l) nor potassium excretion (range 0.6–1.1 meq·l$^{-1}$·min$^{-1}$) differed between strains or across times of hypoxic exposure (data not shown). Plasma sodium was significantly less than the sea-level value on days 1 and 14, when data from H and M rats were pooled (there was a significant main effect of the duration of altitude exposure). Serum sodium on day 14 was also less than sea-level control, but this failed to achieve statistical significance ($P = 0.054$). Plasma sodium was significantly less in the M than in the H rats on day 14 only. The fractional sodium reabsorption was significantly less than sea-level values on day 1, when results from H and M rats were pooled at each exposure time. Sodium excretion did not differ between strains or among exposure times. However, the largest sodium excretion occurred on day 1 in both strains, which is consistent with the lower fractional sodium reabsorption at that time. A reduction in sodium reabsorption often occurs in the first 24–48 h of acclimatization to high altitude (18). The GFR was constant at all altitudes and similar in the two strains. The changes in renal excretory function are statistically significant but of small magnitude, and the work of the kidney was maintained during hypoxic exposure.

Effect of 10.5% inspired O$_2$ on systemic and renal tissue oxygenation in conscious H and M rats. Table 2 summarizes $P_{A\text{O}_2}$, renal venous $P_{O_2}$ (Prv$O_2$), Ca$O_2$, renal venous $O_2$ content (Crv$O_2$), renal V$O_2$, and COD measured or calculated in H and M rats at sea level and during hypoxic exposure. There were no strain differences in any of these variables at sea level. $P_{A\text{O}_2}$ fell precipitously at the onset of hypoxic exposure in both rat strains. $P_{A\text{O}_2}$ in the H rats decreased as the hypoxic exposure persisted, whereas $P_{A\text{O}_2}$ in the M rats re-
out the 30-day hypoxic period. In contrast, PrvO₂ rose the initial hypoxic exposure and remained low through-

mained relatively stable and even increased over the remainder of the hypoxic exposure. As a result, the mean value of PaO₂ was −41 Torr in the H rats and 48 Torr in the M rats at the end of 30 days. PrvO₂ was similar at sea level in H and M rats and fell at the onset of hypoxia. PrvO₂ values paralleled the arterial values similar at sea level in H and M rats and fell at the onset of hypoxia. PrvO₂ was similar at sea level in the two strains and fell at the onset of hypoxia. PrvO₂ rose in both strains, but the rise was more rapid in the M rats. Renal venous CO₂ exceeded the sea-level value on day 14 in M rats and on day 30 in H rats. Renal blood flow was a constant function of Hct in both strains and increased gradually as the hypoxic exposure progressed and Hct rose (C. D. Thron, J. Chen, J. C. Leiter, and L. C. Ou, unpublished observations). The renal COD, the product of renal blood flow and CaO₂, fell at the onset of hypoxic exposure but increased steadily as Hct rose. The renal COD did not differ between strains, and in both strains the renal COD was significantly less than the sea-level value on days 1 and 3 and greater than the sea-level value by day 30 of hypoxic exposure. The mechanism whereby equivalent renal O₂ delivery was maintained differed in the two strains: hemoglobin was greater and PaO₂ was lower in the H rats; the reverse was true in the M rats. Renal VO₂ was similar in H and M rats at sea level and at each particular altitude, but when pooled across all altitude conditions, renal VO₂ was significantly lower in the M rats (main effect of strain).

EPO levels increase during hypoxia (low PaO₂ and low tissue Po₂) and carbon monoxide exposure (normal PaO₂ but low tissue Po₂). Hence, the hypoxic sensor for EPO synthesis seems to respond to tissue PO₂ (23). PrvO₂ closely approximates renal tissue Po₂ (37), and values of EPO mRNA, renal EPO, and plasma EPO have been plotted as functions of PrvO₂ in Fig. 5. The EPO and PrvO₂ values were obtained from different sets below sea-level values in both strains, PrvO₂ values were greater in M than in H rats by day 14, and this persisted to the conclusion of the study. The hemoglobin concentration was similar in the strains at sea level, rose in both strains, and was significantly greater than the sea-level value from day 3 until the conclusion of the study. The increase in hemoglobin was greater in H rats from day 14 onward. CaO₂ fell in both strains at the onset of hypoxia but rose steadily until day 30, when CaO₂ had returned to the sea-level range. The restoration of CaO₂ to the sea-level range was achieved by different mechanisms in each strain, as discussed below. CrvO₂ was similar at sea level in the two strains and fell at the onset of hypoxia. CrvO₂ rose in both strains, but the rise was more rapid in the M rats. Renal venous CO₂ exceeded the sea-level value on day 14 in M rats and on day 30 in H rats. Renal blood flow was a constant function of Hct in both strains and increased gradually as the hypoxic exposure progressed and Hct rose (C. D. Thron, J. Chen, J. C. Leiter, and L. C. Ou, unpublished observations). The renal COD, the product of renal blood flow and CaO₂, fell at the onset of hypoxic exposure but increased steadily as Hct rose. The renal COD did not differ between strains, and in both strains the renal COD was significantly less than the sea-level value on days 1 and 3 and greater than the sea-level value by day 30 of hypoxic exposure. The mechanism whereby equivalent renal O₂ delivery was maintained differed in the two strains: hemoglobin was greater and PaO₂ was lower in the H rats; the reverse was true in the M rats. Renal VO₂ was similar in H and M rats at sea level and at each particular altitude, but when pooled across all altitude conditions, renal VO₂ was significantly lower in the M rats (main effect of strain).

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![Fig. 4. Changes in plasma (A) and renal tissue (B) EPO levels in H (○) and M (●) rats during acute and chronic exposure to a simulated altitude of 5,500 m. *P < 0.05 compared with M rats at same time point.](image)

Table 1. Parameters of renal function in rate at sea level and during hypoxia

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sea level</th>
<th>Hypoxia Day 1</th>
<th>Hypoxia Day 3</th>
<th>Hypoxia Day 14</th>
<th>Hypoxia Day 30</th>
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<td>H</td>
<td>M</td>
<td>H</td>
<td>M</td>
<td>H</td>
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<tr>
<td>Urine Output, µl·min⁻¹·100 g body wt⁻¹</td>
<td>7.4±3.0</td>
<td>9.1±5.1</td>
<td>142.4±1.2</td>
<td>142.8±0.9</td>
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<td>Plasma Na, meq/l</td>
<td>H</td>
<td>M</td>
<td>H</td>
<td>M</td>
<td>H</td>
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<tr>
<td>Excretion, µequiv·min⁻¹·100 g body wt⁻¹</td>
<td>0.6±0.2</td>
<td>0.8±0.4</td>
<td>99.4±0.2</td>
<td>99.5±0.1</td>
<td>1,025±130</td>
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<tr>
<td>Na Reabsorption, %</td>
<td>H</td>
<td>M</td>
<td>H</td>
<td>M</td>
<td>H</td>
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<td>GFR, µequiv·min⁻¹·100 g body wt⁻¹</td>
<td>H</td>
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Values are means ± SD; number in parentheses is number of rats. GFR, glomerular filtration rate. *Significant difference between strains at a particular time, P < 0.05. †Significantly different from sea-level control when values from H and M rats were pooled (a main effect of altitude and time), P < 0.05.
of animals studied under identical conditions at equivalent times. In all cases the curves were hyperbolic with a threshold between 40 and 50 Torr and an asymptote between 19 and 29 Torr. The hyperbolic equations were statistically significant, and, even for the worst fit, 58% of the variation in kidney EPO levels could be attributed to the variation in PrvO2.

Effect of 10.5% inspired O2 on ventilatory response in conscious H and M rats. To determine the cause of the accentuated hypoxemia in the H rats during hypoxic exposure, minute ventilation (Ve) at sea level and at a variety of times during the hypoxic exposure was measured in H and M rats. The results of measurements of Ve, PaO2, and arterial PCO2 (PaCO2) are displayed in Fig. 6. There were no differences in Ve and PaO2 in the two rat strains at sea level. Ve increased and PaCO2 decreased similarly in response to hypoxic exposure in the H and M rats, despite the marked strain differences in PaO2. Ve remained similar in the H and M rats as the H rats became more hypoxemic late in the hypoxic exposure period. However, we have never detected any differences in hypoxic or hypercapnic ventilatory responses at sea level or at any time during exposure to simulated high altitude (28). Furthermore, we have seen in previous studies greater ventilatory responses associated with more severe hypoxemia in the H than in the M rats after chronic exposure to hypoxia (7) and after monocrotaline treatment (5).

**DISCUSSION**

In the present study we examined the molecular and genetic mechanisms of polycythemic responses to hypoxia in H and M rats during chronic hypoxic exposure.

### Table 2. Arterial and renal blood-gas values in rats at sea level and during hypoxia

<table>
<thead>
<tr>
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<th>H</th>
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<th>Renal VO2, µmol min⁻¹ 100 g body wt⁻¹</th>
<th>Renal COD, µmol min⁻¹ 100 g body wt⁻¹</th>
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<tr>
<td><strong>Hb, g/dl</strong></td>
<td>14.9</td>
<td>14.4</td>
<td>14.9</td>
<td>14.4</td>
<td>14.9</td>
<td>14.4</td>
<td>14.9</td>
<td>14.4</td>
<td>9.0</td>
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<td>77</td>
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<td>80</td>
<td>77</td>
<td>39.7</td>
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<tr>
<td><strong>PrvO2, Torr</strong></td>
<td>51</td>
<td>48</td>
<td>51</td>
<td>48</td>
<td>51</td>
<td>48</td>
<td>51</td>
<td>48</td>
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<tr>
<td><strong>CaO2, ml/dl</strong></td>
<td>18.6</td>
<td>17.4</td>
<td>18.6</td>
<td>17.4</td>
<td>18.6</td>
<td>17.4</td>
<td>18.6</td>
<td>17.4</td>
<td>22.0</td>
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<tr>
<td><strong>CrvO2, ml/dl</strong></td>
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<td>9.8</td>
<td>10.8</td>
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<td>10.8</td>
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<td>10.8</td>
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| **Values are means ± SD; number in parentheses is number of rats. Arterial values are representative of systemic blood; venous values reflect renal venous blood. Hb, hemoglobin; PaO2 and PrvO2, arterial and renal venous PO2, respectively; CaO2 and CrvO2, arterial and renal venous O2 content, respectively; VO2, O2 consumption; COD, coefficient of O2 delivery (blood flow × CaO2). *Significantly different from control value of that strain, P ≤ 0.05. †Significant difference between strains at that particular time, P ≤ 0.05. ‡Significantly different from control value when values from both strains were pooled (a main effect of altitude), P ≤ 0.05. §M rats had a lower renal VO2 than H rats when values across all exposure times were pooled (a main effect of strain), P ≤ 0.05.**
and the factors thought to stimulate a polycythemic response to hypoxia: the balance between renal O₂ delivery and renal V˙O₂. Because hypoxic exposure results in development of CMS with excessive polycythemia in the H rats but elicits only moderate polycythemia and no apparent ill effects in the M rats, the present results provide a spectrum of hypoxic responses within which to analyze the mechanism(s) underlying the widely different polycythemic responses in healthy and diseased states at high altitude.

Molecular and genetic mechanisms of different polycythemic responses to hypoxia in H and M rats. The present study revealed, for the first time, the sequence of events, starting from enhanced expression of the EPO gene to elevated plasma and renal tissue EPO levels to, finally, the polycythemic responses in rats during chronic exposure to a simulated altitude of 5,500 m. The levels of EPO mRNA that accumulated with 10.5% inspired O₂, as employed in this study, could not be detected by Northern blot analysis but were readily detectable by a more sensitive RNase protection analysis (33). RNase assays are more sensitive than Northern blot analysis in this study, which might further reduce the sensitivity of the particular Northern blot analysis we performed. The exaggerated EPO production and excessive polycythemia, which developed in the H rats during hypoxic exposure, originated from an inordinate and sustained expression of the EPO gene in the kidney (28, 30). Because no EPO mRNA was detectable in liver, heart, or spleen by Northern blot or RNase protection assays under sea-level control and hypoxic conditions in both rat strains, the kidney must be the primary site of EPO production in the adult animals exposed to 10% inspired O₂. This conclusion is supported by the negligible increase in circulating EPO levels in nephrectomized rats of both strains when exposed to severe hypoxia (28). There were no strain differences in the rates of EPO degradation in these two rat strains under control and hypoxic conditions (28). Therefore, the rate of EPO gene expression in the kidney is the major factor determining the levels of EPO mRNA production and the polycythemic response to hypoxia in the H and M rats. This conclusion, however, is at variance with the work of Tan et al. (36). Using a similar RNase protection assay, these authors observed that EPO mRNA increased significantly in the liver and spleen in rats exposed to hypoxia. This discrepancy could be due to the difference in severity of the hypoxic stimulus used in the two studies: 7% O₂ was used in the study of Tan et al., whereas 10.5% O₂ was employed in the present study.

When the animals were exposed to severe hypoxia with or without cobalt treatment, circulating EPO and renal tissue EPO mRNA levels were elevated and similar in the H and M rats. M rats were capable of mounting an EPO response; there is no evidence that deficient EPO-generating capacity in the M rats accounted for the different EPO responses reported here. The different EPO responses in the two rat strains appear to be hypoxia specific. Treatment with cobalt, another erythropoietic stimulus, enhanced EPO gene expression and EPO production similarly in H and M rats. Moreover, elevated EPO mRNA and circulating EPO levels in the chronically hypoxic H rats fell abruptly to sea-level control values when the animals were brought down to sea-level conditions. Rather than strain-specific differences in EPO regulation, more severe renal tissue hypoxia in the H than in the M rats during equivalent hypoxic exposure seems to account for the divergent EPO responses observed in this study (Fig. 5, Table 2).

Regulation of EPO gene expression and EPO production. The physiological mechanism regulating EPO gene expression and EPO production remains incompletely understood. Renal tissue oxygenation is undoubtedly the single most important factor (12, 22). The balance between renal O₂ delivery and renal V˙O₂ determines the level of renal tissue oxygenation. Renal V˙O₂ depends on the work of the kidney, particularly sodium reabsorption, which is the primary energy-requiring renal process (22, 38). Inhibition of proximal sodium reabsorption attenuates the EPO response to hypoxia, presumably as a result of decreased renal work and
elevated renal tissue oxygenation, but the inhibition must be sufficient to prevent reabsorption of at least 20% of the filtered load of sodium (10, 11). In the present study, there were only minor changes in fractional sodium reabsorption and, in general, renal function was preserved without strain differences at sea level or under hypoxic conditions. Despite stable excretory renal function under control and hypoxic conditions, hypoxic exposure elicited distinctly different changes in EPO gene expression and EPO production in the two rat strains. The dissociation between measures of renal function and EPO production under hypoxic conditions renders unlikely any significant regulatory role of renal function in the EPO response to hypoxia.

Role of renal tissue hypoxia. Carbon monoxide and anemia stimulate EPO gene expression and EPO production in the absence of changes in PaO2; therefore, the renal hypoxia-sensing process is probably located near a venous or tissue site (20). The hypoxia-sensing process probably occurs adjacent to the EPO-generating sites in the kidney in adult mammals (22, 31). Because PrvO2 approximates the average renal tissue O2 level (37), PrvO2 is an estimate of the primary hypoxic signal determining EPO production. Renal O2 delivery and renal VO2 did not differ in the two rat strains under sea-level control or hypoxic conditions (Table 2). Systemic arterial hypoxemia was more severe in the H than in the M rats, and the severity of hypoxemia increased in the H rats, but not in the M rats, as the hypoxic exposure was prolonged (Fig. 6) (7, 28). Not surprisingly, PrvO2 was lower in the H than in the M rats after 2 wk of hypoxic exposure (Table 2) (28). There is no reason to believe that the EPO stimulus-response characteristics differ between the rat strains during chronic (>24 h) hypoxia: H rats simply experienced greater hypoxic stimulation during equivalent altitude exposure. Furthermore, there can be no effective polycythemic compensation for arterial hypoxemia in the H rats; the arterial values were below the threshold of the EPO response (Fig. 5). Thus more severe renal tissue hypoxia can account for the inordinate EPO gene expression and EPO production and, thereby, the excessive polycythemia in the H rats during chronic hypoxic exposure.

The greatest EPO gene expression in each strain occurred after 6 h of hypoxia. The magnitude of EPO mRNA and plasma EPO at 6 h exceeded expression at any other time within each strain, but the PaO2 values at 6 h were not the most hypoxic values. We have no PrvO2 values at 6 h to correlate with the intense EPO gene expression at 6 h. Therefore, it remains possible that hypoxia-EPO stimulus-response characteristics differ between the acute and chronic phases of the hypoxic exposure. Nevertheless, H rats are more hypoxic than M rats acutely (PaO2 = 44 ± 1 and 46 ± 1 Torr at 6 h in H and M rats, respectively; unpublished observations) and in the later stages of chronic hypoxia. Thus the relationship between EPO expression and hypoxia may differ acutely and chronically, but, in each time period, H rats, in which EPO expression was greater, were consistently more hypoxic than M rats. We have no evidence that the sensitivity of the O2 sensor for EPO expression differs between strains acutely or chronically.

More severe renal tissue hypoxia developed in the H rats during hypoxic exposure as a result of accentuated arterial hypoxemia. Accentuated hypoxemia in patients with CMS is believed to result from hypoventilation (27, 40), even though hypoventilation has not been observed consistently in patients with CMS (6). Impaired pulmonary gas exchange is found in patients with CMS and must contribute to systemic arterial hypoxemia (39). In this and previous studies (28, 30), accentuated hypoxemia in the H rats during hypoxic exposure also resulted from abnormal gas exchange. Ventilatory responses to a simulated altitude of 5,500 m were similar in the H and M rats; PaCO2 fell in response to hypoxic exposure to comparable levels in both rat strains (28), indicative of a comparable alveolar hyperventilation. Severe pulmonary hypertension, one of the characteristic signs of CMS in humans and rats, is associated with vascular remodeling (25) and may impair gas exchange between the alveoli and small pulmonary arteries and capillaries, worsening arterial hypoxemia. Suppression of the development of pulmonary hypertension in the H rats during hypoxic exposure was associated with an increased PaO2 and an attenuated polycythemic response without any change in ventilation (8). In murine CMS, accentuated hypoxemia follows the development of pathologically elevated pulmonary arterial pressures.

Severe blood hyperviscosity associated with excessive polycythemia did not compromise renal blood flow or renal VO2 delivery during hypoxia in H rats. Contrary to the belief that polycythemia might reduce renal blood flow and O2 delivery (13), renal blood flow actually increased during hypoxic exposure as Hct rose and blood viscosity increased (unpublished observations). As a result of increased renal blood flow and increased O2 content associated with the polycythemic response, renal O2 delivery increased beyond the control levels in both rat strains after chronic hypoxia. Nevertheless, renal tissue hypoxia and EPO gene expression persisted in H rats.

Role of negative-feedback control. The early finding that a humoral erythropoiesis-stimulating factor (EPO) increased rapidly on hypoxic exposure and declined toward control levels led to the notion of a negative-feedback control mechanism regulating erythropoiesis. According to this concept, hypoxia stimulates EPO production and erythropoiesis; the resulting polycythemia increases the O2-carrying capacity and O2 content of blood, and this improves tissue oxygenation and turns off further production of EPO (14). The idea of EPO-O2-carrying capacity feedback is inherent in all EPO dose-response curves published: the Hct is plotted as the independent variable (14, 23). Nevertheless, EPO measurements using sensitive radioimmunoassay for EPO and studies of EPO mRNA levels have made it clear that a negative-feedback control mechanism does not account for the regulation of the polycythemic
response to environmental hypoxia (3, 9). The high levels of renal EPO mRNA and circulating EPO apparent at the onset of hypoxic exposure declined within the first 24 h of hypoxia before any detectable polycythemia or increased $O_2$-carrying capacity developed in this and other studies (9). The rapid decline in EPO mRNA during continued hypoxic exposure in the kidney was also unrelated to the rising circulating EPO levels; there is no evidence of direct negative feedback in which EPO might inhibit its own production (9). Furthermore, evidence obtained in the present study does not support the concept of a negative-feedback mechanism, in which a polycythemic response to hypoxia suppresses EPO production. Rather, the data in Fig. 5 indicate that feedback control of EPO gene expression and EPO production operates in terms of PrvO$_2$, and polycythemia is only one of multiple factors modifying PrvO$_2$. The pattern of EPO production was biphasic. At the onset of the hypoxic exposure, there was a sharp increase in EPO production, but in $<$24 h, EPO levels fell substantially but remained above control values. EPO levels rose slowly in the late stages of the hypoxic exposure in the H rats. PrvO$_2$ follows a similar pattern: an initial sharp drop, a rapid recovery to a stable level in M and H rats, and a slow decline after 2 wk of continued hypoxia in H rats (Table 2) (28). The parallel pattern of changes in PrvO$_2$ and EPO levels during hypoxia is consistent with feedback control of EPO synthesis and release as a function of PrvO$_2$. Polycythemia modifies PrvO$_2$ only in M rats after 2 wk of hypoxia. The early changes in PrvO$_2$ reflect ventilatory acclimatization, acute cardiovascular responses to hypoxia, and perhaps some benefit from the hemoconcentration after diuresis in the first days of acclimatization to high altitude (Table 2) (18). Only the late increase in PrvO$_2$ in M rats demonstrates the beneficial effect of polycythemia. In contrast, the PrvO$_2$ remained low in the H strain. This was not due to a lack of $O_2$-carrying capacity (Hct rose above 70%) or low renal blood flow (renal COD was equivalent in the H and M rats and exceeded the sea-level value by day 30 of the hypoxic exposure) but to a low PaO$_2$. Thus renal hypoxic stimulation persists when arterial hypoxemia is below the threshold of the EPO response and, therefore, beyond the capacity of polycythemia to restore renal tissue oxygenation to suprathereshold levels. The accentuated hypoxemia of the H rats, which originates in abnormal pulmonary vascular responses to hypoxia and associated gas exchange abnormalities, creates a situation in which polycythemia develops but has no significant effect on the level of EPO stimulation. Consequently, the signs of CMS develop: profound arterial hypoxemia, persistent production of EPO, and extraordinary polycythemia.

The EPO-PrvO$_2$ response curves (Fig. 5), whether in terms of mRNA, renal EPO, or plasma EPO, have a shape resembling the ventilatory response to hypoxia and the pattern of carotid body discharge in response to hypoxia (24): there is little response until a threshold is reached, and below the threshold the response, ventilation or sinus nerve discharge or EPO production, rises steeply. The mechanism whereby declining $O_2$ levels are sensed and transformed into ventilatory and EPO responses is unknown, but it is conceivable that the renal and carotid sensors share a common mechanism. For example, both may rely on heme proteins (1, 17, 26).

In summary, the present study examined the genetic regulatory mechanisms as well as the physiological significance of the polycythemic response to environmental hypoxia in two rat strains with distinctly different susceptibility to CMS. The results suggest that the polycythemia of CMS derives from pulmonary abnormalities of gas exchange, vascular remodeling, and more severe arterial hypoxemia in the H rats rather than different patterns of EPO synthesis and release to a similar stimulus. EPO levels were appropriate for the levels of renal tissue hypoxia, but PaO$_2$ and PrvO$_2$ were below the EPO response threshold in the H rats, and no polycythemic response can restore renal tissue oxygenation in that setting.

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REFERENCES


