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ORIGINAL ARTICLE

Kidney-synthesized erythropoietin is the main source for the hypoxia-induced increase in plasma erythropoietin in adult humans

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Abstract
Purpose Erythropoietin (EPO) is mainly synthesized within renal peritubular fibroblasts, and also other tissues such as the liver possess the ability. However, to what extent non-kidney produced EPO contributes to the hypoxia-induced increase in circulating EPO in adult humans remains unclear.
Methods We aimed to quantify this by assessing the distribution of EPO glycoforms which are characterized by posttranslational glycosylation patterns specific to the synthesizing cell. The analysis was performed on samples obtained in seven healthy volunteers before, during and after 1 month of sojourn at 3,454 m altitude.
Results Umbilical cord (UC) plasma served as control. As expected a peak ($p < 0.05$) in urine (2.3 ± 0.5-fold) and plasma (3.3 ± 0.5-fold) EPO was observed on day 1 of high-altitude exposure, and thereafter the concentration decreased for the urine sample obtained after 26 days at altitude, but remained elevated ($p < 0.05$) by 1.5 ± 0.2-fold above the initial sea level value for the plasma sample. The EPO glycoform heterogeneity, in the urine samples collected at altitude, did not differ from values at sea level, but were markedly lower ($p < 0.05$) than the mean percent migrated isoform (PMI) for the umbilical cord samples.
Conclusion Our studies demonstrate (1) UC samples express a different glycoform distribution as compared to adult humans and hence illustrates the ability to synthesis EPO in non-kidney cells during fetal development (2) as expected hypoxia augments circulating EPO in adults and the predominant source here for remains being kidney derived.

Keywords EPO · Renal · Altitude

Abbreviations

EPO	Erythropoietin
Glc-NAc	*N*-acetylglucosamine
HA	High altitude
MAIIA	Membrane-assisted isoform immunoassay
PMI	Percent migrated isoform
SL	Sea level
WGA	Wheat germ agglutinin
UC	Umbilical cord

Introduction

Erythropoietin (EPO) governs hemoglobin concentration and thus arterial oxygen content by regulating red blood cell and plasma volumes (Jelkmann 1992; Lundby et al. 2007; Olsen et al. 2011). The classic view is that hypoxia stimulates the synthesis and release of EPO in renal peritubular fibroblasts. Work based on HIF-1 knock-out mice however suggested that astrocytes, which express both EPO and its receptor (Bernaudin et al. 2000), may contribute by as much as 50 % to the hypoxia-induced increase in plasma

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EPO (Weidemann et al. 2009). In divergence hereto we observed no detectable release of EPO from the hypoxic human brain to the blood stream or to cerebral spinal fluid (Rasmussen et al. 2012). In contrast to the HIF-1 knockout study, however, the samples were obtained after 4 and 8 h of hypoxic exposure when the erythropoietic response to hypoxia may not yet have reached its peak, which is usually reached within the first 1–3 days of exposure (Robach et al. 2007).

Besides astrocytes (Masuda et al. 1994) EPO may also be synthesized in the liver and based on EPO mRNA analysis it is generally accepted that this is the main site for EPO production during gestation (Dame et al. 1998; Ohls 2002). Adult rodent liver retains its ability to produce EPO in response to hypoxia or HIF activation (Fried 1972; Kapitsinou et al. 2010; Minamishima and Kaelin 2010). Other cells have also been demonstrated to be capable of synthesizing EPO although only in minor quantities (Haase 2010) and accordingly nephrectomized humans demonstrate elevated EPO levels (Mirand et al. 1968). However, to what extent these non-renal EPO production sites contribute to the hypoxia-induced increase in plasma EPO in adult humans remains elusive. Since the posttranslational glycosylation patterns of the different EPO glycoforms are specific to their synthesizing cells (Lönnberg et al. 2013; Masuda et al. 1994; Wide and Bengtsson 1990), the contribution of the various synthesizing cells to the circulating EPO concentration can be determined by analysis hereof. In the present study, we analyzed EPO glycoform composition of healthy humans exposed to altitude for 1 month and thereby evaluated the contribution of EPO originating from non-kidney cells to the hypoxia-dependent increase in circulating EPO. As control we used EPO purified from umbilical cord (UC) samples since the glycosylation pattern observed here most likely differentiates from that seen in adults (Wide and Bengtsson 1990). Based on our previous study conducted in acute hypoxia (Rasmussen et al. 2012), we hypothesized that EPO originating from the kidney will remain the main source for circulating EPO in hypoxic adult humans.

Methods

Seven healthy male sea-level dwellers with no exposure to altitudes above 2,000 m for the last 4 weeks (26 ± 4 years; 180 ± 1 cm; 76 ± 6 kg) participated in this study which was approved by the Ethical Committee for the Eidgenössische Technische Hochschule Zürich (EK 2011-N-51) and conducted in accordance with the declaration of Helsinki. Prior to the start of the experiments, informed oral and written consents were obtained. After baseline sampling in Zürich (480 m) all subjects were transported by train to the Jungfraujoch Research Station (3,454 m, Bernese Alps, Switzerland) where they stayed for 4 consecutive weeks. The facilities provide normal living conditions, comfortable room temperatures and access to food as consumed at sea level.

Urine and venous plasma samples were collected from all subjects at sea level (SL), and then after 1 (HA1; 17 ± 1 h), 3 (HA3; 65 ± 1 h) and 26 (HA26) days of altitude exposure, and again after 7 (SL + 7) and 14 (SL + 14) days upon return to sea level. All samples were stored at −80 °C.

Total plasma EPO concentrations were determined by means of a solid-phase sandwich ELISA kit and concentrations were determined based on the standard provided with the kit (Human Erythropoietin Quantikine IVD ELISA Kit, Quantikine, R&D Systems, Minneapolis, USA). Total urine EPO concentrations were determined by membrane-assisted isoform immunoassay (MAIIA) (EPO Quantification Urine Kit, MAIIA Diagnostics, Uppsala, Sweden) as specified by the manufacturer. In short, 0.5 ml of each urine sample was desalted and the EPO concentration determined by lateral flow immunoassay (Lönnberg et al. 2008): As reference, a dilution series of epoetin β (provided in the kit) ranging from 3 to 1,000 ng l^{-1} was measured by the MAIIA method; the obtained standard curve was fitted with a four-parameter logistic routine and from this the EPO concentrations in the urine samples were calculated (Lönnberg et al. 2012a).

The glycoform heterogeneity of the samples was analyzed by an EPO WGA MAIIA kit (MAIIA Diagnostics, Uppsala, Sweden) that previously have been used for detection of recombinant EPO in humans and horses (Lönnberg et al. 2012b; Lönnberg and Lundby 2013; Mørkeberg et al. 2013). In the present study we included umbilical cord (UC) plasma, which is known to contain mainly liver-derived EPO (Dame et al. 1998; Ohls 2002), as positive control for non-renal-derived EPO. For detailed description of the procedure refer to Lönnberg et al. (2012a). In brief, EPO from six UC plasma samples (2 ml) and from the urine samples (20 ml) obtained at SL, HA1, HA3 and HA26 were purified on single-use anti-EPO columns (EPO Purification Kit, MAIIA Diagnostics, Uppsala, Sweden) according to the directions of use. The average purification efficiency for the urine samples was 65 ± 10 %.

The EPO WGA MAIIA isoform distribution kit is based on affinity chromatography—with immobilized lectins [wheat germ agglutinin (WGA)] interacting with the glycosylated EPO forms and *N*-acetylglucosamine (Glc-NAc) used as competing sugar—in combination with lateral flow immunoassay. We established the optimal Glc-NAc concentration to be used in the elution buffer low by running pilot experiments at four different low Glc-NAc

concentrations (5, 10 15, and 20 mM) and one high Glc-NAc concentration (300 mM) on samples containing EPO purified from UC and urine. Epoetin β provided in the kit was included in all experiments and was used as reference (Lönnberg et al. 2012a). For the final determination of EPO glycoform distribution in the samples, 5 and 300 mM of Glc-NAc were used in the elution buffers low and high, respectively. All samples were run in duplicates and under standardized conditions (e.g. temperature, humidity, using the same scanner and reagent lot number). The percent migrated isoform (PMI) values were calculated as the percent of EPO released by the elution buffer low with respect to the total EPO concentration (obtained by elution buffer high).

Results and discussion

Umbilical cord EPO and circulating EPO (urine and plasma) from healthy subjects gave different PMI values at the lower concentrations of Glc-NAc (Fig. 1), verifying the difference in glycosylation patterns (EPO glycoforms) depending on the origin of synthesis.

As expected (Berglund et al. 2002; Robach et al. 2007; Siebenmann et al. 2012) a peak ($p < 0.05$) in urine (2.3 ± 0.5-fold) and plasma (3.3 ± 0.5-fold) EPO was observed on HA1 and although reduced from that point remained elevated at HA26 for plasma by 1.5 ± 0.2-fold. One week after return to sea level also the plasma EPO concentrations had returned to SL values (Fig. 2).

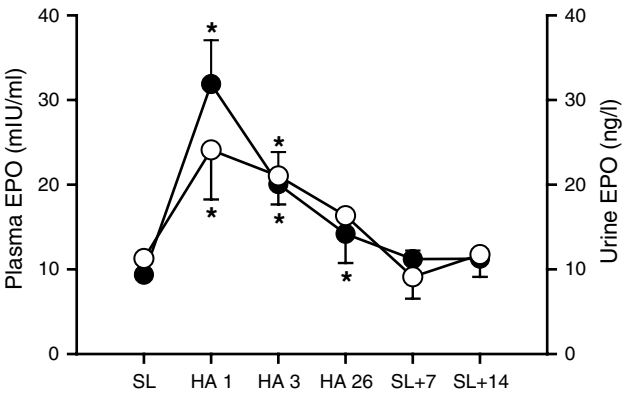

Fig. 2 Plasma (*open circles*) and urine (*filled circles*) EPO concentrations (mIU ml^{-1} and ng l^{-1}, respectively) in seven volunteers studied at sea level (SL) and after 1 (HA1), 3 (HA3) and 26 (HA26) days during exposure to 3,454 m altitude, and again after 7 (SL + 7) and 14 (SL + 14) days upon return to sea level. *$P < 0.05$ as compared to SL. Values are mean ± SEM

The EPO glycoform heterogeneity, expressed as percent migrated isoforms (PMI) (Lönnberg et al. 2012a) in the urine samples collected at altitude, did not differ from values at SL, but were markedly lower ($p < 0.05$) than the mean PMI for the umbilical cord samples (Fig. 3). Samples containing different EPO glycoforms (e.g. recombinant and endogenous EPO) have PMI values that are intermittent of the "pure glycoforms" (Lönnberg et al. 2012a, b, 2008; Lönnberg and Lundby 2013) and therefore a potential contribution of non-renal-derived EPO to the circulating EPO

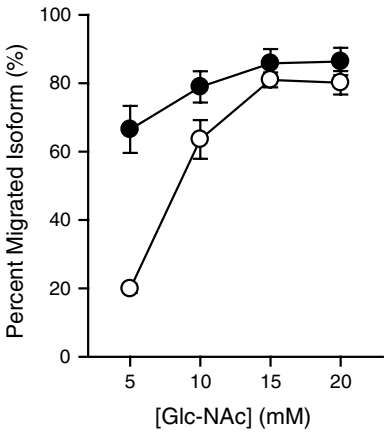

Fig. 1 The percent migrated isoform (PMI) values for umbilical cord EPO (*closed circles*) and circulating EPO from adults (*open circles*) when using different Glc-NAc concentrations in the elution buffer *low*. At the low (5 mM) Glc-NAc concentration, the difference in PMI between the two EPO glycoforms is greatest and due to differences in glycolation in the two populations of EPO. Values are mean ± SEM, $n = 6$ for UC, $n = 3$ for adults

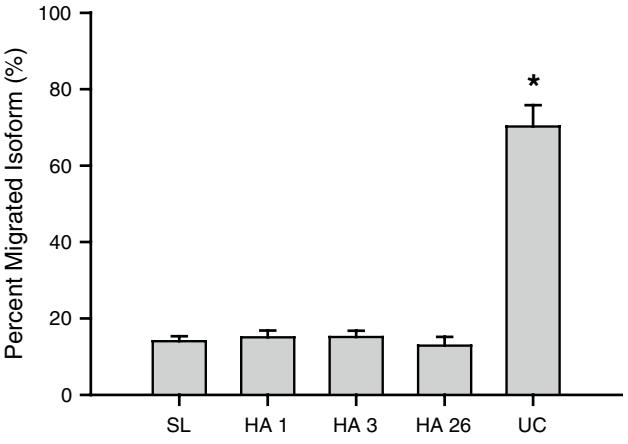

Fig. 3 EPO glycoform heterogeneity in samples obtained before and during acclimatization to high altitude. The PMI values are directly related to the glycoform composition of EPO in a given sample. Hence, for the HA samples an increase in PMI value from SL towards UC would be a consequence of a sample containing both renal and hepatic EPO. Abbreviations as for Fig. 1. Umbilical cord (UC) samples are here shown to have marked higher PMI values. *$P < 0.05$ as compared to the samples obtained in the adult volunteers. Values are mean ± SEM

should have altered the PMI values when comparing SL to HA samples. Since this was not the case in the present study, we demonstrate in healthy humans exposed to high-altitude (3,454 m) hypoxia that the predominant source for the rise in plasma EPO is the kidney. Although we did not include a control for CNS-derived EPO in the present study (currently unavailable), it has been shown that brain-derived EPO is less glycosylated than renal-derived EPO (Masuda et al. 1994), and hence an increase in PMI would be expected if more EPO was to be derived from the brain. Our findings are in line with previous studies on dogs, demonstrating that when exposed to 10 % hypoxia, renal venous EPO content was 63 % higher than arterial EPO content (Abbrecht and Malvin 1966). Although this does not exclude a contribution from other cells, the findings suggest a massive contribution of the kidneys to the circulating EPO response to hypoxia. When Bernhardt et al. (2010) administrated a prolyl hydroxylase inhibitor, which stabilizes HIF expression, kidney transplant patients demonstrated no increase in circulating EPO whereas this was the case in healthy volunteers. Thus, also from a biochemical perspective it seems clear that the kidneys are indeed the predominant organs responsible for the synthesis of circulating EPO when stimulated by HIF, as expected at high altitude. Our study demonstrates the differences in EPO glycoform distribution in neonatal umbilical cord samples as compared to adults, and thereby extends previous tissue mRNA work (Dame et al. 1998; Ohls 2002) suggesting liver cells to be the main site for EPO synthesis during gestation. Thus, although the human liver in the neonatal state has an important role in the synthesis of EPO and although astrocyte-derived EPO may account for up to 50 % of the erythropoietic response in mice when exposed to hypoxia, this does not seem to be relevant for the circulating EPO levels in healthy adult humans when exposed to continuous hypoxia.

Conflict of interest The authors declare not to have any conflict of interest with regard to the study.

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