# MINIREVIEW

# Erythropoietin: Physiology and Pharmacology Update[1,2]

JAMES W. FISHER[3]

*Department of Pharmacology, Tulane University School of Medicine, New Orleans, LA 70112–2699*

**This minireview is an update of a 1997 review on erythropoietin (EPO) in this journal (1). EPO is a 30,400-dalton glycoprotein that regulates red cell production. In the human, EPO is produced by peritubular cells in the kidneys of the adult and in hepatocytes in the fetus. Small amounts of extra-renal EPO are produced by the liver in adult human subjects. EPO binds to an erythroid progenitor cell surface receptor that includes a p66 chain, and, when activated, the p66 protein becomes dimerized. EPO receptor activation induces a JAK2 tyrosine kinase, which leads to tyrosine phosphorylation of the EPO receptor and several proteins. EPO receptor binding leads to intracellular activation of the Ras/mitogen-activated kinase pathway, which is involved with cell proliferation, phosphatidylinositol 3-kinase, and STATS 1, 3, 5A, and 5B transcriptional factors. EPO acts primarily to rescue erythroid cells from apoptosis (programmed cell death) to increase their survival. EPO acts synergistically with several growth factors (SCF, GM-CSF, 1L-3, and IGF-1) to cause maturation and proliferation of erythroid progenitor cells (primarily colony-forming unit-E). Oxygen-dependent regulation of EPO gene expression is postulated to be controlled by a hypoxia-inducible transcription factor (HIF-1$\alpha$). Hypoxia-inducible EPO production is controlled by a 50-bp hypoxia-inducible enhancer that is approximately 120 bp 3′ to the polyadenylation site. Hypoxia signal transduction pathways involve kinases A and C, phospholipase $A_2$, and transcription factors ATF-1 and CREB-1. A model has been proposed for adenosine activation of EPO production that involves protein kinases A and C and the phospholipase $A_2$ pathway. Other effects of EPO include a hematocrit-independent, vasoconstriction-dependent hypertension, increased endothelin production, upregulation of tissue renin, change in vascular tissue prostaglandins production, stimulation of angiogenesis, and stimulation of endothelial and vascular smooth muscle cell proliferation. Recombinant human EPO (rHuEPO) is currently being used to treat patients with anemias associated with chronic renal failure, AIDS patients with anemia due to treatment with zidovudine, nonmyeloid malignancies in patients treated with chemotherapeutic agents, perioperative surgical patients, and autologous blood donation. A novel erythropoiesis-stimulating factor (NESP, darbepoetin) has been synthesized and when compared with rHuEPO, NESP has a higher carbohydrate content (52% vs 40%), a longer plasma half-life, the amino acid sequence differs from that of native human EPO at five positions, and has been reported to maintain hemoglobin levels just as effectively in patients with chronic renal failure as rHuEPO at less frequent dosing. The use of rHuEPO and darbepoetin to enhance athletic performance is officially banned by most sports-governing bodies because the excessive erythrocytosis can lead to increased thrombogenicity and can cause deep vein, coronary, and cerebral thromboses.** Exp Biol Med 228:1–14, 2003

**Key words:** erythropoietin; darbepoetin; hypoxia; erythropoiesis; receptor; oxygen; hypoxia-inducible factor; adenosine; anemia; doping

## History of Erythropoietin (EPO)

It has been almost a century since Carnot and Deflandre (2) postulated that a humoral factor, which they called "hemopoietine," regulates red blood cell production. Their intriguing experiments were carried out in rabbits, where they removed plasma from a donor rabbit after a bleeding stimulus and found that when this plasma was injected into a normal recipient rabbit, a prompt reticulocytosis occurred. Several investigators confirmed the Carnot and DeFlandre experiments (3, 4). However, Erling Hjort (5) reported the most convincing confirmation of Carnot and DeFlandre's work up to that time, which was published in a Norwegian journal in 1936. Hjort reported that erythropoietically active plasma from bled rabbits produced a reticulocytosis when injected into normal recipients in 18 experiments. Krumdieck (6) published very similar findings as Carnot and Hjort in *Proceedings of the Society for Experimental Biology and Medicine* in 1943 in which they found erythropoietic activity in plasma from bled rabbits when injected into

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[3] To whom requests for reprints should be addressed at Department of Pharmacology, SL83 Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112–2699. E-mail: jfisher@tulane.edu

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recipient rabbits. Allan Erslev, a pioneer in EPO research, must be given much credit for publishing his work in the journal *Blood* in 1953 (7) where he injected large volumes of plasma (50 ml per day for 4 days) from donor rats after a bleeding stimulus into normal recipient rats and produced a marked reticulocytosis. One of the most important papers in EPO research is the work of Kurt Reissmann (8), who in 1950, reported in the journal *Blood* his work in parabiotic rats, when one partner breathed an atmosphere of low oxygen tension and the other partner breathed normal air, both partners developed a reticulocytosis, increase in hemoglobin (Hb), and bone marrow hyperplasia. These findings reawakened interest in EPO. Proof that the kidney is the primary site of EPO production (9–12), that peritubular interstitial cells in the kidney are the renal cells that produce EPO (13, 14), and that the liver is a secondary site of EPO production (15, 16) are major advances in EPO research. One of the most important advances in this field occurred when Miyake *et al.* (17) reported purification to homogeneity of human EPO. This made it possible for Lin *et al.* (18) and Jacobs *et al.* (19) to clone the gene for EPO and to develop a transfected cell line in Chinese hamster ovary cells that provided recombinant EPO for use in clinical anemias. A new molecule has recently been synthesized called "novel erythropoiesis stimulating protein (NESP)," which contains a higher content of carbohydrate and provides a new antianemia agent with a longer circulating plasma half-life *in vivo* than native EPO (20, 21).

## Sites of Production of EPO

It is of historical interest to review the work published on sites of production of EPO. Since the kidney was proven to be the primary site of production of EPO (9–12), a vigorous search for the cells in the kidney that produce EPO has been undertaken. With the cloning of the gene for EPO (18, 19), cDNA probes became available for *in situ* hybridization studies on the kidney and other organs to determine the cell that produces EPOmRNA. Koury *et al.* (13) and Lacombe *et al.* (22) used *in situ* hybridization studies to demonstrate that the cells containing EPOmRNA were in a peritubular (interstitial or endothelial) location in anemic mouse kidneys. Fisher *et al.* (14) reported high levels of EPOmRNA in peritubular (interstitial) cells using *in situ* hybridization studies in hypoxic monkey kidneys. Other investigators have also confirmed a peritubular site for EPO production (23, 24). Maxwell *et al.* (25), using *in situ* hybridization and immunohistochemical methods, and Loya *et al.* (26), using hypoxic transgenic mice, reported a tubular epithelial cell site for EPO production. Other investigators postulated a tubular cell site of EPO production (27–29). Mujais *et al.* (27) recently reported EPOmRNA in tubular cells using reverse transcriptase-polymerase chain reaction (RT-PCR) in microdissected isolated nephron segments (medullary ascending limb, proximal convoluted tubules, cortical ascending limb, medullary collecting duct, and cortical collecting tubules). It is not clear whether interstitial or endothelial cells were contained in all of these tubular segments. On the other hand, Da Silva *et al.* (30), using RT-PCR in microdissected nephron segments, could not detect EPOmRNA in any of the above described microdissected nephron segments, but did report significant EPOmRNA in a peritubular location.

## Factors Involved in the Regulation of Erythropoiesis

Red cell production (erythropoiesis) is regulated by several cytokines. Figure 1 illustrates the process of erythropoiesis from the primitive pluripotent stem cell to the mature erythrocyte and the points in this maturation process where regulatory factors act to induce commitment and further maturation of the cells involved in the red cell lineage. The growth factors known to be involved in erythropoiesis

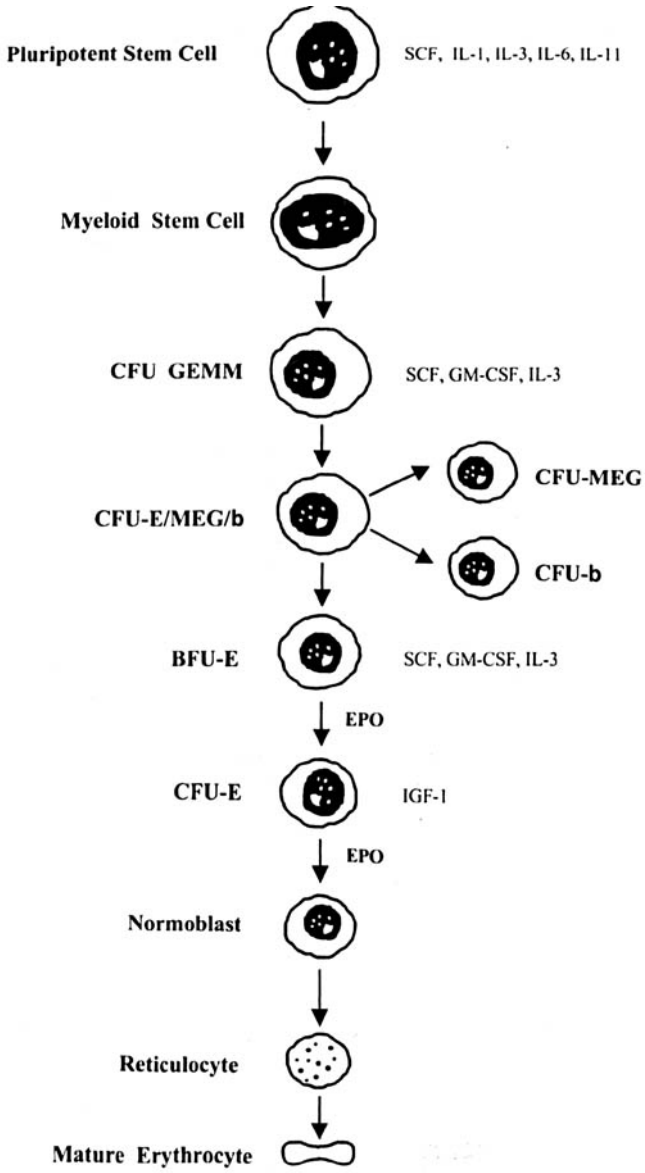


**Figure 1.** The growth factors that influence erythropoiesis from the pluripotent stem cell to the mature erythrocyte. See Ref. 194.

are granulocyte colony-stimulating factor (G-CSF), interleukin (IL)-6, stem cell factor (SCF), IL-1, IL-3, IL-4, IL-9, IL-11, granulocyte-macrophage (GM)-CSF, insulin growth factor-1 (IGF-1), and EPO. EPO acts on the later stages of development of erythroid progenitor cells. EPO acts primarily on colony-forming unit erythroid (CFU-E) to induce these cells to proliferate and mature through the normoblast into reticulocytes and mature erythrocytes (31, 32). The primary target cell for EPO in the bone marrow is the CFU-E. EPO acts synergistically with SCF, GM-CSF, IL-3, IL-4, IL-9, and IGF-1 to cause maturation and proliferation from the stage of the burst-forming unit erythroid (BFU-E) and CFU-E to the normoblast stage of erythroid cell development (33, 34). Thus, EPO acts primarily on apoptosis to decrease the rate of cell death in erythroid progenitor cells in the bone marrow. SCF, IL-1, IL-3, IL-6, and IL-11 provide the stimulus to cause the differentiation of the pluripotent stem cell into the myeloid stem cell and the CFU-granulocyte, erythroid, monocyte, megakaryocyte (GEMM). The CFU-GEMM gives rise to specific CFU for granulocytes, monocytes, megakaryocytes, macrophages, eosinophils, and erythroid cell precursors (35–37).

## EPO Receptor

EPO binds to an erythroid progenitor cell surface receptor to regulate bone marrow erythroid cell proliferation, differentiation, and survival. After the EPO gene was cloned in 1985 (18, 19) and iodinated EPO was made available, the EPO receptor was seen in normal as well as transformed erythroid cells (38). These studies revealed that the number of receptors were less than 1000 per cell (38). The EPO receptor is apparently expressed primarily on erythroid cells between the CFU-E and the pronormoblast stage of erythroid cell development (39, 40). Small numbers of EPO receptors are expressed on BFU-E, and a weak response to EPO is seen in these cells (39). The highest number of EPO receptors is seen on the CFU-E and the pronormoblasts (39, 41). The number of EPO receptors per cell gradually decreases during erythroid cell differentiation, and studies have shown that the reticulocyte and mature erythrocyte do not contain EPO receptors (39–41). The EPO receptor gene has been cloned from murine erythroleukemia cells (42). The EPO receptor is expressed as a protein that ranges from 66 to 78 kD. Protein fragment complementation assays and crystallographic studies indicate that the EPO receptor exists as a preformed dimer (43, 44). One molecule has been demonstrated to activate EPO receptors by dimerization of two EPO receptors (45). EPO binding to the receptor changes the conformation of the EPO receptor, which is necessary for JAK2 activation by a mechanism of self dimerization (46, 47). A high-affinity (kD ~ 1 nM) and a low-affinity (kD ~ 2 μM) binding site for EPO has been demonstrated in the extracellular domain of the EPO receptor (45, 48, 49). EPO-induced intracellular signaling occurs through a rapid tyrosine phosphorylation of several proteins even though the EPO receptor does not possess endogenous tyrosine kinase activity. The several steps in EPO activation of the receptor are outlined in Figures 2 and 3. First, as noted in Figure 2, intracellular signaling occurs by activating the JAK2 tyrosine kinase, which is associated with the EPO receptor constitutively (50). JAK2 is associated with the EPO receptor in the transmembrane region (51). After EPO activates the receptor, eight tyrosine residues in the cytoplasmic domain of the EPO receptor are phosphorylated (52, 53). Docking sites for several intracellular proteins that have Src homology 2 (SH2) domains are provided by these

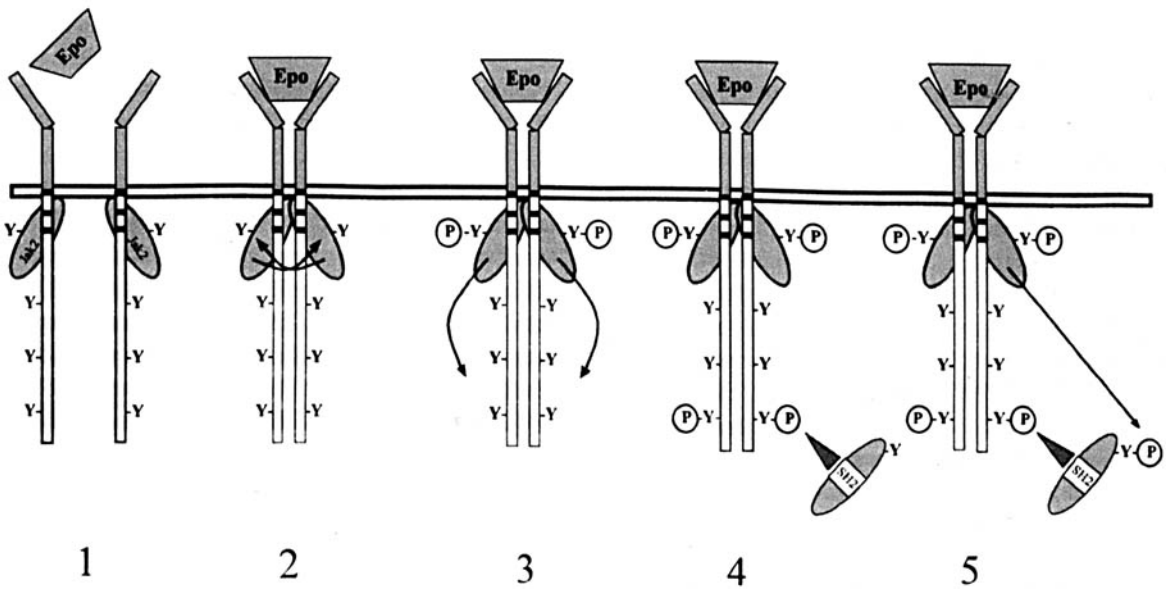


**Figure 2.** The first step in EPO activation of the receptor is dimerization (1); the pre-associated JAK2 kinases are in close contact and activated by transphosphorylation (2); the tyrosine residues of the EPO receptor are then phosphorylated (3, 4), providing docking sites for intracellular signaling proteins with SH2 domains (5). Lacombe C and Mayeux P. Nephrol Dial Transplant 14(Suppl 2):22–28, 1999. By permission of Oxford University Press.

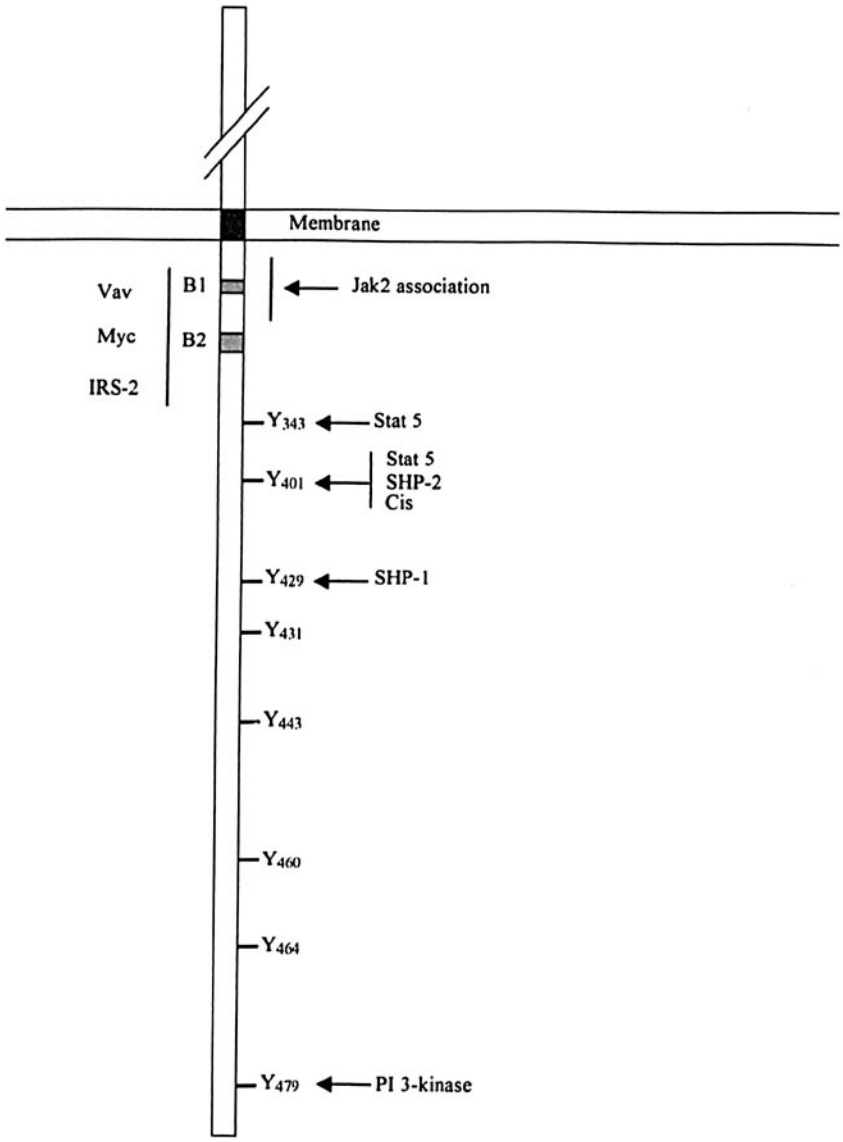


**Figure 3.** Schematic representation of the intracellular part of the EPO receptor and the identified binding sites for signaling proteins. Lacombe C and Mayeux P. Nephrol. Dial Transplant 14(Suppl 2):22–28, 1999. By permission of Oxford University Press.

phosphorylated tyrosines. Constantinescu *et al.* (54) have provided evidence that a hydrophobic juxtamembrane domain in the EPO receptor is required for intracellular signaling. These data suggest that phosphorylation of EPO receptors, which probably provides docking sites for intracellular signaling molecules, is more important in EPO signaling than JAK2 kinase activation. However, Zang *et al.* (55) reported that transgenic mice without cytoplasmic tyrosine residues have normal erythropoiesis. Von Lindern *et al.* (56) have challenged the notion that EPO receptor dimerization alone is sufficient to activate JAK2 tyrosine protein kinase, and they report that protein kinase C is required for the EPO receptor to activate JAK2. Koury *et al.* (57) have recently reviewed the EPO receptor and how transducers act in clearing intracellular signaling. Figure 3 illustrates the downstream signal transduction pathways for the intracellular part of the EPO receptor. The Ras/MAP kinase pathway, which is involved with cell proliferation (58), has been found to be activated by EPO (59, 60). Phosphatidylinositol 3-kinase (PI 3-kinase) and the EPO receptor have been shown to involve the SH2 domains of the p85 subunit of PI-3-kinase and the last tyrosine of the EPO receptor (61, 62). Tyrosine phosphorylation of the adaptor protein IRS2 and its association with PI 3-kinase may also be involved as an alternate pathway of activation (63). EPO is also well known to activate STAT1, STAT3, STAT5A, and STAT5B (64–69), especially in cytokine-induced signaling pathways (68). The precise mechanism of STAT5 activation in EPO-induced signaling pathways is somewhat controversial in that some investigators have shown a correlation between STAT5 activation and cell proliferation (69, 70), whereas others have not been able to confirm this correlation (71, 72). Phosphorylation of SH2 in response to EPO activation has been demonstrated, which results in stimulation of erythroid cell proliferation (73), whereas SH1 leads to dephosphorylation of JAK2 after EPO receptor activation (74). The stem cell factor (SCF) or c-kit is also known to interact with the EPO receptor, probably through phosphorylation of the

EPO receptor, resulting in enhanced erythroid cell differentiation and proliferation (35). Shan *et al.* (75) reported that JNKs/p38 MAP kinase and ERKs play distinct roles in apoptosis and survival of HCD-57 cells induced by withdrawal or addition of EPO. On the other hand, Jacobs-Helber *et al.* (76) reported that JNK and p38 are activated by EPO, but are not induced in apoptosis after EPO withdrawal in EPO-dependent HCD 57 cells. The work reported by Shan *et al.* (75) has been retracted (77).

## Oxygen-Sensing and Signal Transduction Pathways in Hypoxic Regulation of EPO Gene Expression

Oxygen-dependent regulation of the hypoxia-inducible factor (HIF) transcription factor has been reported to play a significant role in the upregulation of hypoxic cells (78–81). The determination of the molecular basis for oxygen-regulated expression of the HIF-1α subunit controlling the EPO gene and other oxygen regulated genes is one of the most important new findings in hypoxic regulation of EPO gene expression. Nonhypoxic cells do not contain a detectable level of HIF-1α protein, whereas cells exposed to hypoxia show HIF-1α expression within 30 min (82). Hypoxia-inducible EPO gene expression has been demonstrated to be controlled by a 50-bp hypoxia-inducible enhancer that is approximately 120 bp 3′ to the polyadenylation site (82–84). There are three different segments of the 50-bp hypoxia-inducible 3′ enhancer (BS1, BS2, and BS3) (85). The BS1-binding site (nucleotides 4–12) for HIF-1 is a conserved sequence located near the 5′ end of the enhancer (86, 87). The factor binding at nucleotides 19–23 (BS2) may be a constitutive factor necessary for the interaction of HIF-1 with the transcription initiation complex (85). A number of the thyroid-steroid hormone superfamily transcription factors may bind to site BS3 (nucleotides 26–48) (85). In 1995, Wang and Semenza (88) accomplished the molecular cloning of HIF-1. These investigators (88) found that the DNA-binding complex contained two basic helix-loop-helix proteins that they called HIF-1α and HIF-1β. HIF-1β, the aryl hydrocarbon nuclear translocator (ARNT), is involved in the xenobiotic response (89). There have been several models proposed for the molecular mechanism by which reduced cellular $O_2$ concentration leads to increased HIF-1α levels. The first model is that proposed by Goldberg *et al.* in 1988 (90), which postulates that $O_2$ sensing is mediated by a heme protein capable of reversible binding of $O_2$ such that the deoxy state leads to EPO gene expression, whereas the oxy state does not. Thus, the addition of cobalt to the heme ring of the putative sensor results in the loss of $O_2$ binding. On the other hand, carbon monoxide (CO), which inhibits hypoxia-induced EPO expression, is purported to bind to the sensor to stabilize the oxyconformation. Cobalt chloride (divalent anion) and deferroxamine (iron chelator) have been demonstrated to induce EPO mRNA, HIF-1 DNA-binding activity, and expression of reporter genes containing the EPO gene for the hypoxia responsive element (HRE) (83, 87, 91). Cobalt and deferroxamine have been found to induce both the expression of HIF-1α protein and its transactivation (92). However, other investigators have presented data suggesting that cobalt and deferroxamine do not act by the same mechanism as hypoxia (93–95). In addition, CO and nitric oxide have recently been reported to inhibit HIF-1 directly (96, 97). Other alternative models as mechanisms for how $O_2$ sensing is mediated have been proposed, such as conversion of reactive oxygen intermediates (ROI) like superoxide ion, $H_2O_2$, or hydroxyl radical and the level of ROI may determine the magnitude of hypoxic signal transduction and EPOmRNA induction (94). However, diphenylene iodonium (DPI), an inhibitor of NADPH oxidoreductase, blocks the induction of EPOmRNA and HIF-1 DNA binding in cells exposed to hypoxia (98), which is opposite to the model predicted for ROI. Therefore, this does not seem to be a plausible hypothesis. It has also been reported that treatment of purified HIF-1 with diomide or $H_2O_2$ results in complete loss of DNA-binding activity, which suggests that a free (reduced) sulfhydryl group on one or more of the cysteine residues of HIF-1 is required for either DNA binding or dimerization, indicating that cellular redox status may also affect HIF-1 directly (99).

It has recently been suggested that HIF-1α could be oxidatively modified by ROS and that these short-range interactions require the participation of prolyl hydroxylase enzymes that could be involved in oxygen sensing (79, 80, 100). The HIF-modifying enzyme apparently depends upon both oxygen and iron (101). Models have been proposed by Zhu and Bunn (79) and Semenza (102) for oxygen-dependent regulation of the HIF transcription factor that involves the hydroxylation of a specific proline residue within a highly conserved region of the HIFα's internal oxygen-dependent degradation domain. This structural modification is necessary for HIFα binding to Von Hipple-Lindau protein. Ubiquitination of HIFα is necessary for this transcription factor to be degraded by the proteasome, and this ubiquitination of HIF requires direct binding to the β-domain of the Von Hipple-Linadau proteins (103). Two independent regions within the HIF-1α oxygen-dependent degradation domain (ODDD) are targeted for ubiquitination by $E_3$ ubiquitin ligase in a manner dependent upon prolyl hydroxylation (104): a conserved HIF-VHL-prolyl hydroxylase pathway in *C. elegans*, and with the use of a genetic approach to identify EGL-9, a dioxygenase that regulates HIF by prolyl hydroxylation has been defined (105); a conserved family of HIF prolyl hydroxylase (HPH) enzymes that appear to be responsible for a ubiquitin-ligase complex that recognizes a hydroxylated proline residue in HIF (post-translational modification) (106). It has recently been demonstrated that the ARNT partner is recruited to HIF-1α in the nucleus (not before HIF-1α enters the nucleus), and the resulting HIF-1α ARNT heterodimer recognizes HRE's of target genes (107). The mechanism of activation of HIF-1α is a multistep process that includes hypoxia-dependent nuclear import and activation (derepression) of the transac-

tivation domain, resulting in recruitment of the CREB-binding protein (CBP)/p300 coactivation (107). Thus, CBP has been demonstrated in a hypoxia-dependent manner to stimulate the HIF-1α/ARNT-heterodimer on a minimal HRE-containing promoter (107). Semenza (102) has proposed a model for the regulation of HIF-1α expression by cellular oxygen concentration that is shown in Figure 4. The model postulates that when cells are hypoxic, the proline is not hydroxylated and HIF-α escapes degradation (102). Further proof is needed to prove that this model plays a role in EPO gene expression *in vivo*.

Hypoxia signal transduction pathways may also involve changes in phosphorylation in that it has been demonstrated that HIF-1 α protein and HIF-1 DNA-binding activity in hypoxic Hep3B cells were blocked by genistein, a tyrosine kinase inhibitor, or 2-aminopurine, a serine/threonine kinase inhibitor (108). It has also been reported that kinase A, but not kinase C, is involved with HIF-1 α activation and EPOmRNA induction (109). These investigators have found that the transcription factors ATF-1 and CREB-1 bind constitutively to the HIF-1 DNA recognition site (109). Adenosine has been postulated to be a primary regulator of EPO production, acting primarily through kinases A and C and phospholipase $A_2$ (Fig. 5). The first work implicating adenosine in EPO production was the report by Ueno *et al.* (110) in which they reported that theophylline, a nonselective adenosine $A_1A_2$ receptor antagonist, inhibited hypoxia-induced EPO production in mice. Support for our model (Fig. 5) for adenosine involvement in signal transduction pathways for the control of EPO production is: hypoxia has been reported to increase adenosine levels in extracellular fluid (ECF) (111) through its effects in increasing ectonucleotidase activity (112), which results from the increase in breakdown of ATP; adenosine $A_{2A}$ receptor messenger RNA, as well as $A_{2A}$ receptor protein, have been found to be increased in PC12 cells incubated in an hypoxic atmosphere (113); theophylline, an $A_1A_2$ adenosine receptor antagonist, inhibits the effects of hypoxia on EPO production in mice (110) and also produced inhibition of EPO production in patients with erythrocytosis associated with kidney transplantation (114); CGS 21680, a selective adenosine $A_{2A}$ receptor agonist, increased EPO levels in culture media and EPOmRNA levels in Hep3B cells (115); SCH 58261, a selective adenosine $A_{2A}$ receptor antagonist, inhibited EPO levels in culture media of Hep3B cells in response to hypoxia (115); enprofylline, a selective $A_{2B}$ adenosine receptor antagonist, inhibited the increase in EPO levels in Hep3B cell culture medium in response to hypoxia (115); adenosine analogs (116, 117) were reported to increase cyclic AMP and EPO production in Hep3B cell cultures; adenosine $A_{2A}$ and $A_{2B}$ receptor activation have been reported to increase adenylate cyclase, cAMP, and kinase A activity in several cell lines (118); cyclic AMP analogs have been reported to increase HIF-1 activity through kinase A activation (109); and adenosine activation of the kinase C pathway through adenosine $A_{2B}$ receptors (119, 120) and the phospholipase $A_2$ pathway (121, 122) leads to increased expression of EPOmRNA through a non-HIF transcriptional factor.

## Therapeutic Uses of EPO in Anemia

In 1987, Eschbach *et al.* (123) reported the results of combined phase I and II clinical trials of recombinant human EPO (rHuEPO) in which the anemia of end-stage renal disease was corrected. rHuEPO was approved for human use in patients with chronic renal failure (CRF) by the Food and Drug Administration (FDA) in June 1989.

The National Kidney Foundation Dialysis Outcomes Quality Initiative (NKF-DOQI) reported, in 1997 (124), the results of a work group that established evidence based guidelines for the management of the anemia of CRF. In 2000, Eschbach (125) outlined several principles related to anemia management of patients with CRF being treated with rHuEPO:

1. The clinical response of the patient with CRF to rHuEPO does not occur unless doses of >15 U/kg i.v., three times weekly, are administered. Further increase in response is

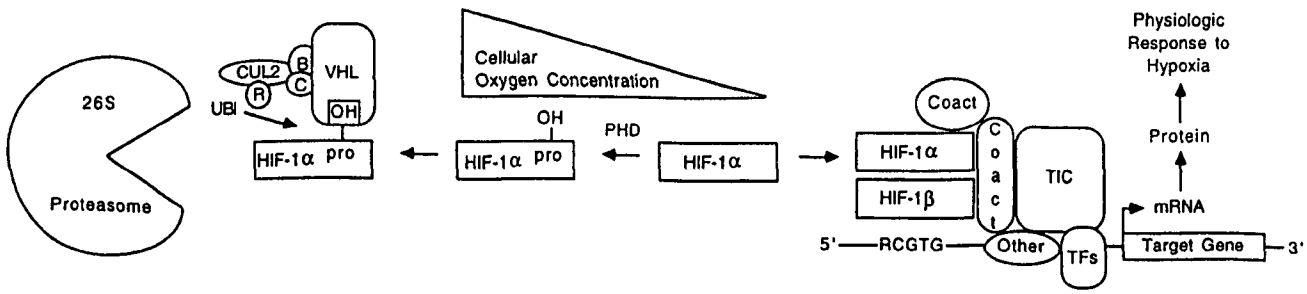


**Figure 4.** Regulation of HIF-1α expression by cellular $O_2$ concentration. $O_2$ availability determines the rate at which HIF-1α is subject to prolyl hydroxylation by PHDs 1-3. Prolyl hydroxylation is required for the interaction of HIF-1α with VHL, which recruits elongins B and C, Cullin 2 (CUL2), and RBX1 (R) to constitute a functional E3 ubiquitin-protein ligase complex. Ubiquitination of HIF-1α targets the protein for degradation by the 26S proteasome. Under hypoxic conditions, HIF-1β dimerizes with HIF-1α, which escapes prolyl hydroxylation, ubiquitination, and degradation. The HIF-1 heterodimer binds to hypoxia response elements containing the core recognition sequence 5′-RCGTG-3′ and recruits coactivator (Coact) molecules, resulting in increased transcription initiation complex (TIC) formation and mRNA synthesis, which ultimately results in the production of proteins that mediate physiologic responses to hypoxia. The battery of HIF-1 target genes that are expressed in response to hypoxia is cell-type specific and is determined by the binding of other transcription factors (TFs), which establish basal rates of transcription. See Ref. 102. Published with permission from Elsevier Science.

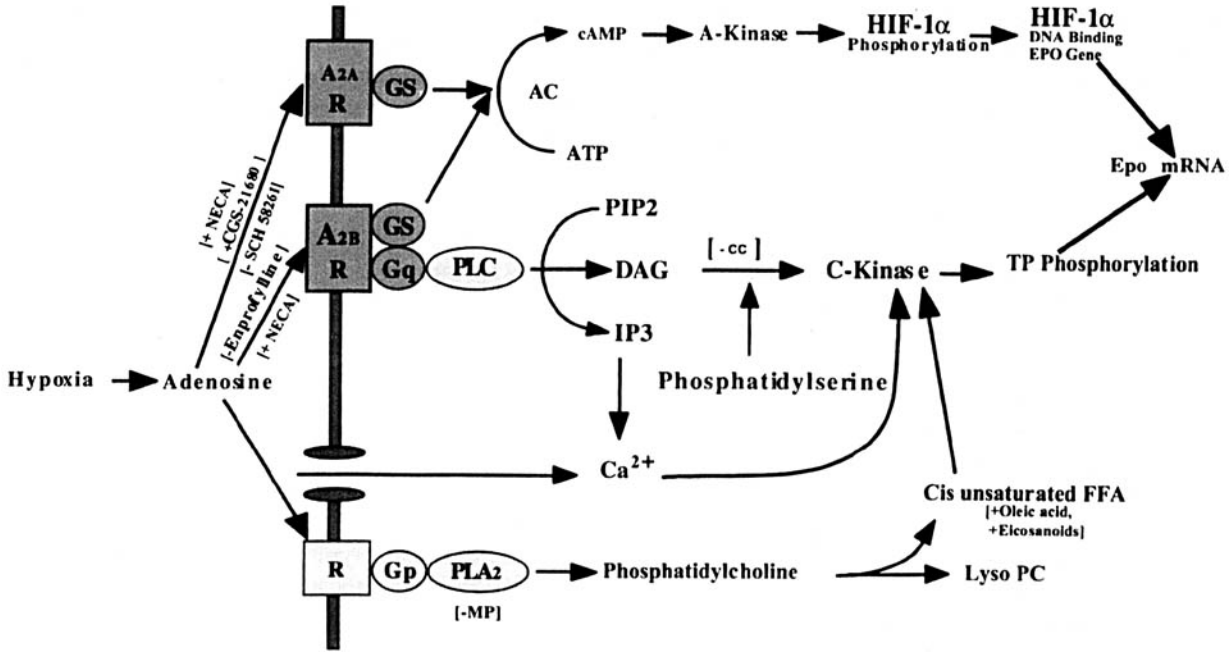


**Figure 5.** Model for adenosine protein kinases A and C, and phospholipase $A_2$ in hypoxic regulation of EPO production. CC, chelerythrine; PLC, phospholipase C; $PLA_2$, phospholipase $A_2$; AC, adenylate cyclase; R, receptor; DAG, diacylglycerol; $IP_3$, inositol trisphosphate; $PIP_2$, phosphatidylinositol 4,5-biphosphate; TP, transcriptional proteins; $G_q$, G protein that activates phosphoinositide-specific PLC; $G_s$, G-stimulating proteins; Gp, G protein that activates $PLA_2$; NECA, 5′-(N-ethylcarboxamido) adenosine; MP, mepacrine; FFA, free fatty acids; PC, phosphatidylcholine. Published with permission from Fisher JW and Brookins J (Ref. 115).

not likely to occur at doses above 500 U/kg i.v. three times weekly (125).

2. On the average, subcutaneous (s.c.) administration is more effective than i.v. or i.p. injections even though only 25% of the s.c. administered dose is absorbed (126).
3. Pharmacokinetic studies show that rHuEPO has a half-life (T½) after i.v. administration of 4–9 hr, whereas the T½ after s.c. injection is >24 hr (127).
4. It is important not to discontinue rHuEPO just because the target Hb has been achieved. The Hb level may fall more abruptly than anticipated if therapy is stopped because the rising Hb suppresses endogenous EPO production (128).
5. Treatment with RHuEPO leads to iron deficiency and it is essential that the patient with anemia of CRF being treated with rHuEPO be followed for symptoms of iron deficiency, e.g., serum ferritin, transferrin saturation, etc.

Even though the best target Hb level that should be achieved in patients with CRF has been somewhat controversial, the European Best Practice Guidelines has recommended that a target hemoglobin of >11g/dl be achieved for ≥85% of patients with CRF (129). To attain this target, the population median will be 12.0–12.5g/dl. They recommend that the target Hb may need to be varied for patients with CRF with specific comorbidities (129). A National Kidney Foundation ad hoc committee recommended a target hematocrit of 33%–38% (130). However, the FDA in the U.S. recommended a hematocrit range up to 36%, and this is the basis for reimbursements of patients treated with EPO in the U.S. Other investigators reported that current evidence-based recommendations suggest a target hematocrit range of 33%–36%, but caution that normalization of the hematocrit in hemodialysis patients with symptomatic heart disease has shown an increase in both mortality and the rate of vascular access thrombosis (131). The value of early management of patients with CRF has been stressed (132). The objective of early referral in patients with CRF is apparently to prevent complications as the disease progresses to the stage where dialysis or transplantation becomes necessary (132). When dialysis is initiated after the development of uremia, the poor nutritional status, severe acidosis, and anemia and poorly controlled hypertension makes the patient more difficult to manage. It has been suggested that nephrology referral should be centered around controlling anemia with rHuEPO, high blood pressure with appropriate drugs (especially converting enzyme inhibitors), nutritional status with adapted protein-calorie intake, and renal osteodystrophy with calcium carbonate or recombinant growth hormone in children (132). It has been suggested that chronic renal disease progression may be slower when anemia is reversed, emphasizing the benefits of early correction of the anemia with EPO (133).

The mechanism of the anemia of CRF is probably due to the inability of the kidneys with compromised renal function to produce sufficient amounts of EPO to meet the demands for new red cell production in patients with uremia. Over the years, there has been some controversy over the

role of the bone marrow response to endogenous EPO in patients with uremic end-stage renal disease. Obviously, the primary cause of the anemia in CRF is due to the lack of sufficient amounts of EPO to maintain steady-state erythropoiesis due to the reduced red cell life span, deficiency of iron and folic acid, hyperparathyroidism with myelofibrosis, aluminum toxicity, blood loss, and inhibition of erythropoiesis caused by "uremic toxins." However, serum levels of EPO in normal human subjects range between 1 and 27 mu/ml (mean 6.2 ± 4.3 mu/ml, $n = 53$), (134). On the other hand, serum levels of EPO in patients with CRF were between 4.2 and 102 mu/ml (mean 29.5 ± 4.0 mu/ml, $n = 36$) (134). Thus, it seems that even though EPO deficiency is the primary cause of the anemia of CRF, the uremic state may blunt the bone marrow response to EPO. Serum levels of EPO in patients with CRF were about five times as high in patients with CRF than in normal human subjects (134). McGonigle *et al.* (135) and Radtke *et al.* (136) reported several years ago a suppressed response of bone marrow cultures to EPO in the presence of plasma from patients with anemia. Macdougall (137) has recently summarized the role of "uremic toxins" in exacerbating the anemia in patients with CRF. Candidates suggested to play a role in uremic inhibition of erythropoiesis are polyamines, parathyroid hormone, and some inflammatory cytokines (137). Radtke *et al.* (136) and Kushner *et al.* (138) provided some support for polyamines as one of several uremic toxins that could be responsible for the suppressed response of the bone marrow to EPO in the patient with anemic CRF. Allen *et al.* (139) reported that when bone marrow from patients with uremia with and without inflammatory disease was cultured with autologous serum, the optimal response to EPO was significantly inhibited. Treatment of parallel cultures with a combination of antibodies to interferon-α (IFN-α) and tumor necrosis factor-α (TNF-α) almost completely restored the response to EPO. They conclude that CFU-E colony formation is inhibited by soluble factors present in the sera of patients with uremia with or without inflammatory disease (139). Erslev and Besarab (140) compared the rate and control of baseline red cell production in hematologically stable patients and uremic patients with anemia. The erythrokinetic rates in the anemic uremic patients were about one-half the rate in normal hematologically stable individuals even though the serum EPO titers were the same or higher in the anemic uremic patients (140). It is also of interest that patients with CRF undergoing ambulatory peritoneal dialysis maintain significantly higher hematocrits than patients on hemodialysis (141, 142). Thus, it seems possible that pharmacological doses of EPO in patients with CRF may overwhelm the bone marrow and correct both the EPO deficiency and the suppressed bone marrow response to EPO.

The cardiovascular effects of EPO and anemia correction has been summarized by Vaziri (143). It appears that EPO-induced hypertension is not due to the amelioration of anemia because a similar rise in blood pressure occurs in EPO-treated iron-deficient animals and humans even though the anemia persists (144, 145). Chronic administration of EPO results in hematocrit independent, vasoconstriction-dependent hypertension that is largely due to elevated resting and agonist-stimulated cytoplasmic calcium concentrations, which leads to resistance to the vasodilatory action of nitric oxide (144, 146). Other factors that may be involved in the hypertensive action of EPO are increased endothelin production (147, 148), upregulation of tissue renin and angiotensinogen production (149), and changes in vascular tissue prostaglandins (147, 150). EPO has also been demonstrated to promote angiogenesis (151–153) and to stimulate endothelial and vascular smooth muscle cells (154). In addition, correction of anemia in patients with CRF may partly prevent or reverse left ventricular hypertrophy in dialysis-dependent and dialysis-independent patients with chronic renal insufficiency (155). It has also been reported that inborn erythrocytosis leads to cardiac dysfunction and premature death in mice overexpressing EPO (156). Finally, it has been reported that neutralizing anti-EPO antibodies and pure red cell aplasia developed in 13 patients with anemia of CRF during treatment with epoetin (157).

Suppression of erythroid progenitor cells in the bone marrow (158) and anemia (159, 160) are frequently seen as side effects of Zidovudine treatment of patients with AIDS. Patients with AIDS with endogenous serum EPO levels <500 mu/ml who are receiving doses of Zidovudine <4,200 mg per week usually respond to rHuEPO therapy (159). However, patients with serum EPO levels >500 mu/ml usually do not respond to RHuEPO therapy (160).

RHuEPO is being used to treat patients with anemia associated with nonmyeloid malignancies where the anemia is due to the effects of concomitantly administered chemotherapy. RHuEPO was reported to be effective in the treatment of patients with cancer receiving cisplatin (161). A study of 3012 patients with nonmyeloid malignancies who were receiving chemotherapy was carried out in a multicenter, open label, nonrandomized study conducted in 600 United States community-based practices (162). Of the study patients, 819 (27.6%) received taxane chemotherapy, 349 (11.8%) received cisplatin chemotherapy, and 682 (23.0%) received carboplatin chemotherapy. Patients were administered 40,000 units of rHuEPO once weekly by the s.c. route, which could be increased to 60,000 units one time per week after 4 weeks, depending upon the hemoglobin response. Treatment was continued for a maximum of 16 weeks. Patients who were accessible for efficacy evaluation (2,964) were observed to have significant increases in Hb levels, decreases in transfusion requirements, and improvements in functional status and fatigue. In another randomized, double-blind, placebo controlled study of 375 patients with anemia receiving nonplatin chemotherapy with solid or nonmyeloid hematologic malignancies were treated with rHuEPO-α at dosages of 150–300 I.U./kg three times per week s.c. for 12–24 weeks. rHuEPO-α significantly de-

creased transfusion requirements and increased Hb (164). An increase in energy level and ability to do daily activities were significantly greater in the rHuEPO-α group when compared with the placebo patients (163).

rHuEPO was studied in a double-blind clinical trail in 316 patients prior to elective orthopedic hip or knee surgery (164). The patients were randomly assigned according to pretreatment Hb levels to receive 300 u/kg, 100 u/kg, or placebo s.c. for 10 days before surgery, on the day of surgery, and for 4 days after surgery (165). The dosage of 300 u/kg rHuEPO significantly reduced the risk of allogenic transfusions. In patients with pretreatment Hb of >10 to <13 g/dl, there was not a significant difference in the transfusion requirements between 100 u/kg rHuEPO and the placebo group. In another study of 145 patients scheduled for orthopedic hip or knee surgery who received 600 u/kg rHuEPO s.c. once weekly for 3 weeks prior to surgery and on the day of surgery or 300 u/kg once daily for 10 days prior to surgery, on the day of surgery, and for 4 days after surgery (166), the increase in Hb in the 600 u/kg weekly group was significantly greater than that observed for the 300 u/kg group.

rHuEPO has been investigated for use in myelodysplastic syndrome (166), preoperative autologous blood donation (167, 168), anemia of pregnancy (169), rheumatoid arthritis (170), anemia of prematurity (171, 172), bone marrow transplantation alone (173), and in combination with G-CSF (174). Evidence-based guidelines for anemia management in patients with CRF have been outlined by the National Kidney Foundation Dialysis outcomes Quality Initiative (125), which supplements guidelines for the clinical use of RHuEPO previously reported (130, 175). In a Phase III multicenter clinical trial (176) of rHuEPO in 333 hemodialysis patients with a median maintenance dose of 75 u/kg i.v. three times a week for approximately 13 months, the adverse effects noted were myalgias, 5%; iron deficiency, 43%; increased blood pressure, 35%; and seizures, 5.4% (176). The patients that did not respond had complicating causes for the anemia, such as myelofibrosis, osteitis fibrosa, osteomyelitis, and acute or chronic blood loss (176). If delayed or diminished response to doses of rHuEPO within the recommended dosing range occurs, the following etiologies should be considered:

1. Iron deficiency: all patients will eventually require supplemental iron therapy.
2. Underlying infections, inflammatory or malignant processes.
3. Occult blood loss.
4. Underlying hematologic disease.
5. Vitamin deficiencies: folic acid or vitamin B-12.
6. Hemolysis.
7. Aluminum intoxication.
8. Osteitis fibrosa cystica.

## Novel Erythropoiesis Stimulating Protein (NESP)

It was well known from previous experiments that sialic acid residues on EPO are responsible for maintaining *in vivo* biological activity of EPO (177). EPO is known to be desialylated *in vivo*, cleared from plasma, and is bound to galactose receptors in the liver (178). Thus, there is a direct correlation between the amount of sialic acid-containing carbohydrates, plasma half-life, and *in vivo* biological activity (177, 178). With this knowledge, it was conjectured that increasing the carbohydrate content of EPO would result in a longer plasma half-life and enhanced biological activity. Thus, a new molecule, NESP, was synthesized using the latest techniques of DNA technology (20, 21). An overview of the efficacy and safety of NESP has been reported by Macdougall (179). NESP contains five N-linked oligosaccharide chains, whereas native erythropoietin contains three oligosaccharide chains. The amino acid sequence of NESP differs from that of native human EPO at five positions (Ala30Asn, His32Thr, Pro87Val, Trp88Asn, and Pro90Thr), which allows for attachment of additional oligosaccharides at asparagine residue positions 30 and 88 (20). NESP contains 22 sialic acid residues compared with 14 sialic acid residues for native EPO. The molecular weight of NESP is 38,500 daltons and native EPO is 30,400 daltons; the total carbohydrate for NESP is 52% and native EPO is 40%. NESP binds to the EPO receptor in an identical manner as native EPO to induce intracellular signaling involving tyrosine phosphorylation by JAK-2 kinase and the same intracellular molecules Ras/MAP-k, P13-k, and STAT-5 as native EPO. In comparing the i.v. pharmacokinetics of NESP and rHuEPO in patients on dialysis (178), the mean terminal half life for NESP was three times longer than for rHuEPO (25.3 vs 8.5 hr). The clearance of NESP was significantly less than that of rHuEPO (1.6 ± 0.3 vs 4.0 ± 0.3 ml/hr/kg); the volume of distribution for NESP and rHuEPO were equivalent (52.4 ± 2.0 vs 48.7 ± 2.1 ml/kg). Given s.c., the mean terminal half-life for NESP was 48.8 hr. Apparently, the half-life for SC rHuEPO is 18–24 hr. When given once weekly i.v., NESP reaches a steady state within 4 weeks and does not accumulate.

The s.c. and i.v. pharmacokinetic profiles of NESP in patients on dialysis support the hypothesis that patients with anemia of CRF require less frequent doses of NESP when compared with rHuEPO (180–182). Three safety and efficacy studies of NESP in patients with anemia of CRF have been completed (183–185). The first two multicenter studies were to evaluate the efficacy of NESP in treating the anemia of CRF in patients on dialysis (183, 184). The third study was to evaluate the safety and effectiveness of NESP in maintaining Hb when administered at less frequent dosing compared with rHuEPO (185).The authors concluded from these studies that NESP is effective in the treatment of anemia of CRF and is safe; that the weekly dose for most patients appeared to be 0.45 μg; and that for both i.v. and

s.c. dosing, once weekly dosing with NESP was possible. It was concluded that NESP can maintain the Hb concentration just as effectively as rHuEPO at less frequent dosing. The adverse effects, withdrawals, and deaths were the same for the NESP as that reported for rHuEPO treatment (184). There have been no reports thus far of antibody production in patients treated with NESP.

In other studies of previously untreated patients suffering from anemia of CRF, NESP at a dosage of 0.45 μg/kg s.c. once weekly was just as effective in increasing Hb as rHuEPO at 50 units/kg two or three times weekly (184). Darbepoetin-α (NESP) has been approved by the U.S. FDA for the treatment of anemia caused by CRF, and may be approved soon for use in cancer patients with anemia associated with chemotherapy (186).

## Blood Doping

Blood doping may be defined as the artificial increase in Hb/hematocrit to enhance performance. The first report of blood doping was in a controlled experiment in 1947 (187). However, blood doping did not gain attention until following the Mexico City Olympics in 1968 in which athletes won endurance races by competition from high altitude training sites, which was a type of physiological blood doping. Ekblum *et al.* (188) showed in a landmark paper that blood doping increased $VO_{2max}$ and running time to exhaustion by 9% and 23%, respectively. Ekblum and Berglund (189) reported a trial that was uncontrolled with rHuEPO in athletes and their results showed that rHuEPO administration improved $VO_{2\ max}$ and running times to exhaustion in conjunction with increases in hematocrits. Jelkmann (190, 191) has reviewed the use of recombinant EPO as an antianemic and performance-enhancing drug. This investigator concludes that rHuEPO doping is not only unethical and illegal, but potentially dangerous. Shaskey and Green (192) have pointed out that the improvements in performance of athletes using EPO as a performance-enhancing drug are not without risk, and the use of exogenous EPO has the potential for increased viscosity of the blood and thrombosis with potentially fatal results. It is important to point out that use of rHuEPO to improve athletic performance can be even more dangerous than invasive traditional blood doping through transfusion. Excessive erythrocytosis can lead to increased blood viscosity and thrombogenicity and can cause deep vein thromboses, pulmonary emboli, coronary thromboses, or cerebral thromboses that can be exaggerated by dehydration. The use of rHuEPO to enhance athletic performance is officially banned by most sports-governing bodies and is to be condemned. There is a need to develop better detection methods using traditional urine testing. Abuse of EPO is currently difficult to detect because the routine assay for EPO is an immunoassay that immunologically cannot distinguish recombinant EPO from natural endogenous EPO (193). Darbepoetin-α (NESP) has been reported recently to be used as a doping agent and was the cause of the disqualification of two athletes performing in the 2002 Winter Olympics in Salt Lake City. Its use as a doping agent is also to be condemned because the excessive erythrocytosis can lead to increased blood viscosity, thromboembolism, and death.

EPO has been reported to activate specific receptors in the cental nervous system and was found to be neurotrophic and neuroprotective in both *in vitro* and *in vivo* models (195, 196). EPO and the EPO receptor have both been reported in the brain cortex, cerebellum, hippocampus, pituitary gland and spinal cord (195). The mechanisms which have been proposed by which EPO produces a neuroprotective effect are (196): reduction in glutamate toxicity, increased production of neuronal anti-apoptotic factors, reduced nitric oxide mediated injury, anti-inflammatory effects, and anti-oxidant properties.


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