ACTA PHYSIOLOGICA

Acta Physiol 2014

On the antioxidant properties of erythropoietin and its association with the oxidative–nitrosative stress response to hypoxia in humans

D. M. Bailey,[1,2] C. Lundby,[3] R. M. G. Berg,[4] S. Taudorf,[4] H. Rahmouni,[2] M. Gutowski,[5] C. W. Mulholland,[1] J. L. Sullivan,[6] E. R. Swenson,[7] J. McEneny,[8] I. S. Young,[8] B. K. Pedersen,[4] K. Møller,[4,9] S. Pietri[2] and M. Culcasi[2]

1 Neurovascular Research Laboratory, Faculty of Life Sciences and Education, University of South Wales, Treforest, UK

2 Aix-Marseille Université, CNRS, Institut de Chimie Radicalaire UMR 7273, Équipe Sondes Moleculaires en Biologie et Stress Oxydant, Marseille, France

3 Center for Integrative Human Physiology, Institute of Physiology, University of Zurich, Zurich, Switzerland

4 Department of Infectious Diseases, Centre of Inflammation and Metabolism, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

5 Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

6 Burnett College of Biomedical Sciences, University of Central Florida, Orlando, FL, USA

7 Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of Washington, Seattle, WA, USA

8 Centre for Clinical and Population Sciences, Queen's University Belfast, Belfast, UK

9 Department of Neuroanaesthesiology, University Hospital Rigshospitalet, Copenhagen, Denmark

Received 6 February 2014, revision requested 14 April 2014, revision received 26 April 2014, accepted 5 May 2014

Correspondence: D. M. Bailey, Neurovascular Research Laboratory, Faculty of Life Sciences and Education, University of South Wales, Treforest CF37 4AT, UK. E-mail: damian.bailey@southwales.ac.uk

Abstract

Aim: The aim of this study was to examine if erythropoietin (EPO) has the potential to act as a biological antioxidant and determine the underlying mechanisms.

Methods: The rate at which its recombinant form (rHuEPO) reacts with hydroxyl ($HO^{\cdot}$), 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) and peroxyl ($ROO^{\cdot}$) radicals was evaluated *in-vitro*. The relationship between the erythopoietic and oxidative–nitrosative stress response to poikilocapneic hypoxia was determined separately *in-vivo* by sampling arterial blood from eleven males in normoxia and following 12 h exposure to 13% oxygen. Electron paramagnetic resonance spectroscopy, ELISA and ozone-based chemiluminescence were employed for direct detection of ascorbate ($A^{\cdot -}$) and *N*-tert-butyl-α-phenylnitrone spin-trapped alkoxyl (PBN-OR) radicals, 3-nitrotyrosine (3-NT) and nitrite (NO_2^-).

Results: We found rHuEPO to be a potent scavenger of $HO^{\cdot}$ (k_r = 1.03–1.66 × 10^{11} M^{-1} s^{-1}) with the capacity to inhibit Fenton chemistry through catalytic iron chelation. Its ability to scavenge $DPPH^{\cdot}$ and $ROO^{\cdot}$ was also superior compared to other more conventional antioxidants. Hypoxia was associated with a rise in arterial EPO and free radical-mediated reduction in nitric oxide, indicative of oxidative–nitrosative stress. The latter was confirmed by an increased systemic formation of $A^{\cdot -}$, PBN-OR, 3-NT and corresponding loss of NO_2^- ($P < 0.05$ vs. normoxia). The erythropoietic and oxidative–nitrosative stress responses were consistently related ($r = -0.52$ to 0.68, $P < 0.05$).

 doi: 10.1111/apha.12313

Conclusion: These findings demonstrate that EPO has the capacity to act as a biological antioxidant and provide a mechanistic basis for its reported cytoprotective benefits within the clinical setting.
Keywords antioxidants, erythropoietin, free radicals, hypoxia, iron chelation.

In its classical role, EPO is the key regulator of erythropoiesis, promoting the survival, proliferation and differentiation of erythrocytic progenitors to increase red blood cell mass for the maintenance of vascular oxygen (O_2) homeostasis (Jelkmann 2011). Broader interest in EPO as a paracrine-autocrine cytoprotective molecule has evolved over recent years which has traditionally been attributed to antiapoptotic signalling (Brines & Cerami 2005). However, emerging evidence suggests that it may equally function as an antioxidant given its ability to attenuate free radical-mediated lipid peroxidation in patients undergoing haemodialysis and those suffering from anaemia of chronic renal failure (Katavetin *et al.* 2007) when administered in its recombinant form (rHuEPO).

While the mechanisms remain unclear, preliminary evidence *in-vitro* suggests that rHuEPO may inhibit metal-catalysed Fenton formation of the hydroxyl radical ($HO^{\cdot}$; Chattopadhyay *et al.* 2000):

$$M^{n+} + H_2O_2 \rightarrow HO^{\cdot} + M^{(n+1)+} + OH^{-} \quad (1)$$

However, to what extent this glycoprotein scavenges $HO^{\cdot}$ directly or limits its formation by inactivating Fenton chemistry subsequent to chelation of M^{n+} (notably Fe^{2+}) during the course of erythropoiesis when iron mobilization is known to increase dramatically, remains unknown (Bailey *et al.* 2006b). Furthermore, there are hitherto no published reports that have documented the apparent second-order rate constant for the reaction of rHuEPO with $HO^{\cdot}$ (k_{rHuEPO}) to compare its scavenging potential against other, more conventional antioxidants.

In light of these findings, an interdisciplinary study taking advantage of combined *in-vitro*/*in-vivo* experimental approaches was undertaken to determine if EPO has the potential to act as a biological antioxidant. First, we sought to determine the underlying mechanisms and rate at which rHuEPO reacts with $HO^{\cdot}$. We hypothesized that rHuEPO would function as an outstanding antioxidant due to its combined ability to directly scavenge $HO^{\cdot}$ including other biologically relevant free radicals and indirectly suppress Fenton chemistry via catalytic iron chelation. Second, we exposed human participants to inspiratory hypoxia for a period of 12 h given that it is an established model for the combined induction of erythropoiesis and oxidative–nitrosative stress, the latter reflected by a free radical-mediated reduction in the vascular bioavailability of nitric oxide (Bailey *et al.* 2009b). While not disassociating cause from effect, we hypothesized that if erythropoiesis does indeed represent an 'acute phase' antioxidant response to hypoxia, we would observe a linear relationship between the systemic rise in the blood–borne concentration of EPO and biomarkers of oxidative–nitrosative stress.

Methods

Ethics

The study was approved by the Scientific Ethics Committee of Copenhagen and Frederiksberg Municipalities, Denmark [file number (KF) 01 290011]. All human procedures were carried out in accordance with the Declaration of Helsinki of the World Medical Association (Williams 2008) and informed consent was obtained from all participants.

Participants and design

Eleven healthy males aged 27 ± 4 years old volunteered for the study. Following a 12 h overnight fast, a catheter was placed under local anaesthesia in the radial artery for the dual purpose of blood sampling and invasive measurement of (mean) arterial blood pressure (MAP). Samples were obtained in normoxia (21% O_2) and following 12 h passive exposure to poikilocapneic normobaric hypoxia (13% O_2). The respective inspirates were achieved by connecting each participant through a tight-fitting mask with a non-rebreathing valve to medical-grade gas directed from compressed gas cylinders into a 500 L meteorological balloon at the prevailing barometric pressure.

Chemicals and reagents

Chemicals were purchased from Sigma-Aldrich® (Poole, Dorset, UK) and Acros Organics (Geel, Belgium). We obtained rHuEPO from an injectable syringe (NeoRecormon; Roche, Mannheim, Germany) that contained 5000 IU of epoietin beta dissolved in 0.3 mL of physiological saline (+10% v/v excipients). All phosphate-buffered aqueous solutions were pretreated with Chelex 100® to remove adventitious metals as verified by the ascorbate test (Buettner 1988).

  doi: 10.1111/apha.12313

In-vitro *experimentation*

Kinetic methods. General—Steady-state competition kinetics experiments were performed to evaluate k_{rHuEPO} where $HO^{\cdot}$ were generated either by photolysis or Fenton chemistry. The ability of rHuEPO to scavenge 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) and peroxyl ($ROO^{\cdot}$) radicals was also assessed as a global measure of antioxidant capacity. Electron paramagnetic resonance (EPR) spectra were obtained at X-band (9.79 GHz) with Bruker (Karlsruhe, Germany) ESP 300 (kinetic experiments) or EMX (other experiments) spectrometers using 100 kHz modulation frequency, 10 mW microwave power (MP) and standard TM_{110} cavities unless otherwise stated.

Determination of rate constants on $HO^{\cdot}$ by EPR spin trapping—2-Hydroxy-5,5-dimethyl-1-pyrrolidinyloxyl radical (DMPO-OH), the DMPO/$HO^{\cdot}$ spin adduct, was produced by photolysing a reaction mixture (0.5 mL final volume) containing a 3% v/v solution of H_2O_2 in the presence of 5 mM 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and varying concentrations of rHuEPO in 150 mM phosphate buffer (pH 7.0). The samples were introduced into 50 μL glass capillary tubes (Hirschmann Laborgeräte, Eberstadt, Germany), sealed with Critoseal® at both ends and tuned within the EPR cavity. Each sample was then continuously photolysed in situ with a 1000 W ultraviolet xenon–mercury lamp (Oriel; Newport Corp, Irvine, CA, USA) and EPR acquisition of DMPO-OH 1 : 2 : 2 : 1 EPR quartets [coupling constants, in Gauss (G): $a_N = 14.98$ and $a_{H\beta} = 14.72$ with a line width of 0.6 G, Fig. 1a) was initiated 5 s after light was activated by signal averaging five scans using the following parameters: magnetic field resolution (*R*), 4096 points; modulation amplitude (MA), 0.70 G; receiver gain (RG), 8×10^4; time constant (TC), 20.48 ms; sweep rate (SR), 1.79 G s^{-1} for a sweep width (SW) of 75 G. In the absence of UV light no significant background DMPO adducts were detected. The general reaction scheme for the trapping of $HO^{\cdot}$ by DMPO in the presence of rHuEPO is detailed below:

I_0(Intensity of control signal):

$$H_2O_2 \xrightarrow{h\nu} 2HO^{\cdot} \xrightarrow{+DMPO} \text{DMPO-OH} \quad (2)$$

I(Intensity of the signal in the presence of rHuEPO):

$$H_2O_2 \xrightarrow{h\nu} 2HO^{\cdot} \underset{+rHuEPO}{\overset{+DMPO}{\rightarrow}} \text{DMPO-OH} \quad (3)$$

Assuming that EPO can compete with DMPO for $HO^{\cdot}$ trapping yielding EPR-silent species then $I < I_0$. The following equation was used to calculate k_{DMPO} (the apparent second-order rate constant for the reaction of $HO^{\cdot}$ with DMPO) and k_{rHuEPO}:

$$\frac{I_0}{I} = 1 + \frac{k_{rHuEPO}}{k_{DMPO} \cdot c_{DMPO}} \cdot c_{rHuEPO} \quad (4)$$

where c_{DMPO} and c_{rHuEPO} are the concentrations of DMPO and rHuEPO respectively.

The slope of the regression plot of I_0/I against c_{rHuEPO} for a constant value of c_{DMPO} (5 mM) was used to calculate k_{rHuEPO} (Finkelstein *et al.* 1980):

$$k_{rHuEPO} = \text{slope} \times k_{DMPO} \times c_{DMPO} \quad (5)$$

The control signal was obtained as above but in the absence of rHuEPO and k_{DMPO} was assumed to be 3.4×10^9 M^{-1} s^{-1} using EPR and the same $HO^{\cdot}$ generation conditions (Finkelstein *et al.* 1980).

Determination of rate constants on $HO^{\cdot}$ by the deoxyribose assay—The inhibition of 2-Deoxy-d-ribose (DR) degradation was employed according to established methods (Halliwell *et al.* 1987). Briefly, the Fenton formation of $HO^{\cdot}$ can be detected by its ability to degrade DR into fragments that on heating with thiobarbituric acid (TBA) generate a pink chromogen:

$$Fe^{3+}\text{-EDTA} + \text{ascorbate} \rightarrow Fe^{2+}\text{-EDTA} + \text{oxidized ascorbate} \quad (6)$$

$$Fe^{2+}\text{-EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{-EDTA} + HO^{\cdot} + OH^- \quad (7)$$

In the presence of excess EDTA, any $HO^{\cdot}$ generated in Eq. (7) becomes accessible to DR and any other potential scavenger including rHuEPO. The ability of rHuEPO to competitively inhibit DR degradation therefore reflects its ability to scavenge $HO^{\cdot}$ from which an apparent rate constant can be calculated (Halliwell *et al.* 1987). The reaction mixture (1 mL final volume) contained DR (2.8 mM) dissolved in KH_2PO_4–KOH buffer (100 μL, 20 mM final concentration, pH 7.4), rHuEPO (500 μL) at varying concentrations, $FeCl_3$ (100 μL, 100 μM), ±EDTA (100 μL, 104 μM), H_2O_2 (100 μL, 1 mM) and ascorbate (100 μL, 104 μM). After 1 h at 37 °C, DR degradation was measured by the reaction of formed malonaldehyde (MDA) with TBA. TBA (1.0 mL, 1% in 50 mM NaOH) and 2.8% trichloroacetic acid (1.0 mL) were added to the reaction mixture and heated at 100 °C for 20 min. After cooling, absorbance was measured at 532 nm against a blank containing only buffer and DR. From the slope of the competition plot for inhibition of DR oxidation by various rHuEPO concentrations, k_{rHuEPO} was

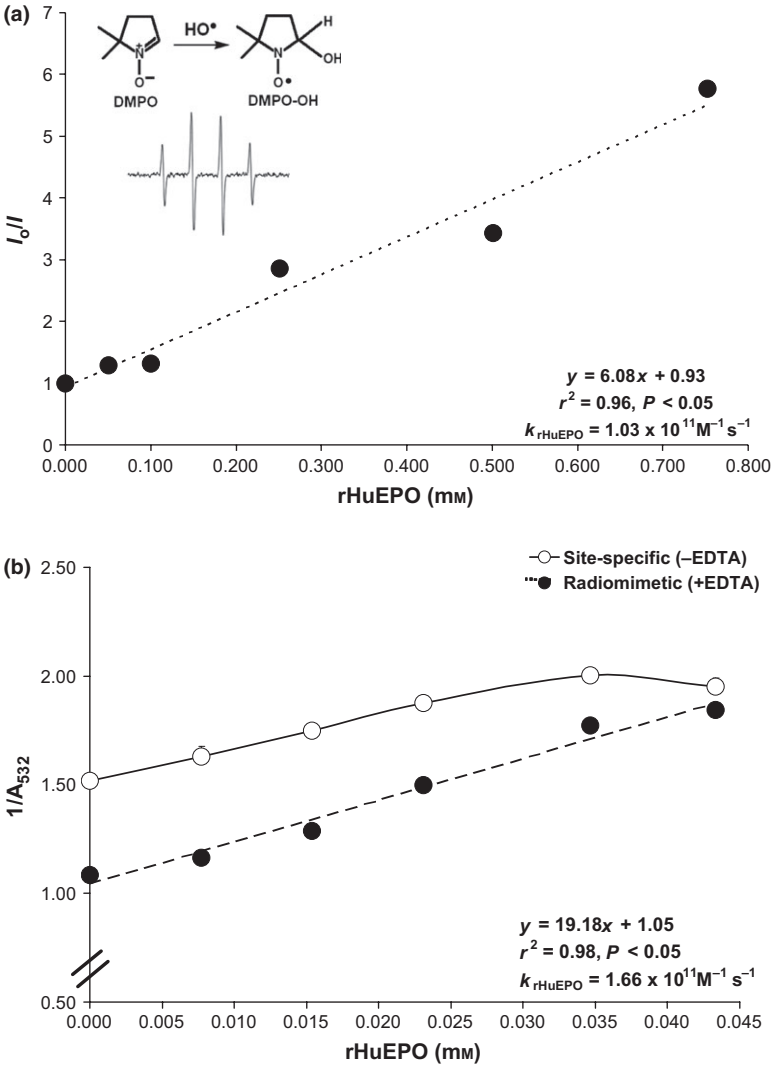

Figure 1 Competition plots of $HO^{\cdot}$ scavenging by rHuEPO. $HO^{\cdot}$ radicals generated in the presence of varying rHuEPO concentrations by: (a) photolysis of H_2O_2 followed by EPR spin-trapping detection using DMPO as the spin trap and (b) a [H_2O_2/Fe^{3+}/ascorbate] Fenton generator in the presence of 2-deoxyribose and in the presence (+; radiomimetic condition) or absence (−; site-specific condition) of EDTA. Corresponding rate constant (k_{rHuEPO}) for the photolytic and radiomimetic methods are shown. Molecular basis of spin-trapping of $HO^{\cdot}$ using DMPO and typical control DMPO-OH EPR spectrum also illustrated (a inset).

calculated according to:

$$k_{rHuEPO} = \text{slope} \times k_{DR} \times c_{DR} \times A^0 \quad (8)$$

where A^0 is the absorbance of the blank, c_{DR} is the DR concentration and k_{DR}, the apparent second-order rate constant for the reaction of $HO^{\cdot}$ with DR, is 3.1×10^9 M^{-1} s^{-1} (Halliwell *et al.* 1987). In experiments aimed at evaluating the ability of rHuEPO to interact with site-specific $HO^{\cdot}$-induced damage to DR, the assay was run as described above while omitting EDTA in the reaction mixtures.

DPPH assay. The free radical scavenging capacity of rHuEPO was evaluated using the $DPPH^{\cdot}$ assay (Prior *et al.* 2005). Briefly, the reaction was carried out using a fresh $DPPH^{\cdot}$ solution (1.5 mL, 0.13 mM final concentration) in methanol (final volume adjusted to 2.25 mL) and rHuEPO (1.22 μM final concentration corresponding to two entire 0.3 mL syringes). The contents were mixed, incubated at room temperature in the dark for 3 min and the corresponding reduction in absorbance measured at 517 nm using an UVmc^2 spectrophotometer (SAFAS, Monaco). Trolox was employed (1.22–25 mM) as the reference antioxidant and corresponding $DPPH^{\cdot}$ scavenging efficiency calculated as:

$$\Delta DPPH^{\cdot} = 100 - \left(\frac{A_{90\,m}}{A_{0\,m}} \times 100\right)\% \quad (9)$$

whereby A_{90m} and A_{0m} refer to the absorbances recorded after 90-min incubation and baseline respectively. The EC_{50} was also calculated, given as the incubation time required to half the initial DPPH concentration.

TRAP assay. The TRAP assay, which determines the potential of a water-soluble antioxidant to inhibit free radical-induced, luminol-dependent chemiluminescence,

was performed in Tris-HCl buffer (0.1 M, pH 7.4) using a modification of reported methods (Bastos *et al.* 2003, Dresch *et al.* 2009). Luminol solutions were prepared daily and kept in darkness at 4 °C until use. The $ROO^{\cdot}$ source 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 0.15 M in 10 mL Tris-HCl) was heated at 37 °C for 30 min, then the temperature was decreased to 25 °C allowing the resulting free radical solution to be used for up to 20 min. The luminescence at 25 °C of samples (3 mL final volume) containing the heated AAPH solution (2 mL), 13 μM luminol (0.5 mL) and 0.5 mL of a Tris-HCl : dimethyl sulphoxide (49 : 1) solution of the test compound to reach the final concentrations of: 0.23–6 μM rHuEPO, 0.1–160 μM fatty-acid-free bovine serum albumin (BSA), 1–10 μM caffeic acid and 1–20 μM 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were measured after 60 seconds stirring (Sirius luminometer; Titertek-Berthold, Pforzheim, Germany). The TRAP (expressed in units equivalent of μM Trolox) was then calculated by determining for each compound a linear correlation between the concentration and the suppression of the area-under-curve (ΔAUC) as compared to the corresponding test compound-free blank (Bastos *et al.* 2003).

In-vivo *experimentation*

With the exception of blood gases (immediate analysis), whole blood was centrifuged at 600 ***g*** (4 °C) for 10 min and the supernatant snap-frozen and stored under liquid nitrogen (N_2, Cryopak CP100; Taylor-Wharton, Theodore, AL, USA) prior to analysis.

Oxygenation. Whole blood was presented anaerobically to a blood gas analyser (ABL OSM; Radiometer Medical, Brønshøj, Denmark) for the measurement of haemoglobin (Hb), haematocrit (Hct), oxyhaemoglobin saturation (SO_2) and partial pressure of oxygen (PO_2). Arterial O_2 content (caO_2 in mM) was calculated as $0.96 \times SaO_2 \times Hb + 0.0013 \times PaO_2$. The intra- and inter-assay co-efficients of variation (CV) for all assays were both <5%.

Oxidative stress. First, the plasma concentration of the long-lived ascorbate radical ($A^{\cdot -}$) was determined as a direct measure of 'global' free radical formation (Buettner & Jurkiewicz 1993). K-EDTA plasma (1 mL) was injected into a high-sensitivity multiple-bore sample cell (AquaX; Bruker Instruments, Billerica, MA, USA) and the characteristic doublet of $A^{\cdot -}$ ($a_{H\beta}$ = 1.76 G) was recorded 12 min after the end of plasma recovery by signal averaging three scans with the following parameters: *R*, 1024 points: MP, 20 mW; MA, 0.65 G; RG, 2×10^5; TC, 40.96 ms; SR, 0.25 G s^{-1}; SW, 15 G. The intra- and inter-assay CV's were both <10%. Second, spin trapping using PBN was employed for the specific detection of lipid-derived radicals formed during the process of lipid peroxidation (Bailey *et al.* 2009b). Aliquots (4.5 mL) of whole blood were added to a 6 mL glass vacutainer (SST) primed with 1.5 mL of PBN dissolved in physiological saline (50 mM final concentration). The vacutainer was gently mixed then placed in the dark to clot for 10 min. Following centrifugation, 1 mL of the serum-adduct was added to a boro-silicate glass tube containing 1 mL of spectroscopic-grade toluene and vortex-mixed for 10 s. The sample was centrifuged at 600 ***g*** for a further 10 min and 200 μL of the organic supernatant added to a (N_2-flushed) precision-bore quartz EPR tube, vacuum-degassed (3× freeze-thaw cycles) to remove O_2 and blocks of 10 incremental EPR scans were recorded using the following parameters: *R*, 2048 points; MA, 0.50 G; RG, 1×10^5; TC, 82 ms; SR, 0.4 G s^{-1}; SW, 50 G. The intra- and inter-assay CV's were both <10%.

Antioxidant status. For ascorbic acid measurements, plasma was stabilized and deproteinated by adding 900 μL of 5% metaphosphoric acid to 100 μL K-EDTA plasma then assayed by fluorimetry based on the condensation of dehydroascorbic acid with 1,2-phenylenediamine (Vuilleumier & Keck 1993). Concentrations of α and γ-tocopherol were determined by high-performance liquid chromatography (Catignani & Bieri 1983, Thurnham *et al.* 1988). The intra- and inter-assay CV's were both <10%.

Nitrosative stress. Plasma NO_2^- was measured by ozone-based chemiluminescence (Bailey *et al.* 2009b). Plasma (200 μL) was injected into tri-iodide reagent (Rogers *et al.* 2007) for the measurement of NO_2^- + *S*-nitrosothiols (not reported here) and 5% acidified sulphanilamide added and left to incubate in the dark at 21 °C for 15 min to remove NO_2^- in a second parallel sample for determination of RSNO. Plasma NO_2^- was calculated as (NO_2^- + RSNO) − RSNO. Plasma 3-nitrotyrosine (3-NT) was employed as a surrogate biomarker of peroxynitrite formation subsequent to the oxidative inactivation of nitric oxide (NO; Pacher *et al.* 2007). This was measured by Enzyme-Linked Immuno-Sorbent Assay (ELISA; Hycult Biotechnology, b.v., Uden, the Netherlands) with a lower detection limit of 2 nM and intra/inter-assay CV of <2 and <5% respectively. All calculations were performed using Origin/Peak Analysis software (OriginLab, Northampton, MA, USA).

Inflammatory stress. White blood cell and differential counts were determined according to standard

laboratory techniques. Duplicate samples (mean calculated) of K-EDTA plasma (100 μL) were analysed for interleukin (IL)-6 using a high-sensitivity ELISA (R&D Systems, Minneapolis, MN, USA). High-sensitivity CRP (hsCRP) was measured using a latex-enhanced immunoturbidimetric assay (Randox Laboratories, Crumlin, UK) on a Cobas FARA bioanalyser (Roche Products, Herts, UK). The intra- and inter-assay CV's were both <15%.

Erythropoiesis. We analysed K-EDTA plasma (100 μL) for erythropoietin (EPO) using hs ELISA (Quantikine IVD; R&D Systems). The intra- and inter-assay CV's were both <10%.

EPR signal quantification. The DMPO-OH adducts were not filtered and simulated using WINSIM software (Duling 1994). For $A^{\cdot-}$ and PBN-adducts, spectra were filtered identically and simulated using BRUKER WINEPR 2.11 (Bruker, Rheinstetten, Germany). Free radical concentrations were determined by double integration of filtered or simulated spectra using ORIGIN 5.0 software (Microcal Software, Inc., Northampton, MA, USA).

Acute mountain sickness. Symptoms of acute mountain sickness (AMS) were assessed using the Lake Louise (LL – self assessment + clinical scores; Roach *et al.* 1993) and Environmental Symptoms Questionnaire Cerebral (ESQ-C; Sampson *et al.* 1983) scoring systems. A visual analogue scale (VAS) (Iversen *et al.* 1989) was employed to examine headache. Clinical AMS (moderate to severe) was diagnosed if a participant presented with a total LL score of ≥5 points in the presence of a headache and ESQ-C score ≥0.7 points (Bailey *et al.* 2006c) at the 12-h hypoxia exposure time-point.

Statistics

Following confirmation of distribution normality using Shapiro–Wilk *W*-tests, data were analysed using paired samples *t*-tests and relationships determined with Pearson Product Moment Correlations. Significance was established at $P < 0.05$ and data expressed as mean ± standard deviation (SD).

Results

In-vitro *study*

Steady-state competition kinetics. Figure 1(a,b) illustrate the competition plots of $HO^{\cdot}$ scavenging by rHuEPO using the photolytic and Fenton-derived $HO^{\cdot}$ generating systems respectively. The corresponding k_{rHuEPO} values obtained from Eqs (5) and (8) were in close agreement and were typical of diffusion-controlled reactions, ranging between 1.03–1.66 × 10^{11} M^{-1} s^{-1}. Control experiments identified that rHuEPO did not interfere with the assessment of DR degradation or react directly with $HO^{\cdot}$ to yield TBA-reactive material. Furthermore, the addition of rHuEPO did not alter the EPR signal intensity of the DMPO-$OH^{\cdot}$ adduct and did not reduce Fe^{3+} to Fe^{2+} to cause artifactual $HO^{\cdot}$ formation.

Chelation of transition metal ions. The finding of lower absorption values in the site-specific (Fig. 1b) vs. radiomimetic DR suggests that rHuEPO was able to inhibit iron ion-dependent DR degradation and thus capable of binding these irons into chelates with a lower ability to form $HO^{\cdot}$ than EDTA. The kinetics of this reaction were complex and a maximum inhibition of 25% was obtained with 0.8 mM rHuEPO.

Free radical scavenging. Figure 2 illustrates that the ability of rHuEPO to scavenge $DPPH^{\cdot}$ was superior to that exhibited by the same concentration of the reference antioxidant Trolox. Indeed, the scavenging kinetics of rHuEPO followed an exponential (as opposed to Trolox's more linear) decay with over twice the reduction in $DPPH^{\cdot}$ observed following 90 min of incubation and equivalent to that achieved with over a 10-fold concentration of Trolox. The EC_{50} was approx. 43 min for rHuEPO and approx. 1 min for (25 μM) Trolox.

TRAP assay. In this assay, rHuEPO behaved similarly to BSA, suppressing chemiluminescence emis-

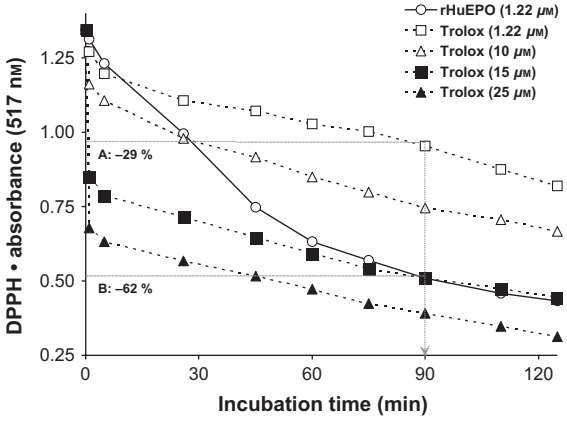

Figure 2 Scavenging of $DPPH^{\cdot}$ (0.13 mM) by rHuEPO as compared to Trolox. Stippled lines compare the $DPPH^{\cdot}$ scavenging efficiency (reduction in $DPPH^{\cdot}$ after 90 min incubation relative to the baseline control) of Trolox (a) and rHuEPO (b) at identical concentrations (1.22 μM).

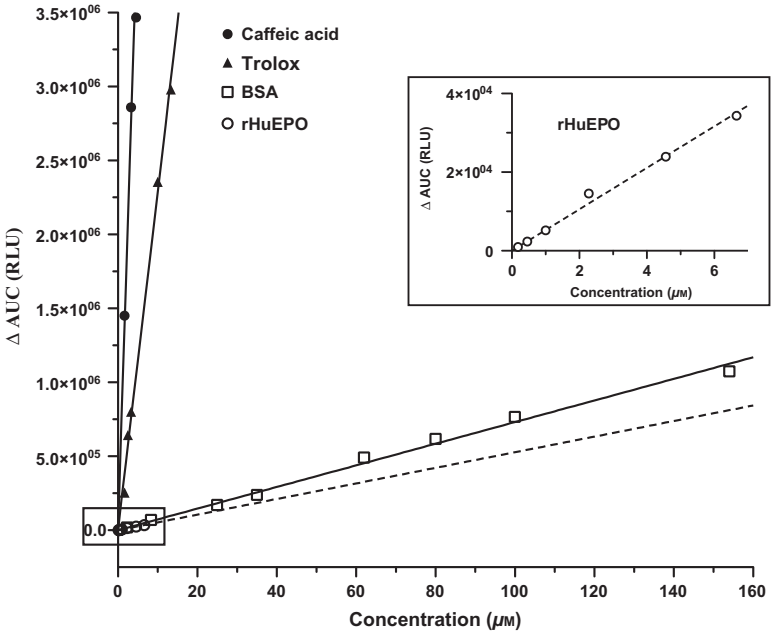

Figure 3 TRAP assay comparing the antioxidant properties of rHuEPO against BSA and reference antioxidants. Inset shows an expansion of the tested rHuEPO concentration range; AUC, area under curve; RLU, relative units equivalent of μM Trolox; BSA, bovine serum albumin.

sion in a concentration-dependent manner and linear correlations with ΔAUC were observed (Fig. 3) that allowed comparison with standard antioxidant Trolox (having TRAP value set at 1), yielding the following TRAP values with rHuEPO consistently shown to be the most effective compound: rHuEPO, 0.022; BSA, 0.032 (consistent with published literature; Pascual & Reinhart 1999) and caffeic acid, 3.77.

In-vivo *study*

Oxygenation. As anticipated, hypoxia resulted in marked hypoxaemia as indicated by a reduction in PaO_2, SaO_2 and corresponding caO_2 (Table 1).

Oxidative stress. Hypoxia was associated with a mild reduction in the systemic (arterial) concentration of ascorbate and γ-tocopherol and corresponding increase in $A^{\cdot-}$ and PBN-adducts (Table 1). In spin trapping experiments using PBN computer simulation of the recorded spin adducts confirmed they exhibited nitrogen and hydrogen couplings of 13.7 and 1.9 G, respectively, consistent with the trapping of alkoxyl (PBN-OR) radicals.

Nitrosative stress. A reduction in NO_2^- and corresponding increase in 3-NT were observed during hypoxia (Table 1).

Inflammatory stress. Hypoxia was also accompanied by a mild inflammatory response confirmed by neutrophilia and combined increases in IL-6 and hsCRP (Table 1).

Erythropoiesis. Hypoxia was associated with an increase in EPO that correlated against the elevation in $A^{\cdot-}$ and PBN-OR (Fig. 4a,b) and corresponding reduction in NO_2^- ($r = -0.52$, $P < 0.05$).

Acute mountain sickness. Hypoxia was shown to induce symptoms of AMS (LL: 3 ± 2 vs. normoxia: 0 ± 0 points, $P < 0.05$; ESQ-C: 0.725 ± 0.711 vs. 0.000 ± 0.000 arbitrary units, $P < 0.05$) and an increase in corresponding headache scores (VAS: 20 ± 17 vs. 0 ± 0 mm, $P < 0.05$). Clinical AMS was diagnosed in three participants.

Mean arterial pressure. Hypoxia did not alter MAP (97 ± 9 vs. normoxia: 96 ± 8 mmHg, $P > 0.05$).

Discussion

Our interdisciplinary experimental approach has revealed several important findings. First, in its recombinant form, EPO was shown to be a potent scavenger of $HO^{\cdot}$ with the capacity to inhibit Fenton chemistry through catalytic iron chelation. Its ability to scavenge other biologically relevant radicals was also found to be superior compared to other more conventional antioxidants. Second, though not disassociating cause from effect, the linear relationships observed between the hypoxia-induced rise in the systemic formation of EPO and biomarkers of oxidative–nitrosative stress in the human study may reflect an acute phase antioxidant defence response that serves to maintain redox homeostasis. Collectively, these findings highlight the potential for EPO to function as a biological antioxidant providing a

Table 1 Metabolic response to hypoxia

Inspirate	Normoxia (0 h)	Hypoxia (12 h)
Oxygenation		
PaO_2 (mmHg)	106 ± 6	50 ± 4*
SaO_2 (%)	99 ± 1	86 ± 3*
caO_2 (mM)	8.46 ± 0.37	7.25 ± 0.46*
Oxidative stress		
Ascorbate (μM)	53.4 ± 11.2	49.8 ± 9.7*
α-tocopherol (μM)	34.0 ± 6.0	34.1 ± 7.6
γ-tocopherol (μM)	2.3 ± 1.0	1.8 ± 0.7*
$A^{\cdot -}$ (AU)	2384 ± 166	3087 ± 276*
PBN-OR (AU)	16 321 ± 2488	19 076 ± 2122*
Nitrosative stress		
NO_2^- (nM)	474 ± 198	291 ± 119*
3-NT (nM)	86 ± 133	102 ± 153*
Inflammatory stress		
Total white blood cells (10^9 L^{-1})	5.1 ± 1.3	8.2 ± 1.3*
Neutrophils (10^9 L^{-1})	2.8 ± 1.1	5.8 ± 1.6*
IL-6 (pg mL^{-1})	1.66 ± 1.93	3.38 ± 2.14*
hsCRP (mg L^{-1})	0.9 ± 0.8	1.8 ± 0.7*
Erythropoiesis		
EPO (mU mL^{-1})	6.2 ± 2.0	22.1 ± 6.3*

Values are mean ± SD; PaO_2, arterial partial pressure of oxygen; SaO_2, arterial oxyhaemoglobin saturation; caO_2, arterial O_2 content; $A^{\cdot -}$, ascorbate radical; PBN-OR, *N*-tert-butyl-α-phenylnitrone spin-trapped alkoxyl radicals; AU, arbitrary units; IL, interleukin; hsCRP, high-sensitivity C-reactive protein; *different ($P < 0.05$).

mechanistic basis for its reported cytoprotective benefits *in-vivo*.

It has been suggested that EPO may exert antioxidant effects through the induction of glutathione peroxidise (Genc *et al.* 2002, Kumral *et al.* 2005) and haeme oxygenase-1, accelerating destabilized haeme degradation and corresponding formation of the antioxidant metabolites biliverdin and bilirubin (Calo *et al.* 2003). Alternatively, EPO may suppress the Fenton formation of $HO^{\cdot}$ given its capacity to mobilize catalytic iron during the course of erythropoiesis (Bany-Mohammed *et al.* 1996, Bailey *et al.* 2006b) though clear evidence is lacking including its ability to function as a direct $HO^{\cdot}$ scavenger.

To provide clearer insight into related mechanisms, we calculated the apparent rate constant for the reaction of rHuEPO with $HO^{\cdot}$ (k_{rHuEPO}) using two different $HO^{\cdot}$ generating systems. The corresponding value observed during photolytic formation of $HO^{\cdot}$ was shown to be diffusion-controlled (1.0×10^{11} M^{-1} s^{-1}) highlighting rHuEPO's ability to function as a direct scavenger. A similar value was obtained following Fenton-formation of $HO^{\cdot}$ (1.7×10^{11} M^{-1} s^{-1}) which was found to be two orders of magnitude greater than that observed for other more established $HO^{\cdot}$ scavengers including salicylate ($6.0–9.6 \times 10^9$ M^{-1} s^{-1}) and mannitol ($1.0–2.0 \times 10^9$ M^{-1} s^{-1}) using the same experimental approach (Halliwell *et al.* 1987).

Precisely why rHuEPO is such a potent $HO^{\cdot}$ scavenger remains unclear. Given that a diffusion-controlled bimolecular reaction between two compounds has a theoretical limit of $k_{DR} = 7 \times 10^9$ M^{-1} s^{-1} assuming a single target site (Alberty & Hammes 1958), the observed k_{rHuEPO} indicates that rHuEPO has approx. 24 target sites for $HO^{\cdot}$. Its large molecular mass (18 396 g M^{-1}) combined with a high sugar and basic amino acid content coupled with charged residues that are characteristic features of small glycopolypeptides may act as 'sinks' for $HO^{\cdot}$ (Chattopadhyay *et al.* 2000). Furthermore, the molecule is highly enriched with sialic acid (Inoue *et al.* 1993), an established $HO^{\cdot}$ scavenger (Ogasawara *et al.* 2007).

However, given its high one-electron reduction potential ($E^{\circ\prime} = +2310$ mV; Koppenol & Butler 1985), $HO^{\cdot}$ can react with most organic molecules at near diffusion-controlled rates. Thus, it is unlikely at the low plasma concentrations encountered (10^{-11} M L^{-1}) that EPO would function directly as a scavenger *in-vivo* especially given the abundance of other endogenous antioxidants and proteins notably albumin that is present at a much higher concentration (700 μM L^{-1}) with an equivalent k_{DR} for $HO^{\cdot}$ ($10^{10}–10^{11}$ M^{-1} s^{-1}; Buxton *et al.* 1988) through metal sequestration and free radical trapping via redox-active methionine and cysteine residues.

A more plausible biological mechanism may relate to EPO's ability to interfere with transition metal ion-dependent $HO^{\cdot}$ formation which may occur in response to hypoxia (Bailey *et al.* 2006c, 2009b) and ischaemia-reperfusion injury (Bailey *et al.* 2006a). When the DR assay is performed without EDTA (i.e. when iron ions are added as $FeCl_3$ and not $FeCl_3$-EDTA), some of the iron ions form a complex with DR (Aruoma *et al.* 1987) that can be reduced to Fe^{2+} by ascorbate and react with H_2O_2 (Aruoma *et al.* 1989). This reaction yields $HO^{\cdot}$ to cause site-specific degradation of DR which can only be inhibited if the target molecule is capable of binding the iron ions thereby reducing their activity in Fenton reactions (Aruoma *et al.* 1987). Thus, our site-specific findings clearly indicate that rHuEPO has the ability to bind iron ions into non redox-active chelates thereby suppressing Fenton formation of $HO^{\cdot}$.

These findings have implications for therapeutic intervention and may provide a complementary mechanism to explain the cytoprotective benefits of rHuEPO treatment in the clinical setting (Katavetin *et al.* 2007).

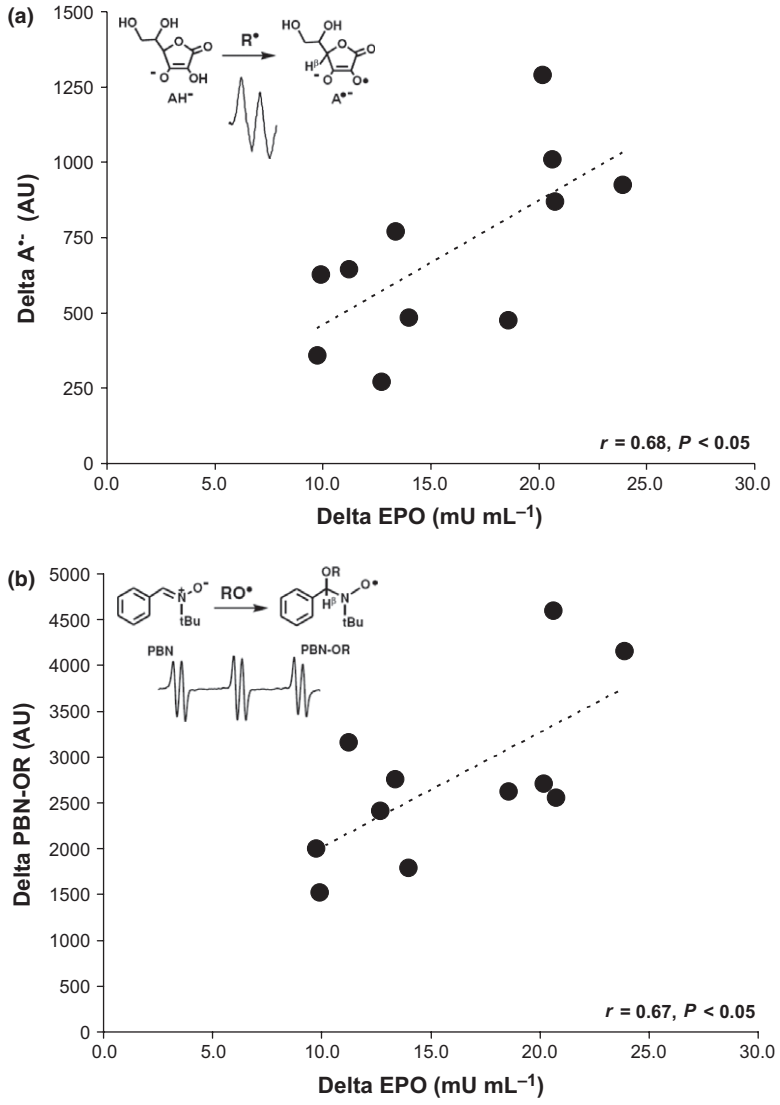

Figure 4 Relationships between the increase (delta: hypoxia minus normoxia) in the arterial concentration of EPO and systemic rate of free radical-mediated lipid peroxidation in the form of (a) ascorbate radical ($A^{\cdot -}$) and (b) PBN spin-trapped radicals generated *in-vivo*. Chemical reactions and typical EPR spectra also illustrated.

When administered at a higher pharmacological concentration as a bolus dose, rHuEPO has clear potential to attenuate oxidant stress despite the co-existence of other endogenous antioxidants including albumin which collectively compete against $HO^{\cdot}$. It would be of future interest to assess the free radical scavenging potential of other exogenous EPO analogues such as darbepoetin which has more glycosylated units and carbamoylated forms that are cytoprotective yet devoid of haemapoetic, pro-thrombotic and vasoconstrictive activities (Hand & Brines 2011).

However, it is worth noting that high-dose exogenous rHuEPO administration causes systemic and cerebrovascular vasoconstriction independent of changes in haematocrit thereby reducing vascular conductance (Rasmussen *et al.* 2012) which has the capacity to impair hypoxic vasodilatation. The mechanisms underlying this response remain to be established but may involve increased endothelin-1 release (Carlini *et al.* 1993) and/or vascular smooth muscle Ca^{2+} uptake (Rasmussen *et al.* 2012). However, the subtle endogenous rise in EPO observed in the present study combined with the lack of increase in MAP argues against the likelihood of such a mechanism.

As a complementary measure, we also chose to assess the free radical scavenging capacity of rHuEPO using the DPPH and TRAP assays, comparing its performance against other more conventional antioxidants. Compared to the water-soluble vitamin E analogue Trolox, the reducing power of rHuEPO towards the stable nitrogen radical $DPPH^{\cdot}$ was shown to be far more marked with an elevated EC_{50} (>30 min). The latter is a favourable feature since 'slow' antioxidants are generally considered less toxic *in-vivo* (Huang *et al.* 2005). Additionally, the TRAP assay further confirmed rHuEPO's supe-

rior scavenging capacity against the more biologically relevant $ROO^{\cdot}$ involved in the propagation of lipid peroxidation.

We extended our mechanistic focus by completing a complementary study in humans to examine the potential relationship between the endogenous formation of EPO and oxidative–nitrosative stress. We employed EPR spectroscopy since it represents the most direct, specific and sensitive technique for the molecular detection and subsequent characterization of free radicals (Swartz *et al.* 2007). Given the low $E^{\circ\prime}$ associated with the $A^{\cdot-}$/ascorbate monoanion (AH^{-}) couple (282 mV; Williams & Yandell 1982), any radical ($R^{\cdot}$) generated within the systemic circulation has the potential to react with this terminal small-molecule antioxidant to form the distinctive $A^{\cdot-}$ doublet ($R^{\cdot} + AH^{-} \rightarrow A^{\cdot-} + RH$; Buettner & Jurkiewicz 1993). Thus, the elevation in arterial $A^{\cdot-}$ provided direct evidence for an increased systemic accumulation of free radicals during hypoxia.

The follow-up spin trapping approach facilitated targeted detection of selected species with the primary adducts tentatively identified as lipid-derived PBN-OR. Given that tissue hypoxia has been shown to liberate catalytic iron (Fe^{2+}), downstream formation of these secondary peroxidants has classically been attributed to the metal-catalysed reductive decomposition of extracellular hydroperoxides. These are typically formed subsequent to primary radical-mediated damage to cell membrane and/or circulating lipoprotein-bound polyunsaturated fatty acids (Fe^{2+} + PUFA-OOH → Fe^{3+} + OH^{-} + PUFA/PBN-OR; Bailey *et al.* 2009b). The PBN-OR detected have the thermodynamic potential in blood (untrapped) to react with NO at a diffusion-controlled rate to form peroxynitrite (PBN-OR + $NO \xrightarrow{k=10^9\ M^{-1}s^{-1}} ONOO^{-}$; Nauser & Koppenol 2002) confirmed indirectly by the combined reduction in NO_2^{-} and elevation in 3-NT. Collectively, these findings indicate that hypoxia increased free radical-mediated lipid peroxidation culminating in oxidative–nitrosative stress which is in broad agreement with the literature (Bailey *et al.* 2009b, 2013).

Cellular studies have identified that an increased mitochondrial formation of free radicals is required for hypoxia-inducible factor-1 DNA binding and subsequent EPO expression (Chandel *et al.* 1998), part of a coordinated transcriptional response that serves to defend systemic oxygenation in the face of hypoxaemic stress (Bailey *et al.* 2009a). In support, albeit contrary to previous studies that have relied on indirect non-specific free radical footprints (Niess *et al.* 2004), our more direct approach has identified consistent relationships between the erythropoietic and oxidative stress response to hypoxia, tentative evidence for cellular O_2 sensing *in-vivo*.

Equally, given its observed potential to act as an antioxidant, the rise in EPO may represent an acute-phase defence mechanism that serves to constrain oxidative stress to maintain redox homeostasis. However, it is important to emphasize that these findings are purely confined to correlational analyses and thus fail to disassociate cause from effect, though they are clearly not coincidental due to chance alone, given the significance of the relationships observed. It was unfortunate that we failed to examine the temporal kinetics of the EPO and oxidative–nitrosative stress responses both during hypoxia and into (normoxic) recovery to provide more detailed insight into the kinetics of the relationships proposed. With regards the latter, the literature suggests that that after peaking at 24–48 h, EPO levels typically continue to rise for approximately 1.5 h following termination of a comparable hypoxic stimulus and after 3 h decline exponentially with an average half-life time of 5.2 h (Eckardt *et al.* 1989). Though beyond the scope of the present study, we hypothesize that consistent relationships would have been observed between the EPO and oxidative–nitrosative stress responses during both the 'on' and 'off' hypoxia transients and that (EPO-mediated) antioxidant protection would have persisted albeit for a limited period into normoxic recovery. This requires confirmation in follow-up studies.

Equally, future studies also need to consider more interventional approaches to determine if rHuEPO supplementation does indeed provide effective prophylaxis in alternative models known to catalyse oxidative–nitrosative stress in the absence of hypoxia, such as acute physical exercise (Bailey *et al.* 2011). Finally, given that hypoxia was also associated with inflammatory stress, it would be pertinent to measure the reactivity of EPO against other oxidants likely to have been formed such as peroxynitrous and hypochlorous acid.

Conclusions

The present findings have provided unique insight into EPO's molecular potential to function as an antioxidant. We identified that in its recombinant form, EPO was able to directly scavenge $HO^{\cdot}$ at a diffusion-controlled rate and further suppress the Fenton formation of $HO^{\cdot}$ through catalytic iron chelation, which likely represents its most important biological action. Combined with a superior ability to scavenge other biologically relevant radicals, these findings collectively emphasize that this glycoprotein hormone exhibits antioxidant potential thereby providing an additional mechanistic basis for its reported cytoprotective benefits *in-vivo*. In support, the links observed between the rise in EPO and biomarkers of oxida-

tive–nitrosative stress in the systemic circulation of humans exposed to hypoxia while not disassociating cause from effect, tentatively suggests that erythropoiesis may also reflect an acute phase antioxidant response that serves to maintain redox homeostasis. This may have important clinical implications for patients suffering from chronic hypoxaemic stress including those with chronic obstructive pulmonary disease or heart failure and patients with renal failure who require EPO therapy.

Conflict of interest disclosure

The authors declare no competing financial interests.

We acknowledge Mrs R Rousing and H Villumsen (University of Copenhagen, Denmark) for their technical assistance and cheerful cooperation of the study participants. Funded by Laboratoire Chimie Provence (formerly UMR CNRS #6264 to DMB), The Danish National Research Council (File Number 22-04-0413), The Copenhagen Hospital Corporation, The Danish National Research Foundation (Grant 504-14), The Laerdal Foundation, The AP Møller Foundation, The Jensa La Cour Foundation, The Larsen Foundation and The Hoejmosegaard Foundation. The study was conducted at the Centre of Inflammation and Metabolism supported by grants from the Danish National Research Foundation (DG 02-512-555) and the Commission of the European Communities (Contract No. LSHM-CT-2004-005272 EXGENESIS).

Author contributions

Damian M. Bailey was the Principal Investigator and recipient of funding. He led the study conception/design, acquisition/analysis/interpretation of data and wrote the first draft of the manuscript. Carsten Lundby, Ronan Berg and Sarah Taudorf made substantial contributions to data acquisition and gave approval to the final version to be submitted. Hidayat Rahmouni, Mariusz Gutowski, Jane McEneny and Ian Young contributed vital reagents and/or analytical tools and analysed data. Clive Mulholland, Jerome Sullivan and Erik Swenson provided important intellectual content and contributed to the first draft of the paper. Bente Pedersen, Kirsten Møller, Sylvia Pietri and Marcel Culcasi obtained funding for human experimentation, designed and performed the study, collected and analysed data and contributed to the first draft of the manuscript.

References

Alberty, R.A. & Hammes, G.G. 1958. Application of the theory of diffusion-controlled reactions to enzyme kinetics. *J Phys Chem* **62**, 154–159.

Aruoma, O.I., Grootveld, M. & Halliwell, B. 1987. The role of iron in ascorbate-dependent deoxyribose degradation – evidence consistent with a site-specific hydroxyl radical generation caused by iron ions bound to deoxyribose molecule. *J Inorg Biochem* **29**, 289–299.

Aruoma, O.I., Chaudhary, S.S., Grootveld, M. & Halliwell, B. 1989. Binding of iron(II) ions to the pentose sugar 2-deoxyribose. *J Inorg Biochem* **35**, 149–155.

Bailey, D.M., Raman, S., McEneny, J., Young, I.S., Parham, K.L., Hullin, D.A., Davies, B., McKeeman, G., McCord, J.M. & Lewis, M.H. 2006a. Vitamin C prophylaxis promotes oxidative lipid damage during surgical ischemia-reperfusion. *Free Radic Biol Med* **40**, 591–600.

Bailey, D.M., Robach, P., Thomsen, J.J. & Lundby, C. 2006b. Erythropoietin depletes iron stores: antioxidant neuroprotection for ischemic stroke? *Stroke* **37**, 2453.

Bailey, D.M., Roukens, R., Knauth, M., Kallenberg, K., Christ, S., Mohr, A., Genius, J., Storch-Hagenlocher, B., Meisel, F., McEneny, J., Young, I.S., Steiner, T., Hess, K. & Bartsch, P. 2006c. Free radical-mediated damage to barrier function is not associated with altered brain morphology in high-altitude headache. *J Cereb Blood Flow Metab* **26**, 99–111.

Bailey, D.M., Evans, K.A., James, P.E., McEneny, J., Young, I.S., Fall, L., Gutowski, M., Kewley, E., McCord, J.M., Moller, K. & Ainslie, P.N. 2009a. Altered free radical metabolism in acute mountain sickness: implications for dynamic cerebral autoregulation and blood–brain barrier function. *J Physiol* **587**, 73–85.

Bailey, D.M., Taudorf, S., Berg, R.M.G., Lundby, C., McEneny, J., Young, I.S., Evans, K.A., James, P.E., Shore, A., Hullin, D.A., McCord, J.M., Pedersen, B.K. & Moller, K. 2009b. Increased cerebral output of free radicals during hypoxia: implications for acute mountain sickness? *Am J Physiol Regul Integr Comp Physiol* **297**, R1283–R1292.

Bailey, D.M., Evans, K.A., McEneny, J., Young, I.S., Hullin, D.A., James, P.E., Ogoh, S., Ainslie, P.N., Lucchesi, C., Rockenbauer, A., Culcasi, M. & Pietri, S. 2011. Exercise-induced oxidative–nitrosative stress is associated with impaired dynamic cerebral autoregulation and blood–brain barrier leakage. *Exp Physiol* **96**, 1196–1207.

Bailey, D.M., Rimoldi, S.F., Rexhaj, E., Pratali, L., Salmon, C.S., Villena, M., McEneny, J., Young, I.S., Nicod, P., Allemann, Y., Scherrer, U. & Sartori, C. 2013. Oxidative–nitrosative stress and systemic vascular function in highlanders with and without exaggerated hypoxemia. *Chest* **143**, 444–451.

Bany-Mohammed, F.M., Slivka, S. & Hallman, M. 1996. Recombinant human erythropoietin: possible role as an antioxidant in premature rabbits. *Pediatr Res* **40**, 381–387.

Bastos, E.L., Romoff, P., Eckert, C.R. & Baader, W.J. 2003. Evaluation of antiradical capacity by H_2O_2-hemin-induced luminol chemiluminescence. *J Agric Food Chem* **51**, 7481–7488.

Brines, M. & Cerami, A. 2005. Emerging biological roles for erythropoietin in the nervous system. *Nat Rev Neurosci* **6**, 484–494.

Buettner, G.R. 1988. In the absence of catalytic metals ascorbate does not autoxidize at pH 7: ascorbate as a test for catalytic metals. *J Biochem Biophys Methods* **16**, 27–40.

Buettner, G.R. & Jurkiewicz, B.A. 1993. Ascorbate free radical as a marker of oxidative stress: an EPR study. *Free Radic Biol Med* **14**, 49–55.

Buxton, G.V., Greenstock, C.L., Helman, W.P. & Ross, A.B. 1988. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals in aqueous solution. *J Phys Chem Ref Data* **17**, 513–886.

Calo, L.A., Stanic, L., Davis, P.A., Pagnin, E., Munaretto, G., Fusaro, M., Landini, S., Semplicini, A. & Piccoli, A. 2003. Effect of epoetin on HO-1 mRNA level and plasma antioxidants in hemodialysis patients. *Int J Clin Pharmacol Ther* **41**, 187–192.

Carlini, R.G., Dusso, A.S., Obialo, C.I., Alvarez, U.M. & Rothstein, M. 1993. Recombinant human erythropoietin (rHuEPO) increases endothelin-1 release by endothelial cells. *Kidney Int* **43**, 1010–1014.

Catignani, G.L. & Bieri, J.G. 1983. Simultaneous determination of retinol and alpha-tocopherol in serum or plasma by liquid chromatography. *Clin Chem* **29**, 708–712.

Chandel, N.S., Maltepe, E., Goldwasser, E., Mathieu, C.E., Simon, M.C. & Schumacker, P.T. 1998. Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. *Proc Natl Acad Sci USA* **95**, 11715–11720.

Chattopadhyay, A., Choudhury, T., Bandyopadhyay, D. & Datta, A. 2000. Protective effect of erythropoietin on the oxidative damage of erythrocyte membrane by hydroxyl radical. *Biochem Pharmacol* **59**, 419–425.

Dresch, M.T., Rossato, S.B., Kappel, V.D., Biegelmeyer, R., Hoff, M.L., Mayorga, P., Zuanazzi, J.A., Henriques, A.T. & Moreira, J.C. 2009. Optimization and validation of an alternative method to evaluate total reactive antioxidant potential. *Annal Biochem* **385**, 107–114.

Duling, D.R. 1994. Simulation of multiple isotropic spin-trap EPR spectra. *J Magn Reson B* **104**, 105–110.

Eckardt, K.U., Boutellier, U., Kurtz, A., Schopen, M., Koller, E.A. & Bauer, C. 1989. Rate of erythropoietin formation in humans in response to acute hypobaric hypoxia. *J Appl Physiol* **66**, 1785–1788.

Finkelstein, E., Rosen, G.M. & Rauckman, E.J. 1980. Spin trapping. Kinetics of the reaction of superoxide and hydroxyl radicals with nitrones. *J Am Chem Soc* **102**, 4994–4999.

Genc, S., Akhisaroglu, M., Kuralay, F. & Genc, K. 2002. Erythropoietin restores glutathione peroxidase activity in 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine-induced neurotoxicity in C57BL mice and stimulates murine astroglial glutathione peroxidase production in vitro. *Neurosci Lett* **321**, 73–76.

Halliwell, B., Gutteridge, J.M. & Aruoma, O.I. 1987. The deoxyribose method: a simple 'test-tube' assay for determination of rate constants for reactions of hydroxyl radicals. *Anal Biochem* **165**, 215–219.

Hand, C.C. & Brines, M. 2011. Promises and pitfalls in erythopoietin-mediated tissue protection: are nonerythropoietic derivatives a way forward? *J Investig Med* **59**, 1073–1082.

Huang, D., Ou, B. & Prior, R.L. 2005. The chemistry behind antioxidant capacity assays. *J Agric Food Chem* **53**, 1841–1856.

Inoue, N., Takeuchi, M., Asano, K., Shimizu, R., Takasaki, S. & Kobata, A. 1993. Structures of mucin-type sugar chains on human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells. *Arch Biochem Biophys* **301**, 375–378.

Iversen, H.K., Olesen, J. & Tfelt-Hansen, P. 1989. Intravenous nitroglycerin as an experimental model of vascular headache. Basic characteristics. *Pain* **38**, 17–24.

Jelkmann, W. 2011. Regulation of erythropoietin production. *J Physiol* **589**, 1251–1258.

Katavetin, P., Tungsanga, K., Eiam-Ong, S. & Nangaku, M. 2007. Antioxidative effects of erythropoietin. *Kidney Int* **72**, S10–S15.

Koppenol, W.H. & Butler, J. 1985. Energetics of interconversion reactions of oxyradicals. *Adv Free Radic Biol Med* **1**, 91–131.

Kumral, A., Gonenc, S., Acikgoz, O., Sonmez, A., Genc, K., Yilmaz, O., Gokmen, N., Duman, N. & Ozkan, H. 2005. Erythropoietin increases glutathione peroxidase enzyme activity and decreases lipid peroxidation levels in hypoxic-ischemic brain injury in neonatal rats. *Biol Neonate* **87**, 15–18.

Nauser, T. & Koppenol, W.H. 2002. The rate constant of the reaction of superoxide with nitrogen monoxide: approaching the diffusion limit. *J Phys Chem A* **106**, 4084–4086.

Niess, A.M., Fehrenbach, E., Lorenz, I., Muller, A., Northoff, H., Dickhuth, H.H. & Schneider, E.M. 2004. Antioxidant intervention does not affect the response of plasma erythropoietin to short-term normobaric hypoxia in humans. *J Appl Physiol* **96**, 1231–1235.

Ogasawara, Y., Namai, T., Yoshino, F., Lee, M.C. & Ishii, K. 2007. Sialic acid is an essential moiety of mucin as a hydroxyl radical scavenger. *FEBS Lett* **581**, 2473–2477.

Pacher, P., Beckman, J.S. & Liaudet, L. 2007. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* **87**, 315–424.

Pascual, C. & Reinhart, K. 1999. Effect of antioxidants on induction time of luminol luminescence elicited by 3-morpholinosydnonimine (SIN-1). *Luminescence* **14**, 83–89.

Prior, R.L., Wu, X. & Schaich, K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agric Food Chem* **53**, 4290–4302.

Rasmussen, P., Kim, Y.S., Krogh-Madsen, R., Lundby, C., Olsen, N.V., Secher, N.H. & van Lieshout, J. 2012. Both acute and prolonged adminsiration of EPO reduce cerebral and systemic vascular conductance in humans. *FASEB J* **26**, 1343–1348.

Roach, R.C., Bärtsch, P., Hackett, P.H. & Oelz, O. 1993. The Lake Louise Acute Mountain Sickness Scoring System. In: J.R. Sutton, J. Coates & C.S. Houston (eds) *Hypoxia and Molecular Medicine*, pp. 272–274. Queen City Printers, Burlington.

Rogers, S.C., Khalatbari, A., Datta, B.N., Ellery, S., Paul, V., Frenneaux, M.P. & James, P.E. 2007. NO metabolite flux

across the human coronary circulation. *Cardiovasc Res* **75**, 434–441.

Sampson, J.B., Cymerman, A., Burse, R.L., Maher, J.T. & Rock, P.B. 1983. Procedures for the measurement of acute mountain sickness. *Aviat Space Environ Med* **54**, 1063–1073.

Swartz, H.M., Khan, N. & Khramtsov, V.V. 2007. Use of electron paramagnetic resonance spectroscopy to evaluate the redox state in vivo. *Antioxid Redox Signal* **9**, 1757–1771.

Thurnham, D.I., Smith, E. & Flora, P.S. 1988. Concurrent liquid-chromatographic assay of retinol, α-tocopherol, β-carotene, α-carotene, lycopene, and β-cryptoxanthin in plasma, with tocopherol acetate as internal standard. *Clin Chem* **34**, 377–381.

Vuilleumier, J.P. & Keck, E. 1993. Fluorimetric assay of vitamin C in biological materials using a centrifugal analyser with fluorescence attachment. *J Micronutr Anal* **5**, 25–34.

Williams, J.R. 2008. The Declaration of Helsinki and public health. *Bull World Health Organ* **86**, 650–652.

Williams, N.H. & Yandell, J.K. 1982. Outer-sphere electron-transfer reactions of ascorbic anions. *Aust J Chem* **35**, 1133–1144.

