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Hypoxia-Stimulated Erythropoietin Secretion in Mice with Different Types of Induced Polycythemia: The Posthypoxic Enigma

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Abstract

Erythropoietin (EPO) is a hormone that is part of a feedback system that adjust the volume of the red cell mass (RCM) to tissue oxygen demands. Increased plasma EPO concentration (pcEPO) accompanies reduced oxygen supply or increased demands, whereas low levels of pcEPO are observed with increased oxygen supply or reduced demands. Therefore, the increment of RCM induced by transfusion (HT mouse) will enhance oxygen supply to tissues and depress EPO secretion when they are subjected to hypobaric hypoxia (HH, a potent stimulus for EPO secretion). When mice made polycythemic by sustained exposure to HH (PH mouse) are re-exposed to the stimulus after a brief period at sea level condition, they react synthesizing EPO as do normocythemic mice. This study was designed to compare HH-stimulated EPO production in mice in which polycythemia was induced by different maneuvers

(exposure to HH [6350 m]) for 2 wks, transfusion of homologous erythrocytes (0.8 ml of packed erythrocytes ip), sustained exposure to air containing 0.06% CO, sustained administration of rHu-EPO (5.5 IU day/2wk) and repeated injections of phenylhydrazyne (60 mg/Kg/3 wk) followed by transfusion. After treatments were completed, all animals were exposed to 337 mmHg for 6 h. Blood collected by cardiac puncture and pcEPO measured by immunoassay (R&D systems). pcEPO was significantly elevated in normocythemic and PH mice in relation to non-exposed controls, whereas pcEPO was not increased in response to HH in the remaining groups. In another experiment, mice were exposed for 2 wk to different simulated altitudes (0, 2500, 3600, 4600, 5500 and 7300 m). They were re-exposed to HH for 6 hr. Linear regression analysis between the variable X (simulated high altitude) and the variable Y (pcEPO) showed a slope of 563.4 ± 116.1 pg/1000m and an r^2 = 0.887. Data indicate that the enhancement of EPO secretion in response to the hypoxic challenge in polycythemic mice only occurs in mice that have been previously exposed to hypoxia. However, the operating mechanism of hypoxia in this state of EPO hypersecretion remains as an open question.

Keywords: erythropoietin – hypoxia – simulated high altitude – polycythemia – carbon monoxide - phenylhydrazyne

INTRODUCTION

Red blood cells (RBC) have both a finite life span and incapacity for cell renewal. Therefore, they are continuously formed in the erythropoietic organs through a continuous cellular renewal system: this physiological function is called *erythropoiesis*, which was designed to maintain a normal number of RBC in the circulating blood. The circulating RBC form an organ, the *red cell mass* (RCM) or *circulating erythron*, which contributes to the convective oxygen transport that occurs in the circulatory system. Erythropoietin (EPO) is a hormone mainly secreted by the kidneys that is part of a feedback system that has evolved to adjust the volume of the RCM to the tissue oxygen demands (Jelkmann, 1992; Koury, 2005). It is accepted as a regulatory principle that increased levels of plasma EPO concentration (pcEPO) accompany reduced oxygen supply or increased oxygen demands, whereas low pcEPO are typical for conditions with increased oxygen supply or reduced oxygen demands (Fried et al., 1957).

In accordance with this theory, an artificial increase in the circulating RCM after transfusion of red cells will increase oxygen supply to tissues and depress

erythropoiesis through reduced EPO production (Gurney and Pan, 1958) until the "excess" of red cells have been lost by senescence. Therefore, the RCM will attain again a normal value as the consequence of disappearance of senescent RBC in the absence of a significant replacement. This condition, called *transfusion polycythemia*, is easily reproduced in the laboratory in which mice are transfused intraperitoneally with homologous (Jacobson et al., 1957) or heterologous red cells (Conti et al., 1999). In this model, Conti et al. (1999) have shown that transfer of ^{59}Fe-labeled red cells from the peritoneal cavity to the blood compartment begins immediately and proceeds as an uninterrupted fashion. Maximal appearance of radioactivity is seen 48 h after injection when 99.2% of the injected radioactivity is in the circulating blood. This transfer of erythrocytes is responsible for the increase in the hematocrit value after transfusion. Both radioactivity and hematocrit start to decline after 48 h with a t½ of 141 h. During this period, splenic erythropoiesis (taken as an expression of the rate of erythropoiesis, Bozzini et al., 1970) is highly depressed in concordance with a drastic reduction of pcEPO.

When mice are transfused with increasing volumes of RBC to obtain different degrees of polycythemia and are then exposed to hypobaric air (hypoxic hypoxia), a potent stimulus for EPO synthesis, the production of EPO is inversely related to the hematocrit value attained by the animals in response to the volume of cells transfused. Thus, this type of polycythemia does not inhibit hypoxia-stimulated EPO secretion in an all or none fashion (Alippi et al., 1983), being highly correlated to the volume of the RCM. This model has been called "*hypertransfused polycythemic mouse*" (*HT mouse*).

Polycythemia can also be induced in mice by chronically exposing them to air maintained at low atmospheric pressure in a simulated high altitude (SHA) chamber (Cotes & Bangham, 1961). EPO production is then marked stimulated during the first days of exposure, declining thereafter, which induces an enhancement in the rate of erythropoiesis and an absolute increase of the RCM (Bozzini et al., 1994). When hypoxia-stimulated mice are removed from the SHA chamber at the end of the exposure period, they are polycythemic. They are then confronted with normobaric air, being the polycythemic state now unnecessary. The erythropoiesis control system senses then the excess of RBC and stops their production, until the excess is eliminated from the circulation by senescence without a significant replacement. This model has been called "*post-hypoxic polycythemic mouse*" (*PH mouse*).

When PH mice are re-exposed to hypobaric air after a brief period at normobaric air (24 h), they react synthesizing EPO in a magnitude that, in general, is not significantly different from that of normocythemic control mice similarly exposed. In other words, PH mice seem to ignore their polycythemic state (Alippi et al., 1983a; Alippi et al., 1983b; Bozzini et al., 1994).

The different hypoxia-stimulated EPO production demonstrated in HT and PH mice explains the higher response of the latter to stimuli that increase erythropoiesis through increasing EPO secretion, namely testosterone, dexamethasone, isoproterenol (Alippi et al., 1985) or cobaltous chloride (Alippi et al., 1992).

The different EPO response to hypoxia between HT and PH mice, both being equally polycythemic, has been confirmed by Erslev and Caro (1987). It has not been adequately explained in spite of the intense investigation performed during several years. Since several other experimental methods are available to induce polycythemia in mice that operates through different mechanisms, the present investigation was designed to compare hypoxia-stimulated EPO production *(hypoxic challenge)* in mice in which polycythemia was induced by different maneuvers in an attempt to find a model that will reproduce the response of the PH mice to re-exposure to hypoxia and thus obtain some explanation for its paradoxical response.

MATERIALS AND METHODS

Adult female mice of the CF_1 strain weighing 23-26 g were used throughout the experiments. They were maintained under standard vivarium conditions in groups of 10 animals each and fed a pelleted diet during the course of the experiments. The total blood volume was determined by the dilution of in-vivo ^{59}Fe-tagged erythrocytes (Berlin et al., 1949). The volume of the circulating RCM was calculated by multiplying the blood volume by the hematocrit which was determined by micromethod. Hypobaric hypoxia was induced by placing animals into a SHA chamber in which the air pressure was kept at the desired level using a continuous vacuum pump and an adjustable inflow valve. Plasma EPO levels (pcEPO) were estimated by using a commercial monoclonal-based, two-site enzyme immunoassay (Quantikine for mouse/rat Erythropoietin, R&D Systems). The induction of transfusion polycythemia was performed by injecting mice intraperitoneally with the desired volume of homologous washed packed red cells obtained from donor mice. Carbon monoxide (CO)-induced hypoxia was performed by exposure animals to CO at normal atmospheric pressure (Fogh, 1966). Mice were maintained in a Plexiglas 93-dm^3 chamber that permitted the inflow of air containing 0.06% CO. Carbon dioxide and humidity were removed from the air inside the chamber by soda lime and calcium chloride, respectively. Air flow rate was continuously maintained in 0.8 l/min. EPO (Human Recombinant Erythropoietin, Hemax, Bio Sidus, Argentina, 4,000 IU/ml) was dissolved in PBS-HAS (1 mg/ml) and injected subcutaneously. A

hemolytic state was induced by repeated subcutaneous injections of 60 mg/kg of phenylhydrazyne (Erslev and Silver, 1975).
Data were analyzed by one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls Multiple Comparison Test. Regression analysis was performed through a 1999 GraphPad Software (GraphPad Prism V.3). *p*-values equal or less than 0.05 were considered significant. This protocol was reviewed and Approved by the Institutional Ethical Review Committee.

RESULTS

Experiment # 1: Polycythemia induced in mice by transfusion, exposure to sustained hypobaric hypoxia, or chronic EPO administration. Effect on hypoxia-stimulated EPO secretion (hypoxic challenge)
Increasing degrees of polycythemia were induced in groups of female normal mice by 1) intraperitoneal injection of 0.2, 0.4, 0.6, 0.8, 1.2, or 1.8 ml of packed red cells (*HT mice*); 2) intermittent (22 h/d) exposure to air maintained at 525, 450, or 380 mmHg, corresponding approximately to simulated altitudes of 3000, 4200 and 5500 m, respectively, for 2 wk (*PH mice*); and 3) daily injections of 1.0, 2.0, 4.0, 8.0 or 16.0 IU of rHu-EPO for 2 wk (*EPO mice*). Animals from groups 2 and 3 received 2 mg iron dextran to assure enough iron stores for the development of polycythemia. The volume of the circulating RCM was determined 48 h after treatments. Figure 1 (A, B and C) show the results of linear regression analysis between the variables X (volume of red cells transfused, simulated high altitude, or doses of rHu-EPO administered) and the variable Y (volume of the RCM). By reading unknowns from the standard curves, it was estimated that the transfusion of 0.44 ml of packed red cells, the exposure to a simulated altitude of 3740 m, or the administration of 1.19 IU/d of rHu-EPO, increased by 30% the volume of the RCM. The corresponding values necessary to expand the RCM by 80% were 0.8 ml, 6350 m, and 5.55 IU. Polycythemic mice, showing a RCM expanded by 80%, were then established by using the procedures described above. They were exposed to hypobaric air (337 mmHg) for 6 h (*hypoxic challenge*), 48 h later. Blood was collected by cardiac puncture under ether anesthesia, microhematocrits were prepared, and plasma was obtained by centrifugation in a refrigerated centrifuge for determination of pcEPO. Results are shown in Figure 2, in which pcEPO in normoxic (black bars) and hypoxic mice (white bars) are compared. Four observations were relevant: 1) exposure to hypoxia increased pcEPO 7.6 times in normocythemic mice when compared to non-exposed ones (*C mice*); 2) polycythemia, induced either by transfusion, EPO administration, or exposure to hypoxia, depressed pcEPO under normobaric

conditions (C vs HT, EPO, and PH mice); 3) polycythemia, induced either by transfusion or EPO administration, blocked EPO secretion in response to the hypoxic challenge; and 4) hypoxia-stimulated EPO secretion in mice made polycythemic by sustained exposure to hypoxia (*PH mice*), although about 20% lower than that found in normocythemic mice similarly exposed (*C mice*), was significantly higher ($p < 0.001$, 15x) than the values found in mice made polycythemic by transfusion (HT) or EPO treatment (EPO).

Experiment # 2: Hypoxia-stimulated EPO secretion in transfused-polycythemic mice previously subjected to stress erythropoiesis elicited by either pharmacologically-induced hemolytic state or exposed to CO hypoxia

Stress erythropoiesis was induced in female mice by either exposure to air containing 0.06 % CO at normal atmospheric pressure or administration of phenylhydrazyne (PHZ). In the first case, animals were placed in the atmospheric chamber for a two-week period. The percent carboxyhemoglobin (COHb) was 1.73 ± 0.05 in normal and 25.7 ± 0.59 in exposed mice, respectively ($p < 0.001$), at the end of the exposure period. Red cell parameters in normal and exposed mice, that reflect the induction of stress erythropoiesis, were, respectively, as follows: 1) Hematocrit: 43.67 ± 0.67 vs 80.33 ± 0.69 % ($p < 0.001$), 2) Hemoglobin: 14.55 ± 0.22 vs 26.76 ± 0.42 g/dl ($p < 0.001$), and 3) Red cell mass: 2.77 ± 0.08 vs 9.45 ± 0.25 ml/100 g b. wt ($p < 0.001$). In the second case, a compensated hemolytic state (CHS) was induced by repeated injections (2/wk) of PHZ during a three-week period. The evidence for the establishment of a CHS was obtained by hematocrit and reticulocyte count determinations. The hematocrit achieved its lowest value on day 2 after the initial injection of PHZ. It then started to increase and returned to its pretreatment level by the second week of treatment. The reticulocyte count of PHZ-treated mice was elevated through the 3-week injection period. Mice were hypertransfused at the end of the PHZ-treatment period. They were exposed to the hypoxic challenge 48 h later. Blood was collected by cardiac puncture under ether anesthesia, microhematocrits were prepared, and plasma was obtained by centrifugation in a refrigerated centrifuge for determination of pcEPO. Results are shown in Figure 3, in which pcEPO titers in normoxic (black bars) and hypoxic mice (white bars) are compared. Three observations were relevant: 1) exposure to hypoxia increased pcEPO 5.5 times in normocythemic mice when compared to non-exposed ones (*C mice*); 2) both transfusion- and CO-induced polycythemia depressed pcEPO under normobaric conditions. However, values did not reach statistical significance; and 3) polycythemia, induced either by transfusion in previously erythropoietically PHZ-stimulated mice or by CO exposure, blocked EPO secretion in response to hypoxia.

Experiment # 3: Hypoxia-stimulated EPO secretion in post-hypoxic polycythemic mice (PH mice) previously exposed to different simulated high altitude levels

Groups of mice were intermittently (22 h/d) exposed for 2 wks (from Monday of week 1 to Friday of week 2) to air maintained at 1.00, 0.74, 0.64, 0.54, 0.50, or 0.37 torr in SHA chambers, which correspond approximately to altitudes of 0, 2500, 3600, 4600, 5500 and 7300 m, respectively. Seventy two hours later, they were re-exposed to air maintained at 0.40 atm. for 6 h (hypoxic challenge) in order to estimate hypoxia-induced EPO production. A group of hypertransfused (HT) mice were also included; these mice were transfused 72 h before the hypoxic challenge. Blood was collected by cardiac puncture under ether anesthesia immediately after the end of the hypoxic stimulation. After preparation of microhematocrits, plasma was separated for pcEPO determination. Results of this experiment are graphically presented in Fig. 4, left, in which pcEPO in normoxic (black bars) and hypoxic (white bars) mice are compared. The following observations were worthnoting: 1) exposure to hypoxia increased pcEPO 15.2 times in normocythemic mice when compared to non-exposed ones (*C0 white* vs *C0 black*); 2) polycythemia induced by transfusion depressed pcEPO under normobaric (*C0 black* vs *T0 black*) and hypobaric (*C0 white* vs *T0 white*) conditions; and 3) previous 2-wk exposure to increased simulated high altitudes induced a progressive enhancement of the EPO response to hypoxia, that reached significance when the altitude was 5,500 m or more. In these cases, pcEPO in response to the hypoxic challenge was not significantly different from the value observed in normocythemic mice. Fig. 4 right shows the results of linear regression analysis between the variable X (simulated high altitude) and the variable Y (pcEPO) in response to the hypoxic challenge, which showed a slope of 563.4 ± 116.1 pg/1000m and an $r^2 = 0.8870$.

DISCUSSION

Hypoxic induction of EPO synthesis occurs after activation of the hypoxia *inducible factor* (HIF) *transcriptional control system* (Semenza and Wang, 1992; Wang and Semenza, 1995; Wenger, 2000; Wiesener et al., 2003). HIF consists of a heterodimer of α- and β-subunits. The latter is widely distributed and constitutively expressed, whereas the α-subunit is inversely controlled by cellular oxygen supply. Lack of oxygen leads to HIFα stabilization and nuclear accumulation-. HIF-1 has the ability to bind to a hypoxia-response element in the 3'flanking region of the EPO gene (Wang and Semenza, 1995). It could thus be inferred that pcEPO could be taken as a good marker of the degree of tissue hypoxia during exposure of animals or humans to high altitude. EPO concentration in the plasma compartment depends on the balance between EPO formation and EPO disappearance rates. Studies performed

in mice under different physiological conditions strongly suggest that the clearance rate of EPO from the plasma compartment, at least in this species, is not subject to physiological regulation and that pcEPO can be taken as a reflection of the EPO production rate (Lezón et al., 1998). pcEPO can thus be considered as the expression of both the degree of tissue hypoxia and the magnitude of hormone synthesis and secretion.

Polycythemia could be defined as a state of increased RCM and hemoglobin mass. It has been experimentally proved (Bozzini et al., 2005) that it is highly effective in ameliorating tissue hypoxia under SHA conditions, thus giving support to the concept of the important role of the increased hemoglobin mass in nongenetically adapted animals to confer a good degree of adaptation to altitude. This type of polycythemia can be considered as *necessary* because it develops in response to conditions of hypoxia. Other types of polycythemia can be considered as *unnecessary*, when occurs under pathological or experimental conditions not related to hypoxia.

Mice of the different groups in the present study were polycythemic at the time of the *hypoxic challenge* (6-hour exposure to hypobaric air). Some of them showed a *necessary* type of polycythemia, whereas others showed an *unnecessary* one. Independent of the type of polycythemia, all groups of mice showed an almost suppressed rate of erythropoiesis when maintained at sea level atmospheric conditions, immediately after the end of treatments to induce the polycythemic state, and before the hypoxic challenge. Care was taken to have all mice with approximately equal degrees of polycythemia. Under these conditions, EPO formation in response to the *hypoxic challenge* should have been inhibited (Alippi et al., 1963a). This inhibition was observed in all studied groups with the exception of the PH-one, as expected.

The reasons why EPO formation in response to the hypoxic challenge is higher in mice that have been previously exposed to hypobaric hypoxia than in the other groups of polycythemic mice are not apparent. The similitudes and differences between the different methods used to induce the various polycythemic states could partially give some explanation. If we analyze some of the effects of treatments imposed to the experimental mice before the hypoxic challenge, we will observe: 1) that PH-, CO-, and PHz-mice were hypoxic; however, PH-mice suffered from hypoxic hypoxia, while CO- and PHZ suffered from anemic hypoxia; 2) that all of these groups, plus the EPO-one, showed a period of an increased rate of erythropoiesis; 3) that PH-, CO- and PHZ-mice showed a period of increased EPO production; and 4) that only HT-mice were normal during the time in which all other groups were treated.

Therefore, the different EPO response to hypoxia that exists between PH and HT mice is not apparently due to the fact that the former was previously erythropoietically stimulated because of a supernormal production of EPO. Data suggest that the previous exposure to hypobaric hypoxia seems to be the difference between PH and HT mice that is responsible for the higher EPO formation seen in the former than in the latter in response to the hypoxic challenge. Interestingly, the effect of previous exposure to SHA is altitude-dependent.

Interestingly, a human correlate appears to exist for the PH mouse (Smith, 2002). When subjects are exposed to acute hypoxia, they react with increased EPO secretion during the first few days. This response decreases within 1 to 2 weeks of continuous exposure to hypoxia. When the hypoxic stimulus is interrupted by only 3 d, the sensitivity of the system completely recovers even after more than 20 yr of intermittent hypoxia. Therefore, post hypoxic men, like post hypoxic mice, increase EPO production when they are re-exposed to hypoxia in spite of the polycythemic state.

The molecular mechanism involucrated in this phenomenon remains as an open question. Whether the changing sensitivity in the EPO formation system is related to changing sensitivity of the stabilization process of HIF-1α remains to be investigated.

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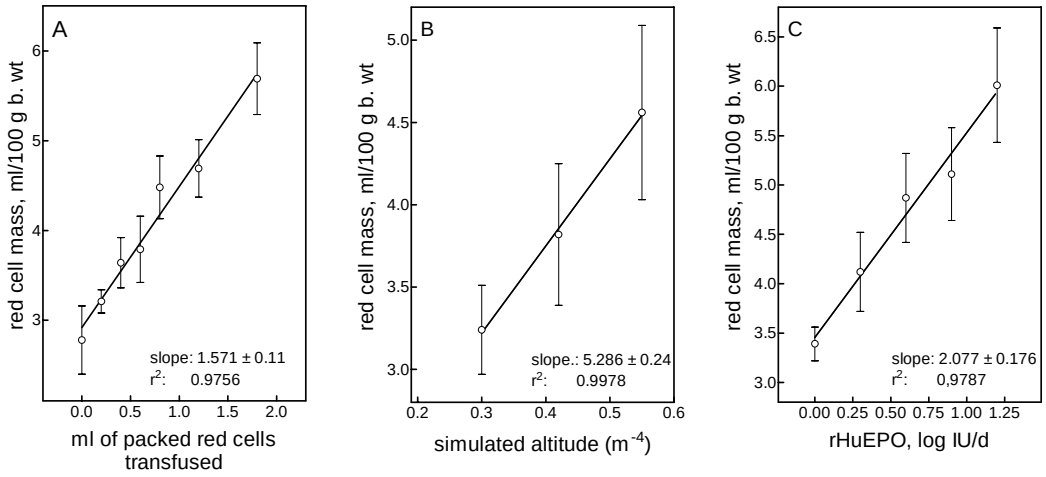
A
red cell mass, ml/100 g b. wt
slope: 1.571 ± 0.11
r2: 0.9756
ml of packed red cells transfused
B
red cell mass, ml/100 g b. wt
slope.: 5.286 ± 0.24
r2: 0.9978
simulated altitude (m-4)
C
red cell mass, ml/100 g b. wt
slope: 2.077 ± 0.176
r2: 0,9787
rHuEPO, log IU/d

FIGURE 1

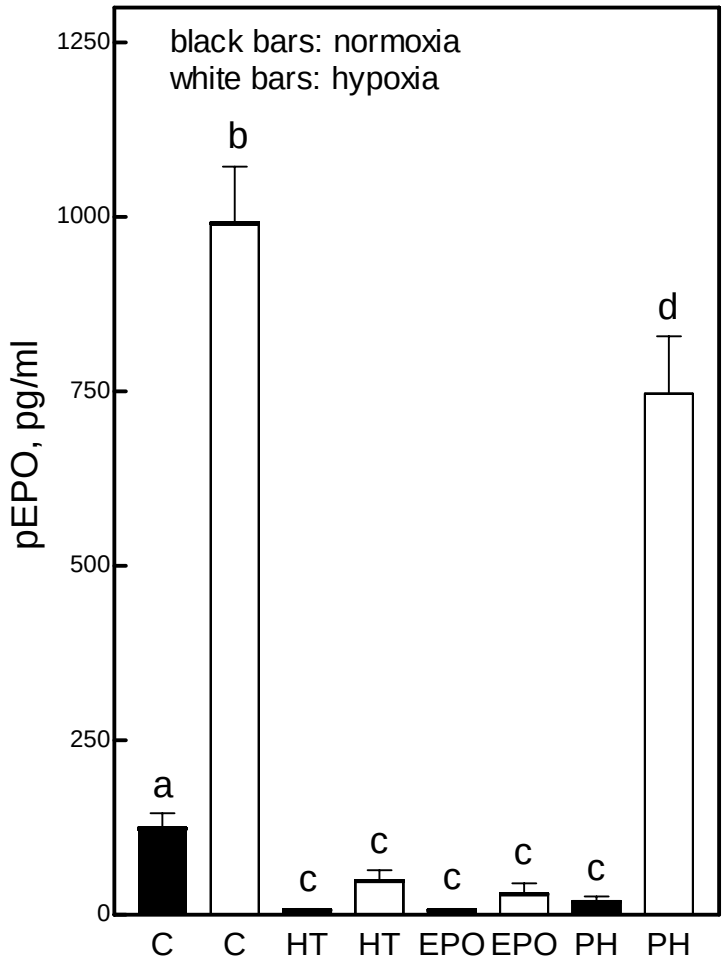
black bars: normoxia
white bars: hypoxia
pEPO, pg/ml
1250
1000
750
500
250
0
a
b
c
c
c
c
c
d
C
C
HT
HT
EPO
EPO
PH
PH

FIGURE 2

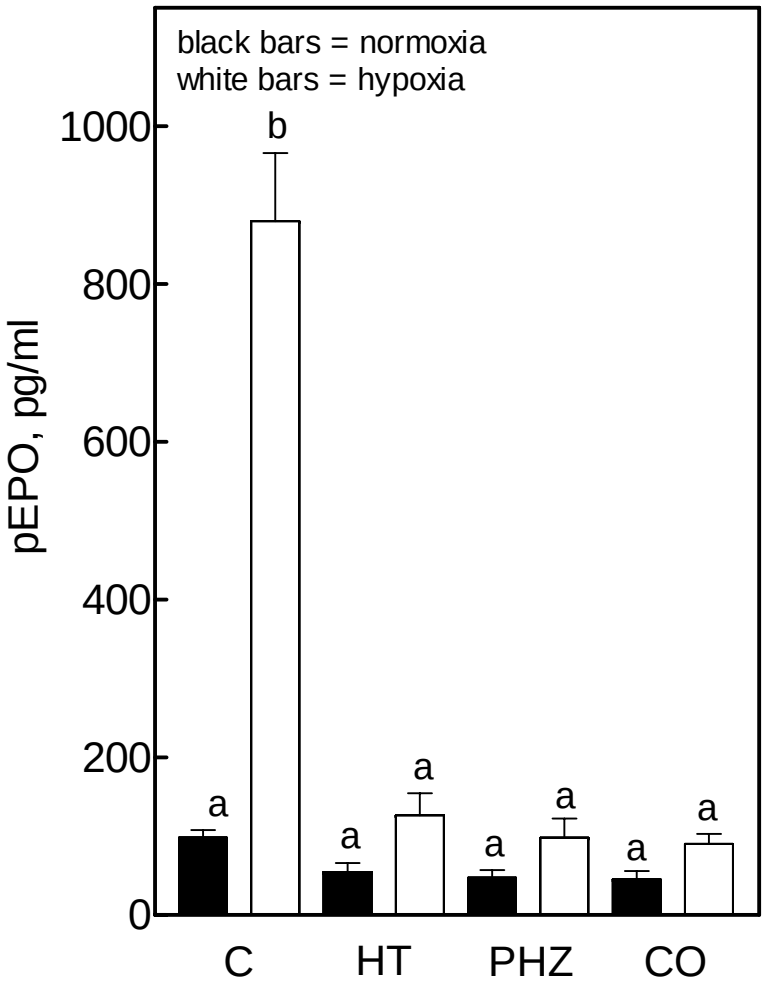
black bars = normoxia
white bars = hypoxia
pEPO, pg/ml
1000
800
600
400
200
0
b
a
a
a
a
a
a
a
C
HT
PHZ
CO

FIGURE 3

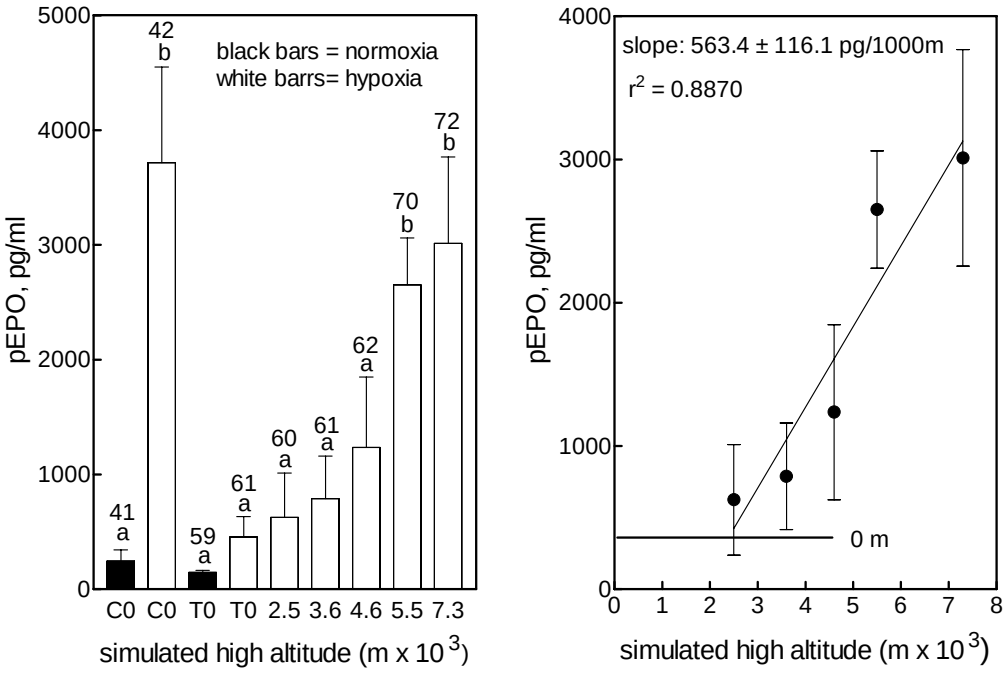
5000
4000
3000
2000
1000
0
pEPO, pg/ml
42
b
black bars = normoxia
white barrs= hypoxia
72
b
70
b
62
a
61
a
60
a
61
a
41
a
59
a
C0 C0 T0 T0 2.5 3.6 4.6 5.5 7.3
simulated high altitude (m x 10 3)
4000
3000
2000
1000
0
pEPO, pg/ml
slope: 563.4 ± 116.1 pg/1000m
r2 = 0.8870
0 m
0 1 2 3 4 5 6 7 8
simulated high altitude (m x 10 3)

FIGURE 4

LEGENDS FOR FIGURES

FIGURE 1: Linear regression analysis between the variables X [volume of red cell transfused (A), simulated high altitude (B), doses of rHu-EPO administered (C)] and the variable Y (volume of the RCM).

FIGURE 2: Plasma EPO concentration in normoxic (black bars) and hypoxic mice (white bars). Hypoxia was induced by 6-hour exposure to hypobaric air (337 Torr) in a simulated altitude chamber. Mice were normocythemic (C) or polycythemic (RCM expanded by 80%). Polycythemia was induced by transfusion of 0.8 ml of packed red cells (HT), by 2-week exposure to a simulated altitude of 6350 m (PH), or by daily injections of 5.55 IU of rHuEPO for 2 weeks (EPO). Each bar represents the Mean ± SD of 10 mice. Different letters at the top of bars denote statistical significant difference.

FIGURE 3: Plasma EPO concentration in normoxic (black bars) and hypoxic mice (white bars). Hypoxia was induced by 6-hour exposure to hypobaric air (337 Torr) in a simulated altitude chamber Mice were normocythemic (C) or polycythemic (HT, PHZ and CO). PHZ mice were hypertransfused at the end of a 3-week period of administration of phenylhydrazyne to induce a compensated hemolytic state. They were exposed to the hypoxic challenge 48 h later. CO mice were exposed to air containing 0.06% CO for 2 weeks for the induction of polycythemia. Each bar represents the Mean ± SD of 10 mice. Different letters at the top of bars denote statistical significant difference.

FIGURE 4: RIGTH: Plasma EPO concentration in normoxic (black bars) and hypoxic (white bars) mice. Hypoxia was induced by 6-hour exposure to hypobaric air (337 Torr). Mice were normocythemic (C) or polycythemic. Polycythemia was induced in T mice by transfusion. It was induced in the remaining groups by exposure to hypobaric air. The simulated high altitudes of the exposure for the different groups are shown in the abscissa. Each bar represents the Mean ± SD of 10 mice. Different letters at the top of bars denote statistical significant difference. Numbers on the top of bars indicate the average hematocrit. LEFT: Linear regression analysis between the variable X (simulated high altitude) and the variable Y (pcEPO) in response to the hypoxic challenge.

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