HIGHLIGHTED TOPIC | Oxygen Sensing in Health and Disease

Antioxidant intervention does not affect the response of plasma erythropoietin to short-term normobaric hypoxia in humans

A. M. Niess, E. Fehrenbach, I. Lorenz, A. Müller, H. Northoff, H.-H. Dickhuth, and E. M. Schneider. Antioxidant intervention does not affect the response of plasma erythropoietin to short-term normobaric hypoxia in humans. J Appl Physiol 96: 1231–1235, 2004; 10.1152/japplphysiol.00803.2003.—Recent research has demonstrated that reactive oxygen species (ROS) participate in intracellular signaling processes initiated during hypoxia. We investigated the role of ROS in the response of plasma erythropoietin (Epo) to short-term normobaric hypoxia in humans. Twelve male subjects were exposed twice to 4 h of normobaric hypoxia (H; inspired oxygen fraction 10.220.33.1 on July 11, 2017 http://jap.physiology.org/ Downloaded from http://jap.physiology.org/ by 10.220.33.1 on July 11, 2017
not recently traveled to altitudes above 1,600 m or been on flights within the last 8 wk before the study. The study was approved by our university Ethics Committee and conducted as a clinical trial.

Because of an injury, an upper respiratory infection, and a vagal reaction during the first experiment in hypoxia, three of the subjects had to be excluded from further participation. During a 9-wk period, a double-blind, placebo-controlled, randomized, crossover trial was completed by the remaining 12 volunteers (age 26.2 yr, 22.1–30.2 yr; height 184 cm, 181–188 cm, body mass 79.8 kg, 74.3–85.2 kg). During this period, subjects were examined at three stages: 1) in week 0 before antioxidant or placebo treatment (control experiment in normoxia), 2) at the end of week 3 [1st experiment in hypoxia (H1)], and 3) at the end of week 9 [2nd experiment in hypoxia (H2)]. A 3-wk washout period followed H1 before the subjects crossed over and received the alternate supplements for the next 3 wk. Supplementation consisted of a combination of two capsules with 400 mg all-rac-tocopherol (α-Toc) each, provided by Woelm Pharma (Bad Honnef, Germany) and one capsule of 600 mg α-lipoic acid (α-LA) purchased from ASTA Medica (Frankfurt, Germany) or corresponding placebo capsules. The participants were instructed to ingest the capsules orally before H1 and H2, respectively.

Experimental procedure. Subjects reported to the laboratory in the morning 7:00 AM. An indwelling cannula was inserted into a forearm vein and kept patent with sterile saline solution (0.9% sodium chloride, Braun, Melsungen, Germany). Exposure to hypoxia or normoxia started at 8:00 AM and lasted 4 h. During the experiments, subjects were sitting in a comfortable position and breathed normal room air [control experiment, inspired oxygen fraction (FIO₂) 20.9%] or a hypoxic gas mixture diluted with nitrogen (H1 and H2: FIO₂ 12.5%) via a tight-fitting face mask. Venous blood samples were drawn: 10 min before (7:50 AM); during the 2nd (10 AM) and 4th h (12 AM); and 1 (1 PM), 4 (4 PM), 8 (8 PM), and 20 h (8 AM) after the end of the experiments. Whole blood aliquots were centrifuged (4°C, 1,000 g, 15 min) immediately after sampling, and plasma aliquots were stored at −80°C until further analyses.

Pulse oximetric O₂ saturation was monitored continuously (monitor SC 6000, Siemens, Erlangen, Germany). Capillary blood samples for blood-gas and lactate measurements were obtained from the hypererized earlobe 20 min before, every hour throughout, and 20 min after exposure to normoxia/hypoxia.

Analytic procedures. Blood gases were determined by a blood-gas analyzer (STAT Profile pHxS, Nova Biomedical, Waltham, MA). Lactate concentrations were measured electrochemically by using a lactate analyzer (EBIO, Eppendorf, Germany). Plasma Epo concentrations were determined by an enzyme-linked immunometric assay using the Immulite System (JPC Biermann, Bad Nauheim, Germany). The manufacturer reports a sensitivity of this procedure of 0.24 mIU/ml. Intra- and interassay coefficients of variation are 6.3 and 6.1%, respectively. Total peroxide concentrations in EDTA plasma were assessed photometrically (PerOx Kit, Immunodagnostik, Bensheim, Germany). Serum α-tocopherol concentrations were determined by means of HPLC (HPLC system Merck, Hitachi L6200, Merck, Darmstadt, Germany) with ultraviolet detection by using a commercial available kit (Chromsystems, München, Germany).

Calculations and statistics. Statistical analyses and graphics were computed by the statistical software package JMP 4.0 (SAS Institute, Cary, NC) and KaleidaGraph (Synergy Software, Abellbeck, Reading, PA). Data are expressed as means and 95% confidence interval (95% CI). To exclude diurnal changes and possible effects of blood sampling on Epo production, we additionally corrected the increases of plasma Epo under hypoxic conditions (ΔEpoH) by the changes of plasma Epo in the normoxic control experiment (ΔEpoN) for every sampling point using the following equation

\[ \Delta\text{Epo}_{\text{corr}} = \Delta\text{Epo}_{\text{H}} - \Delta\text{Epo}_{\text{N}} \]

Corrected changes of plasma Epo (ΔEpo_{corr}) were used to plot an Epo concentration-time curve for each individual and condition, thereby allowing determination of the integrated area under the resulting Epo curves (AUCEpo) by trapezoid calculation.

The changes in plasma Epo and peroxide levels were tested for significance by calculating the individual differences between the different sampling points and baseline (0 h). Differences were regarded as significant at the 5% level if the associated 95% CI excluded the zero difference (9). To evaluate the effect of antioxidant treatment on plasma Epo and peroxide levels as well as on plasma Epo changes, individual differences between verum and placebo conditions were calculated for every sampling point. A treatment effect was considered significant at the 5% level if the 95% CI of the crossover difference did not include zero (9). Linear regression analysis was calculated by the Pearson method.

RESULTS

Pulse oximetric O₂ saturation and capillary PO₂ decreased significantly during both hypoxic trials in a similar extent without any differences between verum and placebo conditions (Fig. 1, Table 1). Blood lactate concentrations were not affected by hypoxia (Table 1). Supplementation with α-Toc and α-LA resulted in a significant increase of serum α-Toc levels

| Table 1. Capillary PO₂ and lactate concentrations before, during, and after exposure to the normoxic and hypoxic experiments (placebo and verum conditions) |
|-----------------|-----------------|-----------------|
| +20 Min Exposure to Hypoxia/ Normoxia (240 min) +20 Min |
| PO₂, Torr N | 77.3 (73.7–81.0) | 73.7 (72.7–79.0) | 75.9 (72.7–79.0) | 75.9 (72.7–79.0) |
|   P | 81.8 (76.5–87.1) | 84.6 (79.4–89.8) | 48.2 (45.1–51.3) | 48.2 (45.1–51.3) |
| V | 79.5 (77.2–81.8) | 83.8 (78.5–89.1) | 45.1 (42.4–47.9) | 45.1 (42.4–47.9) |
| Lactate, mmol/l N | 1.19 (1.16) | 1.05 (0.84–1.19) | 1.49 (1.38–1.60) | 1.49 (1.38–1.60) |
|   P | 1.02 (0.85–1.19) | 1.02 (0.85–1.19) | 1.02 (0.85–1.19) | 1.02 (0.85–1.19) |
| V | 1.02 (0.85–1.19) | 1.02 (0.85–1.19) | 1.02 (0.85–1.19) | 1.02 (0.85–1.19) |

Values are means with 95% confidence interval in parentheses. N, normoxia; P, placebo; V, verum [all-rac-α-tocopherol (α-Toc) and α-lipoic acid (α-LA)]. *Significantly different from baseline (−20 min), †Significantly different from placebo conditions (5% level).
(46.8, 38.2–55.5 nmol/ml) compared with placebo conditions (23.0, 20.3–25.7 nmol/ml). Plasma concentrations of lipid peroxides were not affected by hypoxia but were lower under verum conditions (Fig. 2). Epo kinetics in plasma showed a large individual variability, and the maximum increase ranged between +35 and 201% (Fig. 3). Treatment with α-Toc and α-LA did not affect the hypoxia-induced rise of plasma Epo or the absolute plasma Epo concentrations (Fig. 4). Under normoxic conditions, plasma Epo levels increased significantly by +57% (+15 to +99%) at 8 PM (12 h) and were still elevated at 8 AM on the following day (24 h). Changes in plasma Epo concentrations corrected by the corresponding changes in the normoxic trial and AUCEpo did not show any treatment effect (Figs. 5 and 6). We failed to find a correlation between AUCEpo and the mean Po2 during exposure to hypoxia (Fig. 7).

**DISCUSSION**

To delineate the involvement of ROS in the regulation of Epo production in response to short-term normobaric hypoxia in humans, we conducted the present antioxidant intervention trial. Consistent with other studies (8, 14, 21), plasma Epo increased rapidly during 4 h of normobaric hypoxia. In addition, our findings of a maximum increase of Epo concentration ranging between 35 and 201% confirm earlier reports of a large individual variability of the Epo response to a given hypoxic challenge (3, 10). The observation that the behavior of plasma Epo concentrations was not related to the individual degree of hypoxemia during the hypoxic trials supports the assumption that the variability in the Epo response is mainly due to individual differences on the regulatory level (10). As shown in the animal model, Epo response to acute as well as chronic...
exposure to hypoxia differs dramatically between rodent strains (24), pointing to an involvement of genetic factors.

The major finding of this study was that antioxidant supplementation with α-Toc and α-LA did not affect hypoxia-induced changes in plasma Epo levels. We selected a combination of both antioxidants with respect to synergistic effects of α-Toc and α-LA. α-Toc is well accepted as an effective lipid-soluble, chain-breaking antioxidant (22). Regeneration of α-Toc can be achieved by dihydrolipoic acid, the reduced form of α-LA. In addition to its recycling effects on α-Toc, α-LA acts as an potent antioxidant in both the lipid and aqueous phases of the cell (25), and it has also been reported to be an effective glutathione substitute (12). Despite supplementation with α-Toc and α-LA resulting in a significant rise in serum levels of α-Tocopherol and in attenuated plasma peroxide concentrations, we failed to detect any effects on the behavior of Epo in plasma. Thus our results provide no evidence that ROS play a major role in the regulation of human Epo production in response to short-term hypoxia. Our findings are in line with more recent findings that HIF-1α stabilization does not require cellular redox changes and depends mainly on prolyl hydroxylation (16, 17).

On the other hand, our observation that peroxide concentrations were not affected by hypoxia may provide some indirect information that the extent and duration of hypoxia used do not exert large effects on ROS formation. It can be speculated that this may be due to a still sufficient oxygen delivery to the tissue, because no significant increase was found for lactate levels in capillary blood. However, it has to be taken into account that measurements of plasma markers of oxidative stress, such as peroxide levels, do not allow a valid estimation of redox changes at the cellular level (13).

With respect to more severe hypoxia, as used in most of the in vitro studies, it appeared generally accepted that ROS formation is positively related to tissue PO2 (for review, see Ref. 11). An hypoxia-induced drop in cellular ROS levels has been assumed to allow HIF-1α stabilization and subsequent binding to the Epo gene enhancer (15). However, studies of enhanced hydrogen peroxide formation by mitochondria during hypoxia (2, 5) revealed the opposite. They demonstrated that mitochondrial ROS are required for both HIF-1 DNA binding and expression of Epo. Although cell type-dependent factors and differences in the analytic methods used in these studies may account in part for the divergent findings, the question of whether hypoxia promotes or decreases prooxidant conditions is still unresolved. Similarly, equivocal findings also exist with respect to the effects of antioxidants on Epo production. Cultured HepG cells or perfused rat kidneys exhibited an increased hypoxia-induced Epo production under treatment with a combination of retinol acetate, α-tocopherol, and ascorbic acid (19) or with retinol acetate alone (23). In contrast, treatment of rats with different antioxidants suppressed the hypoxia-induced rise of Epo concentrations in vivo (6), which is in line with findings that mitochondrial ROS are required for mRNA expression of Epo (2). In humans, Hildebrandt et al. (14) reported an augmenting effect of oral treatment with N-acetylcysteine (NAC) on plasma Epo concentrations even in normoxia, which was also apparent during and after subsequent exposure to 6 h of normobaric hypoxia (FiO2, 12.0%). To the contrary and in accordance with our results, Freudenthaler et al. (8) did not detect any influence of NAC or α-LA, both given intravenously, on hypoxia-induced Epo production in response to a similar degree of normobaric hypoxia (FiO2, 13.0% for 6 h).

For interpretation of such inconsistent findings, it appears to be necessary to consider that the hypoxia increase of plasma Epo levels may also be additionally affected by individual and diurnal factors (10, 20). To exclude such influences, we conducted the present study in a crossover design and performed an additional control trial in normoxia. Noteworthy, the behavior of plasma Epo concentrations in the normoxic control trial was subject to substantial diurnal changes, reaching 57% higher values on average at 8 PM compared with baseline. Furthermore, we observed significantly higher Epo levels at 8 AM on the second day compared with those on the preceding day at 8 AM. The latter finding may point to an additional effect of blood sampling (4, 18), which reached a total volume of 180 ml per condition in our study. Thus it seems to be reasonable to consider such additional effects if isolated effects of hypoxia on the Epo response are studied.

In conclusion, our results provide no evidence that ROS play an important modulatory role in the Epo response to short-term normobaric hypoxia in humans. With respect to in vitro data from other groups, we assume that an involvement of ROS in hypoxia-induced signaling processes may be restricted to more severe or prolonged hypoxia. Thus future research in this

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**Fig. 6.** Integrated area under curve concentrations for plasma Epo (AUC_Epo) under verum (α-Toc & α-LA) and placebo conditions. Values are means and 95% CI.

**Fig. 7.** Relationship between AUC_Epo and mean PO2 during hypoxia under verum (filled symbols) and placebo conditions (hatched symbols).
area should address this issue by performing studies on the effect of ROS on human Epo production in response to more prolonged or intermittent hypoxia.

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REFERENCES


