S. RENDIC, S. PICKETT, B. BROMLEY:
Human Recombinant Erythropoietin (rhEPO) - Physiology and Biochemistry
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Human Recombinant Erythropoietin (rhEPO) - 

Physiology and Biochemistry

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1. INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone which is required for maintenance, proliferation and differentiation of the stem cells that produce erythrocytes. It is a member of cytokine family, a group of proteins involved in regulation of cell growth and differentiation. As with other cytokines, the activity of EPO is mediated by formation of extracellular complexes, which results in the transmission of a signal to the interior of the cell. In the case of EPO the level of the circulatory erythrocytes is regulated by stimulating the maturation of late erythroid progenitor cells into proerythroblasts. By this mechanism the hormone stimulates production of red blood cells. Administration of EPO increases reticulocyte counts, haemoglobin levels (from 15 to 54%) and haematocrit (from 17 to 60%) in a dose-proportional manner after intravenous administration. Interestingly, hematocrit (HCT) increased more rapidly after s.c administration compared to patients who received EPO intravenously, and may last about two to six weeks. It has been reported that natural level of serum EPO increased after exercise but the mean hematocrit values were not changed significantly (Dunn, C.J. and Markham A., 1996). Administration of EPO is associated with a significant decrease in serum ferritin (by 74%) which might be reversed with i.v. iron supplementation. (Goldberg, M.A., 1995).

Erythropoietin was purified from the urine of anaemia patients to homogeneity and was cloned from a genomic library in 1983. Recombinant human erythropoietin
(rhEPO), under the name Epoietin Alfa, became available for therapeutic uses in 1985. It was the first human biomedicine produced in heterologous mammalian cells (Chinese Hamster Ovary or HCO cells). In clinics, rhEPO is used for treatment of the anaemia associated with chronic renal failure, cancer, HIV infection, etc. (Takeuchi M. and Kobata, A., 1991; Inoue, N., et al. 1993; Shimosaka, A., 1994). Recombinant EPO is thought to be used by athletes in aerobic sports for purpose of increasing oxygen transport and aerobic power (Stier, R., 1992, Casoni I., et al. 1993, Gareau, R., et al. 1996) most likely by both intravenous (i.v.) or subcutaneous (s.c.) injections. Side effects after EPO administration exerted by athletes might appear after competition is already over (Stier, R., 1992).

Physiologically, EPO is synthesised in the kidney of adults and in the liver of foetus in response to hypoxia due to ischemia, hypoxemia, or anaemia. The production of this hematopoietic factor is regulated by varying partial oxygen pressure in the blood, and is stimulated by cobalt and nickel chlorides, androgens, and prostaglandins (Shoemaker, C.B. and Mitsock, L.D., 1986; Goldberg, M.A., et al. 1988, Inoe, N. et al. 1995, Caravella, J.A., et al. 1996). As the expression of EPO by the kidney and the liver is in reverse relationship to the oxygen delivered to these organs, the rate of EPO gene expression is considered as a direct marker for tissue oxygenation levels and reflects the tissue oxygen tensions in the surroundings of its producer cells (renocortical interstitial cells and hepatocytes). Hypoxia, CO, and divalent metals (e.g. cobalt) increased EPO mRNA levels in the kidneys of rats 60-fold, 140 fold, and 5-fold, respectively, and in livers 11-fold, 11-fold, and 3-fold, respectively (Sandner, P., et al. 1996, Rithalder, T., et al. 1996). Circulating levels of EPO may increase from a normal concentration (which is 15-30 mU/mL) to the values as much as 1000-fold higher after stimulation by severe hypoxia (Dube, S. et al. 1988). Since kidney is the major EPO producing organ in adults (also liver participate at some extent), some diseases of the kidney (e.g. chronic renal failure) cause lack of the hormone and evoke anaemia.
The hormone delivered to bone marrow by circulation binds to specific receptors on target cells thus promoting the differentiation of erythroid progenitor cells (Takeuchi, M., et al. 1990, Inoe, N., et al. 1995). A model of the structure of erythropoietin-receptor complex was made based on evidence that this includes a homodimer of the receptor chain with known sequence (Caravella, J.A., et al. 1996).

**Pharmacokinetics of rhEPO**

The dosage of rhEPO used for treatment of anaemia usually ranges from 100-150 IU kg\(^{-1}\) three times weekly (starting with 20 and 40 IU kg\(^{-1}\) by s.c. and i.v. administration, respectively). Dosage reduction of about 30% is possible during s.c. administration. Reported \(t_{1/2}\) of elimination are from about 5 to 9 and up to 24 hours after i.v and s.c. administration, respectively (Dunn, C.J. and Markham A., 1996; Goldberg, M.A., 1995; Shimosaka, A., 1994). After i.v. administration, peak levels of EPO are attained within the minutes while after s.c. peak levels are reached in 5-24 hours (Goldberg, M.A., 1995). Recent clinical study comparing i.v. and s.c. administration on dialysis patients showed no difference in dose requirement, efficacy of treatment, or in blood pressure when two treatments were compared. Subcutaneous administration has low and variable availability but significantly higher through serum EPO level. In difference, i.v. administration is characterised by a higher EPO peak concentrations (Jensen, J.D., et al. 1996). Urinary excretion of administered rhEPO in healthy control subjects was negligible, less than 1% of the doses as estimated by RIA (Jensen, J.D., et al., 1995).

**2. FACTORS AND AGENTS THAT AFFECT PHYSIOLOGICAL BLOOD LEVEL OF EPO AND/OR ERYTHROPOIESIS**

Physiological factors and pharmacological agents may either decrease or increase the erythropoiesis and/or the erythropoietin level in blood (Tables 1 and 2).
DECREASE OF THE EPO LEVEL.

Theophylline, as a non-selective adenosine antagonist, was shown to suppress the production of erythropoietin by kidney in the renal-transplant recipients. The effect was accompanied by a reduction in both the hematocrit and the red cell mass. The effect was lost with discontinuation of the drug but it returned when therapy with theophylline was committed again. Besides in patients, this effect was observed also in normal subjects. It has been observed that stimulatory effects of beta- adrenergic agonists on both renal erythropoietin and erythroid progenitor cells production could be abolished by beta-2 adrenergic blockade (Jelkmann, W., et al. 1979).

Inhibition of erythropoiesis by inflammatory and infectious diseases was suggested to be mediated by release of cytokines from monocytes in the inflammatory process. Proposed mechanisms were either the inhibition of biosynthesis of EPO or the effect on EPO stimulatory action on erythroid precursor cells in the bone marrow. In vitro synthesis of EPO by HepG2 cells was reduced by cytokines such as interleukin-1β and IL-6, and by tumour necrosis factor-α, TNFα, (Leng, H.J.M., et al. 1996); Table 1.

Table 1. Factors and agents which decrease the level of EPO in blood and/or erythropoiesis.
- Some kidney diseases (e.g. chronic renal failure)
- Administration of theophylline
  (acting as adenosine antagonist).
- Rheumatoid arthritis and some other inflammatory and infectious diseases may inhibit of erythropoiesis by enhanced production of inflammatory cytokines. interleukin-1β and IL-6 and tumour necrosis factor-α, TNFα).
- Non-steroidal anti-inflammatory drugs may reduce EPO level by inhibition of prostaglandin biosynthesis.
INCREASE OF THE EPO LEVEL IN BLOOD

Hypoxia

Hypoxia is a chief stimulus for endogenous EPO production. After hypoxia is sensed in the kidney and, to a lesser extent, in the liver, EPO gene transcription increases leading to increased EPO messenger RNA level, and increased production and secretion of EPO. The secreted hormone travels through the blood to hematopoietic tissues in the bone marrow where it binds to its receptor on erythroid progenitor cells stimulating them to proliferate and differentiate into mature RBCs. These, in turn, increase blood's oxygen carrying capacity and alleviate the hypoxia stimulus leading to a decrease in endogenous EPO synthesis and secretion.

Table 2. Factors and agents which increase the level of EPO in blood and/or erythropoiesis.

- Administration of:
  Adenosine and A₂-receptor agonists
  (e.g. 5’-N-ethyl-carboxamide adenosine),
  Androgenic and anabolic steroids
  (e.g. nandrolone decanoate, testosterone),
  Epinephrine,
  Beta-adrenergic receptor agonists
  (e.g., salbutamol, isoproterenol),
  Cyclic AMP and derivatives,
  Desferrioxamine (an iron helator),
  Divalent cations,
  (e.g. Co²⁺, Ni²⁺, Mn²⁺),
  Prostaglandins A and E.
- Hepatitis
- Major loss of blood
- Changed arterial oxygen tension (hypoxic condition)

**Beta-2 adrenergic agonists**

Beta adrenergic agonists (salbutamol, isoproterenol, albuterol) produce marked stimulation of erythropoiesis. For instance, salbutamol enhances the rate of red blood cell formation and provoked a highly significant erythropoiesis stimulating activity in vivo. Elevation in erythropoietin plasma level by salbutamol (4-8 hours after i.v. drug administration) was explained by direct action on the erythropoietin-producing cells of the kidney. Mechanism proposed is mediation of the renal cyclic adenosine phosphate through adenylate cyclase-cyclic 3',5'-adenosine monophosphate system and an increased release of erythropoietin. Involvement of the beta-2 adrenergic receptors in regulation of erythropoietin synthesis was confirmed by finding that the production of erythropoietin during hypoxia can be attenuated by pre-treatment with agents which block beta-2 adrenergic receptors (in mice and rabbits). However, the effect of beta-2 adrenergic agonists on erythropoietin level of normal persons may not be significant because of feedback effect to kidney which should decrease erythropoietin release (Fink, G.D. and Fisher, J.W., 1977a; Fink, G.D. and Fisher, J.W., 1977b).

After infusion of the selective beta adrenergic agonist albuterol a significant increase in plasma levels of erythropoietin was observed in rabbits. Albuterol also potentiate the effects of hypoxia on erythropoiesis as significantly higher red cell mass and hematocrits were observed when rabbits were treated with albuterol prior to hypoxia. The effect was explained as activation of bone marrow erythroid progenitor cells. Thus, the selective beta-2 adrenergic agonist albuterol is capable of increasing both renal production of erythropoietin in vivo and to stimulate differentiation and proliferation of erythroid committed progenitor cells in bone marrow cultures in vitro. In difference, beta-2 adrenergic antagonist butoxamine inhibited these effects (Jelkmann, W., et al. 1979).
Adenosine and selective A₂-receptor agonists

Adenosine and selective A₂-receptor agonist 5'-N-ethyl-carboxamide adenosine increased both erythropoietin production and adenylate cyclase activity. This finding supported involvement of adenosine receptors in attenuation of erythropoietin synthesis. However, no selective A₂-receptor agonist for human use has been reported so far (Bakris, G.I., et al. 1990).

Androgenic steroids

Androgenic steroids possess stimulating effect on release of erythropoietin from diseased kidney and, therefore, have been used for treatment of anaemia patients on dialysis since 1970. The use of androgens have been abandoned with introduction of rhEPO although a comparative study of the effects of synthetic steroid nandrolone decanoate and the erythropoietin in patients on dialysis showed similar results regarding the haemoglobin EPO level after both agents. A lower incidence in the high blood pressure and an improvement in anaemia at lower costs was obtained after nandrolone comparing with the rhEPO treatment (Teruel, J.L., et al. 1996).

As already discussed, expression of erythropoietin may be induced also by prostaglandins (in mice) and divalent cations such as Co²⁺, Ni²⁺ and Mn²⁺ (Shoemaker C.B. and Mitsock L.D. 1986); Table 2.

3. INTERACTIONS OF CYTOKINES

Induction of secretion of endothelin-1 (ET-1) by rhEPO

Endothelins (ETs) comprise a group of three related peptides, named ET-1, ET-2 and ET-3. ETs are significantly expressed by a variety of organs and cell types, however, the physiological role of ETs in the control of cellular and organ function is not clear enough. ETs (in particular ET-1) are potent vasoconstrictors and are speculated to play a role in the control
both blood pressure and organ perfusion. In addition, they are considered to have a growth factor activity. There are evidences that hypoxia as well as rhEPO administration enhances the gene expression of ET-1 in several tissues. ETs may also be involved in the hypoxic vasoconstriction in the lung. In fact, it was found that hypoxia enhances ET-1 gene expression in the lung and in endothelial cell cultures (cells from human umbilical veins). Experiments performed in rats suggested that hypoxemia and tissue hypoxia modulate stimuli for the expression of the ET-1 gene in the kidney and in the liver. In addition, hypoxemia was found to be stimulus for both the EPO and ET-1 gene expression in the kidneys and livers but different oxygen signalling pathways were suggested. Whilst in rats hypoxia and anaemia increased EPO mRNA levels in the kidneys up to 150-fold and in the livers up to 20-fold, ET-1 mRNA levels increased maximally fourfold under the same conditions (Ritthaler, T., et al. 1996).

Major complications of i.v. rhEPO administration reported in patients are hypertension and vascular thrombotic events. These effects may result from an increase in peripheral vascular resistance, increased blood viscosity, increments in cytosolic calcium in vascular smooth muscle and platelets, and from a direct presser effect. Results from a comparative clinical study on dialysis patients showed no difference in blood pressure when different routes of rhEPO administration were applied (Jensen, J.D., et al. 1996). However, another study performed on hemodialysis patients demonstrated a higher plasma endothelin-1 (ET-1) levels after i.v. rhEPO when compared with s.c. rhEPO administration (Carlini, R.G., et al. 1993a).

In vitro studies demonstrated that rhEPO stimulated vascular endothelial cells in culture and increased ET-1 release in a time dependent fashion. Release of ET-1 by cells of human umbilical veins was increased by approximately 90%. Based on these results it was suggested that increased levels of ET-1 and a high EPO plasma levels in patients after i.v. administration of rhEPO may provide an explanation for appearance of the side effects connected with the EPO administration (Dunn, J. and Markham, A., 1996, Carlini, R.G. et al., 1993b).
**Induction of secretion of atrial natriuretic peptide (ANP) after endothelin administration**

Cardiac atria are thought to participate in the regulation of fluid volume, electrolyte homeostasis and blood pressure that cause diuresis, natriuresis and blood pressure reduction. Peptides called atrial natriuretic peptides (ANP) are released into circulation in response to hypoxia, pulmonary vasoconstriction, increase in atrial pressure followed by a strong diuresis, natriuresis, and cyclic GMP level elevation in plasma and urine (Stasch, et al., 1989, Westendorp, R.G.J., et al. 1993). For instance, during the placebo infusion the plasma concentration of ANP increased from 13.8±1.0 pmol/L at sea level to 19.6±2.3 pmol/L at the highest simulated altitude. In the same time cGMP concentration increased from 2.1±0.2 to 3.7±0.5 nmol/L (Westendorp, R.G.J., et al. 1993).

Vasoconstricting peptide endothelin was found to stimulate the ANP release *in vitro* in a dose dependent manner. After *in vivo* injection of endothelin in rats, an increase in ANP plasma level as well as that of cyclic GMP was observed. For instance, injection of 0.3 μg/kg of endothelin in rats caused a 2.6-fold increase of ANP and about 4-fold increase of cGMP in the plasma (Stasch, J.-P., et al. 1989). It was concluded that ET-1 is the most potent secretagogue of ANP from atrial myocytes both *in vivo* and *in vitro* (Ruskoaho, H., 1992; Stasch, J.-P., et al. 1989).

**Induction of secretion of ANP by rhEPO**

*In vitro* experiments demonstrated stimulating effect of EPO on ANP secretion from isolated atrium (Porat, O., et al. 1996.). In addition, at physiological concentrations, ANP potentate EPO-induced erythropoiesis *in vitro*, and was found to be a potent secretagogue of EPO from EPO-producing renal carcinoma cells. Other factors which enhance the level and production of ANP are presented in Table 3.
Table 3. Factors and agents which enhance synthesis of ANP

- Cardiac diseases
- Diabetes mellitus
- Hypertension
- Hypoxia
- Nephrectomy
- Salt intake
- Water deprivation
- Beta-adrenergic agonists
- Catecholamines (α1-adrenergic agonists)
  (epinephrine, norepinephrine, phenylephrine)
- Endothelin
- Glucocorticoids
  (dexamethasone)
- Growth factors
  (endothelin, ET-1)
- Mineralocorticoids
  (deoxycorticosterone acetate)
- Prostaglandins
- Thyroid hormones

(Ruskoaho, H., 1992)

Porat, O., et al. 1996, reported that infusion of low doses of ANP increased the endogenous circulating ANP levels by two fold, and significantly improved pulmonary gas exchange in healthy human subjects exposed to hypoxia. Because of this and other effects (e.g., improvement of passage of red blood cells through capillaries and oxygen delivery to the tissue) it was proposed that ANP may improve lung and hearth function during hypoxia as well as blood oxygen-carrying capacity (Porat, O., et al. 1996.).

Kokot, F., et al. 1995, reported significant elevation of ANP after EPO administration in hemodialysed (HD) patients after 3 and 6 months of treatment. However, following long term erythropoietin therapy (s.c. administration, 12 months) the ANP levels decreased to the control
level, and as the EPO level was still enhanced, the ANP/EPO ratio decreased (Table 4). The ANP/EPO ratio in both group of patients (EPO treated and EPO untreated) was significantly higher comparing to healthy persons. At the same time the ferritin concentration and the plasma transferrin saturation decreased after EPO administration.

Table 4. ANP and EPO levels, and ratios in non-treated (1) and *EPO-treated (2) hemodialysed (HD) patients

<table>
<thead>
<tr>
<th></th>
<th>Healthy non-treated</th>
<th>HD Patients (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>ANP pg/mL</td>
<td>13.2</td>
<td>(1) 217</td>
</tr>
<tr>
<td></td>
<td>(2) 243</td>
<td>651</td>
</tr>
<tr>
<td>EPO mU/mL</td>
<td>18.1</td>
<td>(1) 20.6</td>
</tr>
<tr>
<td></td>
<td>(2) 14.0</td>
<td>41.9</td>
</tr>
<tr>
<td>ANP/EPO</td>
<td>0.73</td>
<td>(1) 10.5</td>
</tr>
<tr>
<td></td>
<td>(2) 17.4</td>
<td>15.5</td>
</tr>
</tbody>
</table>

*Doses 20-40 IU/kg, s.c., 3 x w, for 12 months (Kokot F. et al. 1995).

4. EPO - METABOLISM, STRUCTURE, AND ACTIVITY

Wendell, R.F. and Waldman, T.A., 1964, observed that the urinary level of EPO is roughly proportional to the serum level, suggesting that the urinary excretion was derived from the serum.
It was also suggested that the urinary excretion of endogenous erythropoietin accounts less than 10% of the total daily elimination of the hormone. In addition to urinary excretion, the liver and the bone marrow has been suggested as sites of EPO degradation.
The renal clearance of recombinant EPO in patients accounted for less than 3% of the total body clearance. Urinary excretion of rhEPO in healthy control subjects, as reported by Jensen, J.D., et al. 1995, was negligible, less than 1% of the dose as estimated by RIA. Based on the clinical experiments it was concluded that renal excretion is of minor significance for overall clearance of physiological and rhEPO (Nielsen, O.J., et al. 1990). The mechanisms by which recombinant EPO is excreted are still unknown.

**EPO - structure and metabolism**

Extensive hepatic degradation of recombinant erythropoietin was reported by rat liver subcellular fractions *in vitro* and during rat liver perfusion experiments. In the later experiments a large amount of small molecular weight degradation products were detected (Nielsen, O.J., et al., 1990). The catabolism of serum glycoproteins is mediated by asialoglycoprotein binding lectin (the hepatic cell binding protein for galactose, the penultimate saccharide in the EPO structure) in liver cells. It was, therefore, suggested that the liver may be involved in the degradation process of rhEPO after removal of terminal sialic acid (N-acetyl-neuraminic acid) residues from the structure and that continuous *in vivo* desialation is a rate limiting step in degradation of circulating EPO (Nielsen, O.J., et al., 1990; Dunn, J and Markham, A., 1996). I.v. administration of both $^{125}$I labelled recombinant EPO and recombinant desialated EPO in rats showed rapid plasma clearance of the desialated EPO with $t_{1/2}$ about 2 min. The bulk of the desialated hormone was accumulated in the liver where it was readily catabolised in its breakdown products which were released into the plasma. At the end of the perfusion experiment (180 min) a number of small degradation products were detected in perfusate. The products identified in the supernatant of homogenised tissue after perfusion of the desialated EPO indicate specific and extensive lysosomal digestion. The products were identified predominantly in cytosol, and lysosomes were suggested as a location of catabolism in rat liver cells. Extensive exocytosis of small degradation products was observed 60 min after beginning of perfusion. Degradation products were not identified.
Study of \textit{in vivo} metabolism of labelled desialated and nondesialated rhEPO in rats showed rapid elimination from plasma of the former hormone ($t_{1/2}$ of 2 min) and its accumulation in different organs (liver, spleen, kidney, narrow). In difference, the plasma clearance of the labelled fully glycosilated recombinant hormone was slow with $t_{1/2}$ about 180 min and negligible accumulation in organs until 30 min of administration. It was also suggested that the kidney is, at least in part, involved in catabolism of the hormone. (Spivak, J.L. and Hogans, B.B., 1989).

\textbf{EPO - structure and activity}


The sialic acid (N-acetyl-neuraminic acid) is required for expression of the hormone activity \textit{in vivo} but not \textit{in vitro}. It was shown that asialo-EPO, a less sialated biologically produced EPO, and a glycosidase treated EPO, showed similar or even higher \textit{in vitro} activity than urinary EPO but they have had much lower activity \textit{in vivo}. It was, therefore, suggested that the sialic acid protects the hormone against clearance from the circulation by hepatic asialoglycoprotein binding lectin (Jensen, J.D., et al. 1995, Takeuchi, M., et al. 1990, Fukuda, M.N., et al. 1989).

The lack of the activity of asialo-EPO \textit{in vivo}, due to the rapid elimination from the circulation of desialated EPO by the liver galactosyl receptors, was suggested by Fukuda, M.N., et al. 1989, and Jensen, J.D., et al. 1995. When the terminal sialic acid is removed from the oligosaccharide a galactose residue becomes the new terminal sugar. These galactose-terminated-glycoproteins my than be recognised by receptors present on the surface of
hepatocytes, enter the cells by endocytosis followed by lysosomal digestion (Fukuda, M.N., et al. 1989).

The sugar chains in the structure of EPO are therefore suggested to be important for both biological activity and catabolism of the hormone.

Recently, the US scientists designed a small peptide called *EPO mimetic peptide 1 (EMP1)* that can mimic the action of erythropoietin.

*Structure of the peptide:* The cyclic peptide contains 20 amino acids, MW ~2.1 kDa (x2 as a dimer) with no sequence or structural homology to EPO.

*Activity:* The peptide is a full agonist of EPO, binds to and activates erythropoietin receptor. EMP1 competes with EPO for the EPO receptor inducing similar events and cell cycle progression in EPO responsive cells *in vitro.*

*Affinity of binding* of EMP1 is weaker than EPO (by factor 1000) and less potent in animal studies. Both EMP1 and EPO have a significant erythropoietic activity in mouse *in vivo:*

Reticulocytes increased by:
- 15% after EPO, 40 units;
- 6% after EMP1, 2 mg.


5. QUESTIONES FOR FURTHER COSIDERATION

1. The clinical data presented indicate change of the blood level and interactions between EPO and other cytokines. The best documented *in vivo* and *in vitro* interactions are between EPO, ANP, and ET-1.

2. The question rises whether blood concentrations of EPO, ANP, and ET-1 and/or their ratios could be developed as indicators for administration of the cytokines and/or their derivatives.
Acknowledgements

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