TOPICAL REVIEW

Effects of recombinant human erythropoietin in normal humans

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This review describes some of the physiological effects of recombinant human erythropoietin (EPO) in healthy humans. At the blood level EPO increases the arterial O_2 content not only by increasing red blood cell volume, but also by an equally important decrease in plasma volume. Well before that, EPO causes a prompt decrease in plasma levels of renin and aldosterone. Renal clearance studies suggest that EPO decreases renal proximal tubular reabsorption rate leading to activation of the tubuloglomerular feedback mechanism and a fall in glomerular filtration rate. Thus, treatment with EPO may result in suppression of endogenous EPO production through a decrease in intrarenal oxygen consumption. EPO elevates the arterial blood pressure even in healthy subjects. The receptor for EPO is present in many tissues. However, the functional effects of EPO in the skeletal muscle seem limited, and although it has been speculated that non-erythropoietic effects of EPO (angiogenesis, shift in muscle fibre types, cognitive effects) may be responsible for the increase in exercise performance, this has not been confirmed. EPO-induced haemodynamic effects call for careful monitoring during the administration period. The metabolic, hormonal and renal effects of EPO do not seem to range beyond physiologically acceptable limits and are reversible. Taken together, EPO seems safe to use for experimental purposes in healthy volunteers.

During the last decade many new and exciting functions have been attributed to erythropoietin (EPO), and many of these are related to non-erythropoietic effects (Jelkmann, 2007). This review outlines some of the most physiologically relevant functions and mechanisms of EPO with an emphasis on studies conducted in healthy subjects. Several functions of EPO, i.e. inhibition of inflammation and apoptosis, anti-oxidant effects and stimulation of angiogenesis, may be of potential use in the treatment of pathological conditions but to address the clinical aspects is beyond the scope of the present review. Along with news of potentially beneficial effects attributed to EPO, reports have also been advanced indicating that the mortality and morbidity rates are increased in some patient groups when treated with EPO (Phrommintikul *et al.* 2007; Ehrenreich *et al.* 2009; Pfeffer *et al.* 2009; Dicato & Plawny, 2010). Thus, the question arises whether it can be considered safe to conduct studies with EPO in normal subjects.

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Table 1. Effects of EPO injections in healthy subjects on haematocrit (htc), haemoglobin concentration ([Hb]), body weight and arterial blood pressure

Injection period 1	Injection period 2	Iron supplementation	Pre-htc (%)/Hb (g dl^{-1})	Post-htc (%)/Hb (g dl^{-1})	Changes in body mass	Blood pressure (mmHg)	Reference
3/week for 5 weeks @ 50 IU kg^{-1}	3/week for 3 weeks @ 20 IU kg^{-1}	Oral supplement	41.9/14.2	48.6/16.8	No change	—	(Russell *et al.* 2002)
3/week for 5 weeks @ 50 IU kg^{-1}	3/week for 3 weeks @ 20 IU kg^{-1}	Intravenous infusion	43.6/15.0	49.1/17.1	No change	—	(Russell *et al.* 2002)
10/3 weeks @ 60 IU kg^{-1}	1/week for 9 weeks @ 60 IU kg^{-1}	Oral supplement	42.0/14.2	49.0/17.1	No change	+9	(Lundby *et al.* 2007)
Daily for 26 days @ 50 IU kg^{-1}	—	Oral supplement	44.4/15.1	47.9/15.9*	No change	—	(Audran *et al.* 1999)
3/week for 4 weeks @ 60–77 IU kg^{-1}	—	Oral supplement	42.7%	50.8%*	No change	—	(Birkeland *et al.* 2000)
3/week for 6 weeks @ 20–40 IU kg^{-1}	—	—	44.5%	49.7%	No change	No change§	(Berglund & Ekblom, 1991)
1/week for 4 weeks @ 150 IU kg^{-1}	—	No supplement	43/15.8	49/16.9	No change	No change	(Wilkerson *et al.* 2005)
Every second day/2 weeks @ 65 IU kg^{-1}	1/week for 2 weeks @ 65 IU kg^{-1}	No supplement	43.9%	46.8%	No change	+4	(Lundby *et al.* 2008a)
3/week for 4 weeks @ 50 IU kg^{-1}	—	Oral supplement	44.4/14.6	48.1/16.0	No change	—	(Connes *et al.* 2003)
12/26 days @ 50 IU kg^{-1}	—	Intramuscular injections	14.8 g dl^{-1}	15.9 g dl^{-1}	No change	—	(Parisotto *et al.* 2000)
12/26 days @ 50 IU kg^{-1}	—	Oral supplementation	15.0 g dl^{-1}	16.6 g dl^{-1}	No change	—	(Parisotto *et al.* 2000)

*The protocol was discontinued for two subjects because htc increased to over 50%. §Blood pressure was increased during submaximal exercise.

The mechanisms by which EPO increases haematocrit and O₂ transport capacity

In healthy individuals with haematocrit values in the normal range, injections of around 60 IU kg^{-1} one to three times per week for 4–14 weeks increase the haematocrit to just below 50% (Table 1). The main mechanism for the increase in haematocrit was long thought to rely exclusively on augmentations in red cell mass, but it was demonstrated that EPO also decreases plasma volume (Lundby *et al.* 2007). In fact the relative contributions of an augmented red cell mass and a concomitant decrease in plasma volume to the increase in blood haemoglobin levels were almost equal over a 13 weeks period. This response may require a normal renal function and may not be observed in patients with kidney diseases. The plasma volume-reducing effects of EPO was confirmed in other studies (Lundby *et al.* 2008a; Olsen *et al.* 2010). It is noteworthy that the change in plasma volume preceded the increase in red blood cell volume. A preliminary study suggested that the newly formed erythrocytes undergo apoptosis once EPO stimulation is stopped, so that the EPO-induced increase in red cell mass does not last the expected 120 days (Chang *et al.* 2009). In contrast, however, and based on direct measures of red cell mass following the termination of EPO stimulation, it would appear that haemoglobin concentration [Hb] is normalized rather rapidly due to a restoration in plasma volume without an effect on red cell mass (Lundby *et al.*

2008a; Olsen *et al.* 2010). This implies that although [Hb] and haematocrit are promptly returned toward baseline values after the EPO administration period, red cell O₂ transport capacity may still remain elevated. How long red cell mass remains elevated following EPO treatment is at present unknown.

The influence of EPO on blood pressure

When treatment with EPO in anaemic states became widely appreciated it was recognized that long-term administration of EPO in patients with chronic kidney disease provokes arterial hypertension. The possible mechanisms for this effect, which occurs independently of EPO's haematopoietic effect, have been recently reviewed (Krapf & Hulter, 2009). As shown in Table 1, however, in healthy subjects EPO injections sufficient to increase the haematocrit apparently do not increase the blood pressure. In these studies, blood pressure was usually not the main target investigated and was assessed by the use of sphygmomanometry. In contrast to these studies, the few studies assessing blood pressure by intra-arterial pressure transducers reported a small increase in resting values (Lundby *et al.* 2007). During exercise, haemodilution following EPO treatment may revert the augmented blood pressure response (Lundby *et al.* 2008c). This suggests a significant role of enhanced blood viscosity in EPO-induced hypertension during exercise. EPO-induced

hypertension may, however, also be unrelated to viscosity, as acute injections of high EPO doses (30,000 IU day^{-1} for 3 days) increases both resting arterial blood pressure and the blood pressure response to exercise in normal subjects (Rasmussen *et al.* 2010). EPO treatment with prolonged low-dose administration or high doses for 3 days both induced an increase in total peripheral resistance, and, as assessed by transcranial Doppler measurements and cerebral arterio-venous differences in oxygen content, this vasoconstriction also included the cerebrovascular circulation. The mechanisms remain unclear, but may involve EPO-induced release of endothelin (Bode-Böger *et al.* 1996) and inhibition of eNOS-mediated production of NO (Wang & Vaziri, 1999; Scalera *et al.* 2005). EPO has also been shown to possess direct vasoconstrictive effects in isolated renal resistance vessels (Heidenreich *et al.* 1991) and human placental arteries *in vitro* (Resch *et al.* 2003). Thus, in normal subjects both long-term administration of low doses of EPO and short-term bouts of high-dose EPO may produce arterial hypertension and intracerebral vasoconstriction.

Metabolism: a functional regulating role of EPO?

In haemodialysis patients it was demonstrated that EPO treatment over a one year period lowered the body mass index (BMI) from 22.7 to 22.1 if food intake was not increased (Allegra *et al.* 1997). Because resting metabolic rate is not altered after prolonged EPO treatment in healthy individuals (Lundby *et al.* 2008*c*), and since leptin, which is secreted by the adipose tissue in proportion to the fat stores (Rosenbaum & Leibel, 1999), is decreased after 3–6 months of EPO treatment (Kokot *et al.* 1998) it could be that EPO induced a reduction in BMI by a fat tissue-reducing effect. In this regard, supra-physiological EPO doses may protect against obesity in mice if on a high fat diet (Hojman *et al.* 2009). Furthermore, trials in diabetic, chronic renal disease patients have shown that EPO improves several metabolic parameters including fasting glucose level and insulin sensitivity as measured by euglycaemic hyperinsulinaemic clamp and intravenous glucose tolerance test (Borissova *et al.* 1993; Allegra *et al.* 1996). These findings are, however, not supported by experiments where very high EPO doses (400 IU kg^{-1}) were applied in combination with insulin clamp but did not show effects on glucose or fat metabolism at the skeletal muscle level in healthy humans (Christensen *et al.*, unpublished). It was concluded that EPO injections were not accompanied by distinct alteration in the pattern of substrate metabolism or insulin sensitivity. None of the studies listed in Table 1 have confirmed the early reports of EPO-induced changes in body composition, and taken together, EPO in normal subjects does not seem to have major metabolic effects.

Skeletal muscle

Recently the receptor for EPO was reported in biopsies obtained from human skeletal muscle (Lundby *et al.* 2008*b*; Rundqvist *et al.* 2009). Whether this also implies a functional role or not has been questioned (Sinclair *et al.* 2010). Nevertheless, it seems that EPO injections in healthy volunteers may affect the human skeletal muscle both direct and indirectly. With acute injections of 15,000 IU the mRNA levels of VEGF, HIF-1α, IGF, ferroportin, MyoD and myogen remained unchanged in biopsies obtained 2, 4, 6 and 10 h after the EPO injections, whereas minor inductions were observed in myoglobin, epo-r, transferin receptor and MRF4 (Lundby *et al.* 2008*b*). Although this implies that EPO has at least some direct effects on the skeletal muscle, the physiological consequences of this seem very limited because long-term EPO treatment did not have any effect on the absolute number of capillaries, fibre-type distribution, cross-sectional area of the fibres (Lundby *et al.* 2008*b*), or on the majority of proteins involved in acid–base balance, except for a decrease in the Na$^+$,K$^+$-pump subunit α2 (Juel *et al.* 2007). Also, in the later study, no difference in muscle mass (or body composition) was reported following DEXA scans (Lundby *et al.* 2008*b*). EPO may, however, indirectly affect the human skeletal muscle. It has been hypothesized that EPO injections would cause skeletal muscle iron stores to decrease in order to make iron available for red cell formation (Robach *et al.* 2007). However, on the contrary, following injections with EPO, skeletal muscle accumulates iron despite systemic iron deficiency secondary to EPO-induced erythropoiesis and hepcidin-promoting suppression of iron store mobilization (Robach *et al.* 2009). The exact mechanism for this remains at present unknown.

The renin–angiotensin–aldosterone system and renal function

Production of endogenous EPO is regulated by the renin–angiotensin system (Dunn *et al.* 2007). When normal human subjects are given angiotensin II the plasma levels of EPO increase (Freudenthaler *et al.* 2000; Gossmann *et al.* 2001), and, conversely, inhibition of angiotensin-converting enzyme decreases plasma EPO (Pratt *et al.* 1992). The changes in EPO synthesis are probably mediated through an angiotensin II receptor-1-dependent pathway (Gossmann *et al.* 2001). We have shown the presence of a contra-productive pathway by which treatment with EPO promptly, and before any changes in haematocrit, blood volumes and blood pressure can be detected, down-regulates circulating levels of renin and aldosterone (Lundby *et al.* 2007; Olsen *et al.* 2010).

Concomitant clearance studies revealed that the EPO-induced decrease in renin and aldosterone was associated with a fall in glomerular filtration rate (GFR). Furthermore, lithium clearance, used as an index of proximal tubular outflow, increased indicating a 10–16% decrease in proximal tubular reabsorption of sodium and water (Olsen *et al.* 2010). Changes in end-proximal delivery of tubular fluid to the macula densa produce inverse changes in renin release (Briggs *et al.* 1990) and thus the suppression of plasma renin levels may be secondary to direct effects of EPO on proximal tubular reabsorption. In addition, a decrease in proximal tubular reabsorption activates the tubuloglomerular feed-back mechanism causing a parallel decrease in GFR (Holstein-Rathlou, 1991). The molecular mechanisms for EPO's effect on the proximal tubule remain unknown but may involve inhanced release of renal endothelin-1 (Bode-Böger *et al.* 1996) which in low doses attenuates sodium reabsorption in the proximal tubule (Clavell *et al.* 1995). Tubular reabsorption of sodium is the main oxygen-consuming process in the kidney and around 70% of the filtered load is reabsorbed in the proximal tubule. By inhibiting proximal tubular reabsorption, which in turn results in rapid declines in GFR and renin/aldosterone

levels, EPO directly reduces the major oxygen-consuming factor in the kidney, reduces the filtered load, and decreases angiotensin II- and aldosterone-dependent reabsorption in more distal nephron segments. The expected result will be an increase of the oxygen tension in the environment of renal EPO-producing cells, in this way initiating an appropriate signal for down-regulation of endogenous EPO synthesis when circulating levels of recombinant EPO are high (Fig. 1). In support of such a feedback system, evidence exists to indicate that prolonged administration of recombinant EPO results in a suppression of urinary excretion of endogenous EPO (Lasne *et al.* 2002), and also the renal effects of recombinant EPO fit well with the hypothesis that the kidney operates as a 'critmeter' to regulate the EPO synthesis and body haematocrit through the metabolic signal of renal tissue oxygen pressure (Donnelly, 2003).

The reduction in plasma volume induced by EPO may be caused by natriuresis secondary to the hyporeninaemic hypoaldosteronism (Lundby *et al* 2007; Krapf & Hulter 2009). The renal sodium loss necessary to account for the associated decrease in plasma volume is small and perhaps the net effect of EPO is to cause a negative sodium balance during the treatment period.
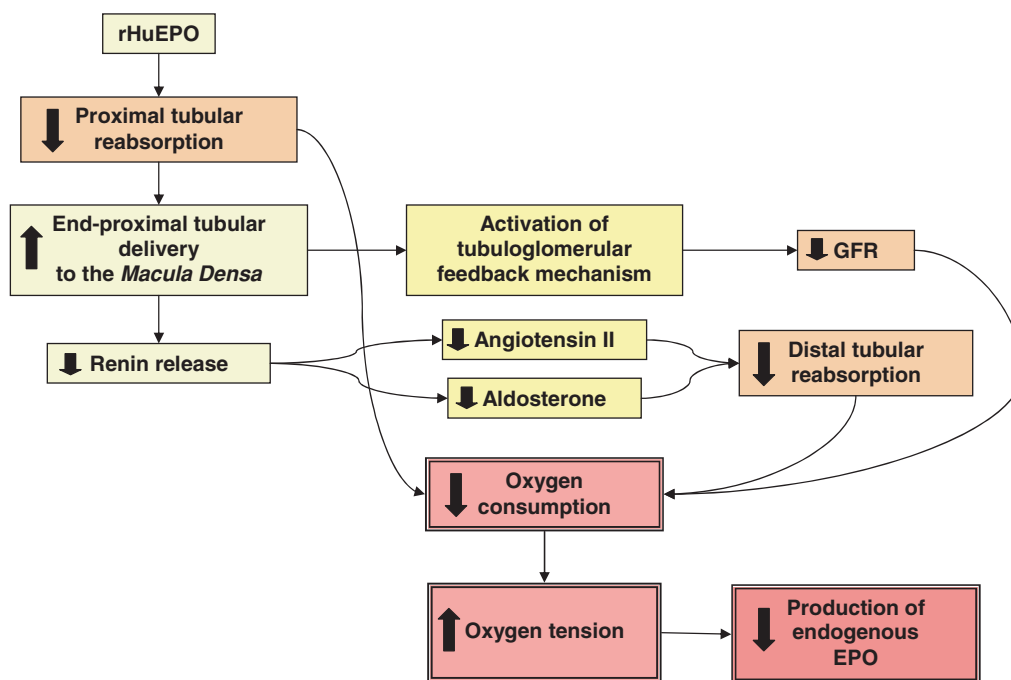

Figure 1. How high levels of circulating recombinant EPO may result in suppression of endogenous EPO synthesis secondary to a decrease in intrarenal oxygen consymption, by intrinsic renal effects
(1) EPO decreases reabsorption of sodium and fluid in the proximal tubule, thereby directly reducing the major oxygen-consuming process in the kidney; (2) increase in end-proximal tubular delivery to the macula densa decreases renin release and subsequent angiotensin II- and aldosterone-dependent reabsorption in more distal nephron segments; (3) decreased proximal tubular reabsorption activates the tubuloglomerular feed-back mechanism producing a fall in GFR and reduction of the filtered load; (4) the resulting increase in renal oxygen partial pressure in the environment of interstitial fibroblast-like cells down-regulates the hypoxia-inducible factor-2-dependent production of endogenous EPO.

Exercise capacity

Exercise performance is the result of multiple factors. Most studies investigating the effects of EPO on performance have evaluated maximal oxygen uptake ($\dot{V}_{O_2max}$). When haematocrit is increased from base values to around 50%, $\dot{V}_{O_2max}$ is increased by 8–12% (Audran *et al.* 1999; Birkeland *et al.* 2000; Russell *et al.* 2002; Thomsen *et al.* 2007; Lundby *et al.* 2008*a*,*c*; Robach *et al.* 2008). The performance-enhancing effects of EPO on $\dot{V}_{O_2max}$ at different altitudes has been investigated and it was demonstrated that EPO provided the greatest advantage at medium altitudes (1500–2500 m) (Lundby & Damsgaard, 2006; Robach *et al.* 2008). One study has assessed the performance gains at submaximal exercise intensities, and demonstrated improvements of more than 50% in a time to exhaustion test (Thomsen *et al.* 2007). Exercise performance could be increased by factors other than those attributed to changes in red blood cell volume. In subjects haemodiluted isovolumically (Lundby *et al.* 2008*c*), i.e. restoring blood parameters to pre-EPO treatment values following 13 weeks of EPO treatment, however, $\dot{V}_{O_2max}$ was similar to the values obtained prior to any injections. Since in this study the EPO dosages were probably too small to cross the blood–brain barrier, and since EPO induces the perception of superior physical fitness (Ninot *et al.* 2006), Rasmussen and co-workers (Rasmussen *et al.* 2010) injected 30,000 IU in three consecutive days to test whether EPO crossing the brain–blood barrier, and hence potentially interacting with the CNS, could increase submaximal exercise performance. Analysis of spinal fluid revealed a massive increase in EPO following the injections, and hence proved that EPO had in fact crossed the blood–brain barrier. Also in this study, however, no changes in exercise performance were noted, and surprisingly the rate of perceived exertion during the exercise became worse. Also in this regard, it was elegantly demonstrated that the O_2 kinetics during the onset of exercise are not altered by EPO (Wilkerson *et al.* 2005), something that had been proposed a few years earlier (Connes *et al.* 2003). Thus, there is no evidence indicating that EPO should increase exercise performance by mechanisms other than increasing oxygen transport capacity. One could speculate that an increase in haemoglobin mass (nHb) may positively influence repeated sprint performance because of increased buffer capacity, but this remains unexplored.

Cognitive function

Although EPO did not reduce the central fatigue during exercise (Rasmussen *et al.* 2010), evidence now exists to suggest that high doses modulate neural processing and may improve cognitive function 3 and 7 days after the administration of EPO (Miskowiak *et al.* 2008*a*,*b*).

40,000 IU of intravenously administered EPO lead to better performance in healthy subjects in a test of verbal fluency 7 days after injection of EPO (Miskowiak *et al.* 2008*a*). Concomitant use of functional magnetic resonance imaging demonstrated that EPO has an enduring effect on neurotrophic signalling in neural networks often affected in neuropsychiatric diseases (Miskowiak *et al.* 2008*a*,*b*). Also a beneficial effect of 12 weekly injections of 40,000 IU of EPO on cognitive function has been reported in patients with schizophrenia (Ehrenreich *et al.* 2007).

Is it safe to conduct studies with EPO in healthy volunteers?

EPO has emerged as a pleiotropic substance with multiple targets of action. The question arises whether the newly discovered effects of EPO hamper its use in physiological research studies beyond the risk associated with known haematopoietic effects. Of greatest concern is the fact that EPO may increase arterial blood pressure even in healthy subjects. It is noteworthy also that short-term administration of high EPO doses may cause peripheral and intracerebral vasoconstriction. Although the effect is small it may call for careful monitoring of the blood pressure during the administration period. The metabolic, hormonal and renal effects of EPO are small and do not seem to range beyond physiologically acceptable limits. Furthermore, the effects observed thus far have been fully reversible. Taken together, the new evidence may call for more information to subjects participating in EPO trials but does not push the stop button.

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