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Unexpected hypoxia-dependent erythropoietin secretion during experimental conditions not affecting tissue oxygen supply/demand ratio

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Unexpected hypoxia-dependent erythropoietin secretion during experimental conditions not affecting tissue oxygen supply/demand ratio. Although a great deal of evidence supports the hypothesis that plasma erythropoietin (EPO) levels of mammals are related to the oxygen supply to the tissues relative to their oxygen needs, several observations millitate against its inherent simplicity. This study presents our results obtained from *in vivo* experiments that suggest that hypoxia-dependent EPO production can be altered by conditions which apparently do not modify the tissue oxygen supply/demand ratio. Hypoxia-dependent EPO production rate (EPO-PR), derived from plasma EPO titers and plasma EPO half-lives, were estimated in both transfused-polycythemic and normocythemic mouse models subjected to different treatments. From calculations of the O_2 carrying capacity of blood and body O_2 consumption, it was assumed that the tissue supply/demand ratios were similar in both experimental and control mice of the same model at the time of induction of EPO production. The following observations were worth noting: (1) EPO-PRs in transfused polycythemic mice whose erythropoietic rates were stimulated by intermittent exposure to hypobaria (0.5 atm, 18 hr/day × 3 weeks), phenylhydrazine administration (40 mg/kg at weekly intervals × 3 weeks) or repeated rh-EPO injections (1500 U/kg 3 times a week × 3 weeks) before transfusion were more than five times higher than in comparably polycythemic mice whose erythropoietic rates were not stimulated previously; and (2) EPO-PR in response to hypobaric hypoxia was 2.08 times normal in normocythemic mice with cyclophosphamide (100 mg/kg) induced depression of erythropoiesis, and 0.33 times normal in normocythemic mice with rh-EPO (400 U/kg × 2) induced enhancement of erythropoiesis. Although the results obtained in polycythemic mice are difficult to explain, those from normocythemic mice suggest the existence of a feedback mechanism between EPO-responsive cells and EPO-producing cells. Both demonstrate the existence of experimental conditions in which modulation of the hypoxia-dependent expression of the EPO gene appears to occur. This modulation would be dependent on factors other than oxygen.

Erythropoietin (EPO) is a 30.4 kDa glycoprotein that appears to be the key regulator of red cell production [1]. It is mainly synthesized by endocrine renal cells through the expression of an EPO gene apparently in response to transcription-regulating factors. Studies conducted on hepatoma cells have shown that one of these factors appears to be constitutively expressed, which explain the presence of immuno-EPO in the plasma of mice made severily polycythemic by hypertransfusion [2]. Another transcription-regulating factor was named hypoxia-inducible factor 1 (HIF-1) [3]. It is only present in extracts from hypoxic cells and may be responsible for the hypoxia-dependent expression of the EPO gene.

According to the old hypothesis of Fried et al [4], that EPO synthesis depends on the convective oxygen supply to tissues relative to their oxygen needs, it seems evident that the EPO synthesis rate is negatively correlated to oxygen availability, namely tissue pO_2. A structure, possibly a hemoprotein, has thus been proposed that senses the oxygen tension and initiates a signal that turns on the expression of the EPO gene [5, 6].

Although Fried's hypothesis has received strong experimental and clinical support, several studies [7–10] militate against its inherent simplicity. The purpose of this communication is to present results obtained from *in vivo* experiments that suggest that the oxygen-dependent EPO production rate (EPO-PR) can be altered by conditions which apparently do not modify the oxygen supply/demand ratio.

Methods

Adult female CF1 mice were used throughout. They were fed a standard rodent chow and water *ad libitum*. Hypobaric hypoxemia was induced by exposing mice to air maintained at 50% atmospheric pressure in a simulated high altitude chamber. Recombinant human EPO (rh-EPO) (Bio Sidus, Argentina; 400 to 1500 U/kg/dose), cyclophosphamide (Endoxan Asta, 100 mg/kg/dose) or phenylhydrazine (20 to 40 mg/kg/dose) were injected to stimulate or depress erythropoiesis or to induce a compensated hemolytic state, respectively. Erythropoiesis was measured by the percent of a tracer dose (0.2 μCi) of ^{59}Fe incorporated into the circulating red cell volume (24 hr). Plasma disappearance of rh-EPO was determined by injecting 30 mice i.v. with 600,000 cpm of ^{125}I-labeled hormone with subsequent bleeding of the animals in groups of five every hour for six hours. The radioactivity in 100 μl of plasma was measured and plotted semilogarithmically versus time. Plasma EPO (pEPO) levels were determined by radioimmunoassay, as previously described [11]. The EPO-PR was calculated from pEPO values according to the equation

$$PR = (EPO_f - EPO_o \cdot e^{-kt})/(1 - e^{-kt}) \cdot K$$

where EPO_o and EPO_f are the basal plasma level and the plasma level at the end of the exposure to hypobaria, respectively. PR is the production rate of the hormone ($K = \ln 2/EPO\ t_{1/2}$). Oxygen

consumption was determined with an OXIMAX fully computerized system (Columbus Instruments, Columbus, OH, USA).

Results

Experiment 1. Hypoxia-dependent EPO production in the transfused-polycythemic mice: Effect of previous stimulation of erythropoiesis

Erythropoiesis was stimulated during a three week period in normal mice by induction of hypobaric hypoxemia (18 hr/day), by administration of phenylhydrazine (initial dose of 40 mg/kg on the first week, followed by doses of 20 mg/kg at weeky intervals), or by injection of rh-EPO (1500 U/kg three times a week). Both control mice with normal erythropoiesis and mice with enhanced erythropoiesis in response to the phenylhydrazine-induced hemolytic state were made polycythemic by injecting them with packed red cells on two consecutive days. The hematocrit was measured in all mice of the remaining groups at the end of the period of stimulation of erythropoiesis. When necessary, mice were transfused in order to keep the hematocrit level around 0.65. It was done so because the erythropoietic response of transfused-polycythemic mice to acute hypoxemia was negatively related to the degree of polycythemia [12]. Mice from each group were exposed to hypobaria (14 hr) four days after the end of the period of erythropoietic stimulation or after transfusion, depending on the specific group. They were bled immediately after hypoxic stimulation. Plasma rh-EPO $t_{1/2}$ was also measured in all groups. The value of 182.8 + 14.4 minutes found in normal mice was not significantly modified by treatments. Figure 1 shows the EPO-PR during hypoxemia derived from all experimental groups. Two observations are worth noting: (1) EPO-PR was depressed in mice with normal erythropoiesis after transfusion (NC vs. PC); and (2) EPO-PRs in transfused polycythemic mice whose erythropoiesis were previously stimulated by intermittent hypoxemia, hemolytic state or rh-EPO administration were more than five times higher than in comparably polycythemic mice whose erythropoieses were not stimulated before exposure to hypobaria (P-PH, P-PHZ and P-EPO vs. PC).

Experiment 2. Hypoxia-dependent EPO production in normocythemic mice at different rates of erythropoiesis

Erythropoiesis was either stimulated by the s.c. injection of 400 U/kg of rh-EPO on two consecutive days, or depressed by the i.p. injection of 100 mg/kg of cyclophosphamide. Mice with normal erythropoiesis were used as controls. Hematocrit level, RBC-59Fe uptake, pEPO $t_{1/2}$ and pEPO concentration were measured in mice from the three groups three days after treatments. The hematologic parameters in the three experimental models are shown in Table 1. It appears evident that the oxygen-carrying capacity of blood, the pEPO titer and the pEPO half lives were not significantly different among groups in spite of significant variations in erythropoiesis. When mice from the three groups were exposed to hypobaria for six hours three days after treatments to stimulate EPO formation, the derived EPO-PR, as shown in Figure 2, were 208% of normal in mice with cyclophosphamide-induced depression of erythropoiesis and 33% of normal in those with EPO-induced enhancement of erythropoiesis.

Discussion

Hypoxia-dependent EPO production was estimated in both polycythemic and normocythemic mouse models previously subjected to different treatments. It was assumed that the tissue oxygen supply/demand ratios were similar in both experimental and control mice of the same model at the time of the hypoxic induction of EPO production. The assumption was based on calculation of the oxygen-carrying capacity of blood and body oxygen consumption. In the presence of equal oxygen supply/demand ratios any change in hypoxia-dependent EPO formation could be attributed to influences other than oxygen availability and/or consumption.

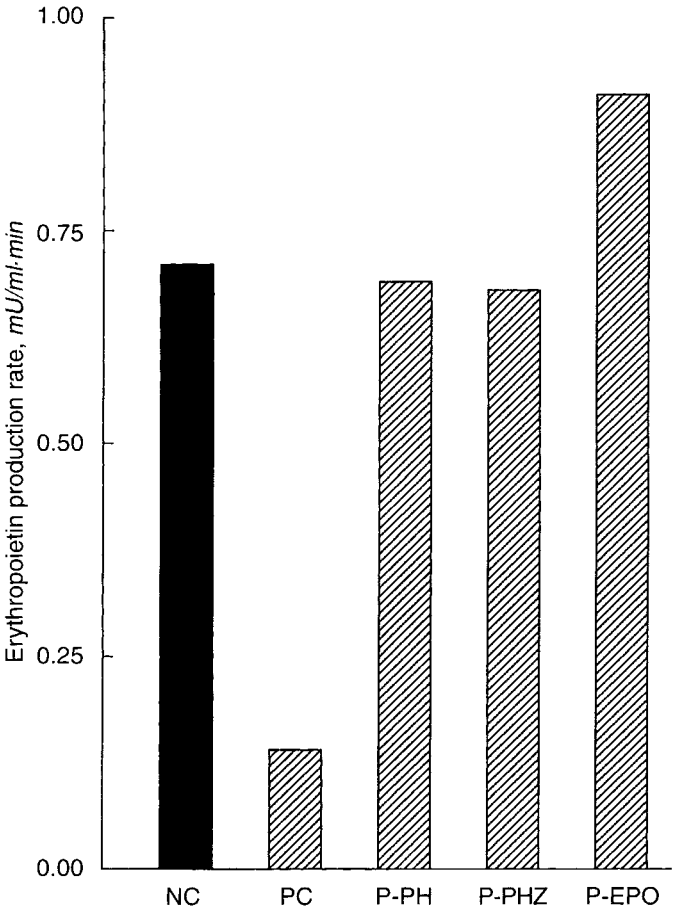

Fig. 1. *Erythropoietin-production rate, calculated from average increase in plasma EPO levels and plasma EPO half-lives, in response to exposure to hypobaria (0.5 atm, 14 hr) in normocythemic mice (NC) and in transfused polycythemic mice whose erythrocyte production rates were either not previously stimulated (PC) or stimulated during a 3-week period by intermittent exposure to hypobaria (P-PH), by phenylhydrazine administration (P-PHZ) or by repeated rh-EPO injections (P-EPO).* Symbols are: (■) normocythemic; (▨) polycythemic.

Hypoxia-stimulated EPO production was drastically depressed in transfused-polycythemic mice. However, this effect of polycythemia was no longer observable when mice were erythropoietically stimulated prior to transfusion, EPO production in such polycythemic mice being as high as that found in normocythemic mice. Prior to transfusion, erythropoiesis was stimulated by intermittent exposure to hypobaria, by phenylhydrazine-induced hemolysis or by repeated rh-EPO injections. Two features were common to treatments, namely enhanced erythropoiesis and supranormal plasma EPO titers. One or both, therefore, could be responsible for the described findings. However, this does not contribute significantly to explain the genesis of the phenomenon.

Table 1. Hematologic parameters, labeled recombinant EPO half-lives and oxygen consumption in mice with normal, depressed or stimulated erythropoiesis

Group	Hematocrit	RBC-^{59}Fe uptake	piEPO	pEPO $t_{1/2}$	O_2 consumption
	%		*mU/ml*	*min*	*liter/kg/hr*
Normal	42.3 ± 2.1	26.1 ± 1.8	28.3 ± 5.4	182.8 ± 14.4	3.7 ± 0.4
Depressed	41.6 ± 0.8	1.3 ± 0.1[a]	24.6 ± 2.3	198.2 ± 8.6	3.1 ± 0.3
Stimulated	43.4 ± 1.6	34.2 ± 2.0[a]	30.3 ± 3.2	178.6 ± 9.2	3.9 ± 0.6

Values are mean ± SEM.
[a] Statistically significant ($P < 0.05$) compared to normal values

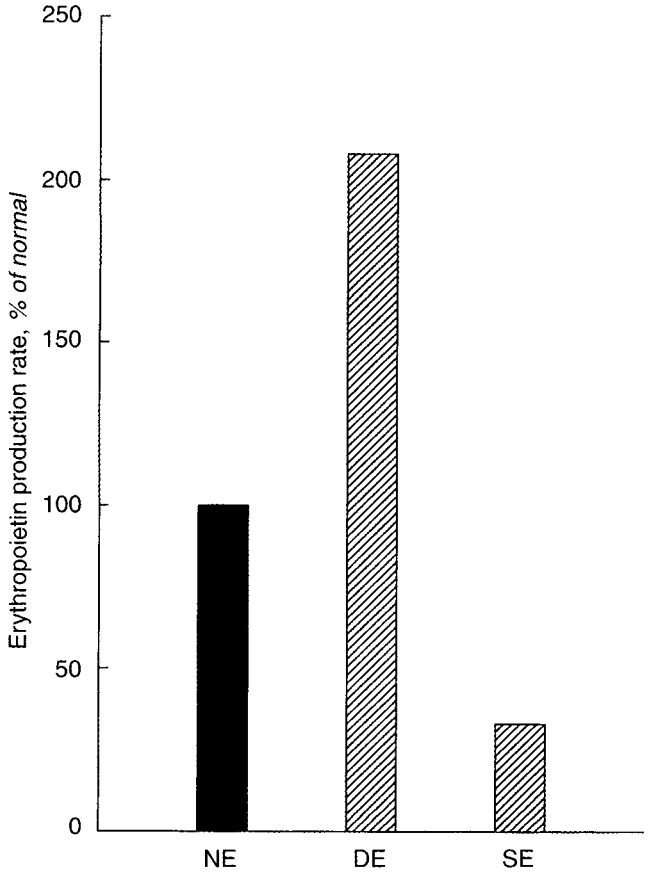

Fig. 2. *Erythropoietin-production rates, calculated from average increase in plasma EPO levels and plasma EPO half-lives, in mice with normal (NE), depressed (DE) or stimulated (SE) rates of erythropoiesis under hypoxic conditions (exposure to 50% atmospheric pressure for 4 hr).*

Recently, the stimulatory effect of rh-EPO on the EPO gene expression has been reported [13] which could help to explain the results obtained.

Hypoxia-dependent EPO production was also estimated in normocythemic mice during the course of either stimulation or depression of erythropoiesis induced by administration of rh-EPO or cyclophosphamide, respectively. Animals so treated showed normal values of oxygen-carrying capacity of blood, plasma EPO titer, plasma EPO half-life and body oxygen consumption at the time of stimulation of EPO formation in spite of significant alterations in the red cell production rate. Hypoxia-dependent EPO-PR was inversely related to the level of erythropoiesis occurring in the animals during exposure to hypobaric hypoxia. No evidence exists on the nature of the operating mechanism. However, data suggest that a functional link could exist between the EPO-responsive cells in the erythropoietic organs and the EPO-synthesizing cells that could modulate the hypoxia-dependent expression of the EPO gene.

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