Serum erythropoietin levels in healthy humans after a short period of normobaric and hyperbaric oxygen breathing: the “normobaric oxygen paradox”.

Running head: EPO increase after oxygen breathing

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Abstract

Renal (peritubular) tissue hypoxia is a well known physiological trigger for erythropoietin (EPO) production. We investigated the effect of rebound relative hypoxia after hyperoxia obtained under normo- and hyperbaric oxygen breathing conditions. A group of 16 healthy volunteers were investigated before and after a period of breathing 100% normobaric oxygen for 2 hours and a period of breathing 100% oxygen at 2.5 ATA (atmospheres absolute) for 90 minutes (hyperbaric oxygen). Serum EPO concentration was measured using a radioimmunoassay (RIA) at various time points during 24-36 hours. A 60% increase (P<0.001) in serum EPO was observed 36h after normobaric oxygen. In contrast, a 53% decrease in serum EPO was observed at 24h after hyperbaric oxygen. Those changes were not related to the circadian rhythm of serum EPO of the subjects. These results indicate that a sudden and sustained decrease in tissue oxygen tension, even above hypoxia thresholds (for example after a period of normobaric oxygen breathing), may act as a trigger for EPO serum level. This EPO trigger – the “normobaric oxygen paradox” - does not appear to be present after hyperbaric oxygen breathing.

Key Words: Erythropoietin stimulus, EPO, normobaric oxygen paradox, hyperbaric oxygen, hypoxia.
Introduction

Erythropoietin (EPO) induces red blood cell production by activating red bone marrow progenitor cells, and is used therapeutically in chronic anaemia, and as illegal pharmacological means to increase blood oxygen transport capacity in athletes. Renal tissue hypoxia is the only widely accepted trigger for EPO production (7, 23) even if new oxygen-sensitive sites have been recently proposed (3, 16, 57). This renal tissue hypoxia trigger has been well established through models of reduced oxygen delivery during anemia (29), reduced renal perfusion (39), hypobaric or normobaric hypoxia (11, 15, 28). In one report, haemoconcentration following sport activities (42) has been reported to increase EPO secretion. There seems to be no agreement on the existence of a circadian variation in EPO level (27, 41). If a circadian rhythm would exist, the exact timing and magnitude of the nadir and zenith are not unequivocally established (26, 55).

Previous observations in breath-hold divers have led us to hypothesize that another triggering mechanism might exist, independent from renal tissue hypoxia (2). After a series of deep breath-hold dives (5 times to a depth of 40 m), two out of 5 divers showed a marked increase of serum EPO levels. Rather than an absolute hypoxia, a relative hypoxia can be held responsible for this observation: indeed, during descent to depth, intra-alveolar oxygen tensions augment (according to Boyle’s and Dalton’s law) and (as the breaking of the breath-hold is usually caused by increased arterial carbon dioxide concentration) during ascent from depth the oxygen tension returns to (low) atmospheric values. During these experiments, no severe alveolar hypoxia was observed after surfacing. Recent data from a Swedish group confirm our previous findings in apnea divers (9). Along the same line, a Spanish study reported that a short exposure to intermittent hypobaric hypoxia showed increased EPO level (44).
We hypothesised that a sudden and sustained decrease in tissue oxygen level, aside
from an absolute low level of tissue oxygen tension, could also act as a trigger for EPO
production. To investigate this hypothesis, we elicited a sudden and sustained decrease in
arterial oxygen tension, from hyperoxic back to normoxic conditions under two different
oxygen pressures.

Materials and Methods

Subjects:

Sixteen healthy volunteers (10 males and 6 females), aged 22 to 47 yrs, participated in
this study after Medical Ethics Committee approval and written informed consent. The
subjects were asked not to smoke, and not to take any medication or perform strenuous
physical exercise 24 hours before and during the entire study protocol (40, 50, 51).

Experimental protocol:

One week before the start of the experimental protocols, all subjects had undergone
serial blood sampling in order to establish a baseline circadian EPO concentration curve.

Then, in a first experiment, the subjects breathed 100% normobaric oxygen (15 lpm in
a “non-rebreather” face mask) for 2 hours. To obtain a more thorough tissue denitrogenation,
they were asked to perform moderate physical exercise (20 knee bends) every 10 minutes
during the whole procedure (20, 53). Oxygen breathing was controlled by constant monitoring
of the following clinical signs: mask fit, movement of the three one-way valves on the mask,
movement of the reservoir bag and moisture formation on the transparent mask during
expiration. Furthermore, transcutaneous oxygen tension (PTcO₂) was measured sequentially
in all subjects using a Radiometer TCM3 monitor (Radiometer, Copenhagen, Denmark) (Fig. 1a).

One week later, the same subjects, but one male subject who could not participate due to problems pressure-equalizing his ears, were exposed to hyperbaric oxygen for 1.5 hours after compression to 2.5 ATA in a multiplace hyperbaric chamber (Haux GmbH, Karlsbad, Germany), breathing 100% oxygen via a face mask (Laerdal, Copenhagen, Denmark). During the compression and decompression phases (15 minutes each) subjects were breathing compressed environmental air (21% oxygen). The total duration of the hyperbaric exposure was thus also 2 hours (Fig. 1b). This protocol corresponds to a standard hyperbaric oxygen treatment and is routinely used in the clinical setting of this author’s (PG) HBO center. The duration of normobaric oxygen breathing was arbitrarily chosen to be of the same magnitude, in order to obtain a more standardized “dose-response” observation.

Analyses:

Venous blood samples were taken immediately before testing, at the end of oxygen breathing (0 time) and thereafter at 2, 4, 5, 7, 10, 24 and 36 hrs after normobaric oxygen breathing and at 2, 4, 5, 7, 10, 24 hrs after hyperbaric oxygen (for technical reasons, blood sampling was not possible at 36 hrs after hyperbaric oxygen). The total quantity of blood taken during the whole protocol was less than 30 ml, and did not have any influence on the haematocrit level. Blood was immediately centrifuged (10 minutes at 3000 rpm), and the serum was frozen immediately to –80°C for a maximum of 24 hours before analysis. Serum EPO concentration was determined using a radioimmunoassay (EPO-Trac 125I RIA, INCSTAR, Stillwater, USA). Haematocrit and hemoglobin concentration were measured before and after oxygen breathing (over 36 h) using a Advia®120 Automated Hematology System (Bayer Diagnostics, Tarrytown, NY, USA) (42). Body impedance was measured before and after oxygen breathing using a Tanita TBF-310-GS scale (Tanita Europe GmbH,
Sindelfingen, Germany) to test the eventual difference in body fat mass as the tissular
denitrogenation can be modified by fat mass (1).

Standard statistical analyses were performed including mean, standard deviation, and
analysis of variance (ANOVA) for repeated measures to test the between and within subject
effect after Kolmogorov Smirnov test for normality. The post-tests Bonferroni or Dunnett
comparison were applied versus the control values. Taking the initial value as “100%”,
procentual variations were calculated for each parameter (Hb, Hct and EPO), thereby
allowing an appreciation of the magnitude of change rather than the absolute values.

Results

All subjects had baseline haematocrit (45.7 ± 2.4%) and hemoglobin values (15.3 ±
0.96 g/dl) within the normal population range. Body fat mass percentage was 18.8% ± 6.4%.
None of these parameters were significantly altered after the normobaric or hyperbaric
oxygen breathing period (Fig. 2a and 2b).

During the baseline measurements, a circadian variation of serum EPO concentration
was found, with the nadir situated around 14.00 Hrs (8.96 ± 2.1 UI/l) and the zenith around
22.00 Hrs (12.1 ± 3.5 UI/l); owing to a great individual variability, this circadian variation
however did not reach statistical difference.

During the normobaric oxygen-breathing period, an initial decrease in serum EPO
concentration was followed by a significant increase at the 24 and 36 hours measuring points
(Fig. 3a). This increase was statistically significant, towards the initial (pre-oxygen breathing)
values and the mean variation of the corresponding circadian rhythm value (Fig. 3b). There
was a “lag time” of about 8 hours after cessation of oxygen breathing before the start of the
increase, which is consistent with the time lapse needed for transcription and secretion of EPO by the renal peritubular cells (48). The regression graph shows a significant correlation \((r=0.93, p=0.0001)\) between the time after cessation of oxygen breathing and the EPO concentration (Fig. 4a).

After hyperbaric oxygen, a marked and sustained decrease in serum EPO concentration was observed, lasting up to 24 hours (Fig. 3a and 3b). For logistical reasons, no blood samples could be obtained after 36 hours, but at 24 hours the curves did not show a tendency to normalization \((r=0.61, p=0.038)\) (Fig. 4b).

**Discussion**

A significant increase in serum EPO levels was measured after breathing 100% normobaric oxygen for 2 hours. This increase was not related to a circadian EPO variation, nor could it be explained by changes in haematocrit level or whole body dehydration. The sequence of EPO transcription, “de novo” synthesis and release after application of a hypoxic trigger has been timed at 4-8 hours (48). This is consistent with the time lag observed in our study.

By breathing 100% oxygen for two hours, a significant nitrogen washout is obtained, thereby increasing the relative oxygen content in all body tissues (34). The cessation of this oxygen breathing results in a rapid decline in oxygen partial pressure in blood and tissues (see Fig.1). The effects of modifications of arterial oxygen pressure on the oxygen status of the cortical and medullar renal tissue have been documented (13), although much less than the effect of changes in renal perfusion pressure (4, 10, 30, 31, 38). Briefly, although normobaric hyperoxia induces a reduction of renal blood flow, this difference is minor and does not modify the cortical or medullary blood flow or perfusion pressure in a significant way (13).
Since renal tissue pO2 is the net result of a balance between oxygen delivery and oxygen consumption (which, in turn, is determined, in the normal kidney by renal blood flow and the glomerular filtration rate respectively (10)), intramedullary pO2 is increased (25).

After cessation of oxygen breathing, the arterial oxygen pressure falls within minutes to a normal baseline level. This decline has been documented using transcutaneous oxygen (PTcO2) measurements, a reliable method to investigate cutaneous and subcutaneous oxygen tension variations. In the non-compromised cardio-pulmonary patient, PTcO2 values have been shown to correlate with arterial oxygen tension (22, 43, 46). Because the change in renal oxygen tension induced by altering the oxygen breathing, which could be called a “relative hypoxia”, persists over a prolonged period (since it is actually the return to the normal oxygenation state), it could constitute a sufficient trigger for increasing EPO transcription and secretion. It has been reported that hypoxic periods of 45 minutes, but not of 30 minutes’ duration, could trigger EPO production in humans (27).

Surprisingly, hyperbaric oxygen breathing did not induce a greater increase in serum EPO concentration. On the contrary, hyperbaric oxygen appears to be a very effective depressor of serum EPO levels, maintaining low EPO levels up to 24 hours after hyperbaric oxygen breathing. The effect of hyperbaric oxygenation on the renal cortical and medullary oxygen tensions has, to our knowledge, not been studied. Indirectly, one can assume however that these oxygen tensions are proportional to the arterial oxygen tension. Indeed, hyperbaric oxygenation reduces tissue blood flow by as much as 20-30%, including renal blood flow (37), but despite this hyperoxic vasoconstriction, peripheral oxygen pressure is maintained at a higher level (about three times the level reached during normobaric oxygenation) (33). This is due to the increased quantity of oxygen dissolved in the plasma (Henry’s Law of physics). Furthermore, it has been shown that during (normobaric) hyperoxia, autoregulation of the renal blood flow is enhanced, preserving medullary blood flow even during severe reductions of renal perfusion pressure (13). It is thus safe to assume that during hyperbaric hyperoxia,
renal tissue oxygen tension is proportionally increased. One might hypothesize that high oxygen pressures persist in the kidney after cessation of hyperbaric exposure. An argument in favour of this hypothesis could be the prolonged vasoconstriction after stopping hyperbaric oxygen breathing (37). However, arterial oxygen tensions after cessation of hyperbaric oxygen breathing fall rapidly (within minutes) to normal atmospheric values and although tissues have a variable delay in decrease of oxygen tension, high intra-tissular oxygen tensions rarely persist for more than 1 hour (47). Therefore, some other process is probably involved.

The mechanisms whereby oxygen sensing leads to activation and transcription of EPO, are still incompletely understood. Only in recent years, the central role of Hypoxia-Inducible Factor 1 alpha (HIF-1α) has been discovered and involved in this process (for a review, see (17) and Fig. 5a). HIF-1α is continuously produced, but continuously inactivated by its binding to another protein, Von Hippel Lindau tumor-suppressor protein (pVHL), and by subsequent ubiquitous metabolization by hydroxylation of proline residues. The configuration changes leading to the binding and thus the destruction of HIF-1α are regulated by a redox mechanism, with glutathione playing a central role. The oxidized form of glutathione, GSSG, which is known to play a major role in the neutralisation (scavenging) of oxygen free radicals (OFR), interacts at least to two possible sites on the HIF-1α molecule, thereby allowing its inactivation (32).

It has been shown that selective inhibition of γ-Glutamyl Cysteine Synthetase (γ-GCS) (which results in GSH depletion) in the alveolar perinatal epithelium abrogates hypoxia-induced nuclear localization, stabilization and activation of HIF-1 (18, 19). A “redox cycle” exists, whereby GSH is oxidized to GSSG and back, in order to “scavenge” the oxygen free radicals. The disruption of this cycle, e.g. by inhibition of Glutathione Reductase, leads to an accumulation of GSSG which effectively inhibits HIF-1α activation (18, 19). Normally, less than 0.2% of GSH exists as GSSG (35). Maintenance of this glutathione equilibrium (and
by inference, the shuttling between reduction and oxidation states) is thus a prerequisite for HIF-1α stabilization.

As the oxygen levels in the cell decrease, a higher proportion of GSSG is reduced to GSH; the GSH-to-GSSG ratio increases, and this directly or indirectly inhibits the HIF-1α/pVHL binding. As a consequence, HIF-1α can exert its DNA-binding and transcriptional activities, inducing EPO and several other proteins, such as Vascular Endothelial Growth Factor (VEGF), NO Synthetase 2, Plasminogen Activator Inhibitor and proteins involved in glycolytic metabolism (17).

Hyperoxygenation (Fig. 5b) leads to an increase in intracellular oxygen free radicals and thus to an increased formation of GSSG (which will suppress EPO gene expression by inactivation of HIF-1α). Additionally, hyperoxygenation induces “de novo” synthesis of GSH by activating γ-GCS. After cessation of hyperoxygenation, this increased stock of GSH, together with the (slow) reduction of GSSG to GSH produces a “surplus” of intracellular GSH (increasing the GSH-to-GSSG ratio), a situation which induces a higher level of activation of HIF-1α (19). This scenario might be responsible for the observed “normobaric oxygen paradox”: increased EPO production after normobaric oxygen breathing.

There have been no previous reports of a possible “refractory period of the oxygen sensor” after high arterial oxygen concentration (Fig. 5c). However, during a normal hyperbaric oxygen treatment session, a substantial quantity of oxygen free radicals is generated, resulting in a temporary depletion of scavenging enzymes such as GSH. This depletion of GSH is due to an increased conversion to GSSG (36). Hyperbaric oxygenation may lead to even more increased oxidative stress than expected on the basis of the oxygen concentration alone, by induction of cytokines (17, 56). Even if some “extra” GSH is produced by stimulation of γ-GCS, all of this GSH is rapidly oxidized. Upon cessation of HBO, GSSG needs to be reduced back to GSH. This process is rather slow (Glutathione
Reductase works at the expense of NADPH, and is thus limited by the conversion rate of glucose. It now takes longer before sufficient GSH levels are attained to re-establish the optimal GSH-to-GSSG ratio; thus the inactivation of HIF-1α is maintained for a longer period of time. This is a logical explanation for the sustained decrease in EPO production after hyperbaric oxygen breathing, as opposed to the “normobaric oxygen paradox”.

Conclusions

This investigation has demonstrated a previously unreported triggering mechanism for EPO production in healthy humans, which is unrelated to an absolute decrease in oxygen delivery to the renal peritubular cells or other tissues (57). Despite a stable erythropoiesis under our experimental conditions, the magnitude of change in EPO levels is thus that this mechanism could possibly be used to induce an increase in red blood cell production with repeated exposures (21). It has been shown that repeated 90-minute exposures to hypobaric hypoxia (which each induce an increase in EPO levels of the same magnitude) lead to erythropoiesis over a period of 3 weeks (45). As EPO is now known to be a member of the cytokine family and has effects other than erythropoiesis (12), this mechanism may play a role in the therapeutic effect of hyperbaric oxygen therapy. Incidentally these mechanisms may also, as proposed by others, provide pathways for potential neuroprotective (5, 6, 8, 24, 54) and other cellular repair and regeneration effects of EPO stimulation (14, 49, 52).
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References


Figures

**Figure 1**: Transcutaneous oxygen pressure during a. normobaric oxygen breathing and b. hyperbaric oxygen breathing

**Figure 2**: Procentual variation of a. hematocrit and b. hemoglobin over 36 hours after 2 hours of normobaric oxygen breathing and the control group.
Figure 3: Evolution of mean (± SEM) serum EPO concentration (UI/l) before and after 2 hours of 100% normobaric oxygen breathing (NBO) over 36 hours, and hyperbaric oxygen breathing (HBO) over 24 hours. Time 0 = end of oxygen breathing. (*: p<0.05 ; ** : p<0.01; ***: P<0.001). Variation of serum EPO expressed as absolute values (a) or as percent of corresponding value (b) in control group not breathing oxygen.

Figure 4: Linear regression curves of percentage variation of plasma EPO concentrations after normobaric oxygen breathing (NBO) or hyperbaric oxygen breathing (HBO). There is no tendency to normalization in the HBO group at 24 hrs, suggesting that the suppression of EPO may last longer than 24 hrs, possible 36 hrs.
Figure 5: Proposed mechanism of oxygen levels and EPO transcription (the “normobaric oxygen paradox”).

a. Exposure of mammalian cells to hypoxia activates a transcriptional response pathway mediated by Hypoxia Inducible Factor (HIF). These responses include the up-regulation of genes involved in erythropoiesis (EPO production), angiogenesis, and glycolysis. HIF is a heterodimeric transcription factor composed of a hypoxia-inducible α-subunit and a constitutively expressed β subunit, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). HIF1-α is continuously produced, but also continuously ubiquitinated through reactions involving Von Hippel Lindau (VHL) protein and Prolyl-Hydroxylase and then degraded by proteosomes; this reaction needs oxygen free radicals (OFR), some of which are neutralized by Glutathione (GSH): thus, intracellular HIF1-α concentrations remain low. During hypoxia, GSH can neutralize all oxygen free radicals, HIF1-α and β dimerization can occur and proceed to the EPO gene expression (19).

b. During normobaric hyperoxia, OFRs stimulate GSH production (Glutathione Synthetase) and thus, upon returning to normoxic conditions, all OFRs are neutralized by the increased intracellular GSH. This induces EPO gene expression similarly to hypoxia, and this situation could be called the “normobaric oxygen paradox”.

c. During hyperbaric hyperoxia, because of the excess presence of OFR and the limited increase of GSH production, most of the GSH is converted to GSSG; after return to normoxia, and during the time needed for GSSG to be converted back to GSH (limiting enzyme: Glutathione Reductase), ubiquitination of all intracellular HIF-α occurs, effectively blocking the transcriptional response pathway for a sustained period of time.