Erythropoietin: Multiple Physiological Functions and Regulation of Biosynthesis

Ryuzo Sasaki,¹ Seiji Masuda, and Masaya Nagao

Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan

Erythropoietin (Epo), which is produced by the kidney in the adult and by the liver in the fetus, increases red blood cells by supporting the survival of erythroid progenitor cells and stimulating their differentiation and proliferation via binding to Epo receptor (EpoR). The main signal in the control of Epo production is oxygen; hypoxia stimulates Epo production through activation of Epo gene transcription. Tremendous progress in our understanding of molecular mechanisms of Epo action on erythroid cells and regulation of the Epo production has been made by manipulation of cDNAs and genes of Epo and EpoR. Studies on hypoxic induction of Epo gene transcription led to the identification of hypoxia-inducible factor (HIF-1), a transcriptional factor, that functions as a global regulator of hypoxic gene expression. Paracrine Epo/EpoR systems that are independent of the endocrine erythropoietic system (kidney/bone marrow) have been found in the central nervous system and uterus. Novel functions of Epo at these local sites and tissue-specific regulation of Epo production including a newly found potent regulator (estrogen) have been proposed. The tissue-specific regulation rationalizes the specific functions of Epo produced by individual tissues.

Key words: erythropoietin; kidney; brain; uterus; hypoxia

In the mammalian embryos, erythropoiesis occurs initially in the yolk sac blood islands (primitive erythropoiesis) and then shifts to the fetal liver, and eventually to the bone marrow and also spleen in small animals such as rats and mice (definitive erythropoiesis). Erythropoietin (Epo), a glycoprotein, acts mainly on the late erythroid precursor cells (colony-forming unit-erythroid, CFU-E) through binding to the specific receptor, Epo receptor (EpoR). Epo supports the survival of CFU-E, and stimulates their proliferation and differentiation (Fig. 1).¹,² Homozygous mutant mice in either Epo gene or EpoR gene resulted in embryonic death by E13.5 due to the complete lack of fetal erythropoiesis, indicating that Epo signaling is not required for the primitive erythropoiesis but it is essential for the definitive erythropoiesis in the fetus.³,⁴ There is a body of evidence showing that Epo is also the principal regulator of another definitive erythropoiesis, adult erythropoiesis.¹,²,⁵ The main Epo production site is the kidney in the adult and the liver in the fetus. Under severe hypoxia, the adult liver contributes somewhat to the total production of Epo. A major signal regulating Epo production in these tissues is the oxygen concentration; Epo production is markedly enhanced under hypoxia through mainly transcriptional activation of the Epo gene (Fig. 1) and partly increased stability of Epo mRNA. An increase of Epo in the blood stimulates the formation of red blood cells, resulting in improvement of the oxygen supply and eventually repression of the activated transcription. Complementary DNAs of Epo and EpoR were cloned in 1985 and 1989, respectively.⁶-⁸ These achievements, together with subsequent sequencing of their genes⁹-³³ and supply of recombinant Epo, have brought tremendous progress in our understanding of Epo and EpoR with respect to the structure-function relationship, Epo signal transduction pathway, Epo production sites including identification of Epo-producing cells, and regulation of Epo production.

Until recently erythropoietic stimulation has been believed to be the sole physiological function of Epo. However, Epo has been found to play important roles in the brain and uterus where new sites of Epo production exist.¹⁵-¹⁸ Many excellent reviews on the erythropoietic stimulation of Epo and the oxygen-dependent regulation of Epo production have been published.¹,²,⁵,¹⁹-³¹ In this review, therefore, we first outline the classical function of Epo and the regulation of Epo production. Second, we deal with the new physiological functions of Epo and the tissue-specific regulation of Epo production.

Structure of Erythropoietin

(1) Urinary and recombinant erythropoietin

Human mature Epo consists of 165 amino acid residues. Epo mRNA encodes a protein with 193 amino acid residues including an amino-terminal leader sequence that consists of 27 amino acid residues.⁵,⁷ Removal of the signal peptide would produce the secreted Epo with 166 amino acid residues but analyses of terminal amino acid residues revealed that the carboxy-terminal arginine is missing in both uri-

¹ To whom correspondence should be addressed. Ryuzo Sasaki, Telephone: +81-75-753-6271; Fax: +81-75-753-6274; E-mail: rsasaki@kais.kyoto-u.ac.jp
nary Epo and recombinant Epo. The physiological significance of removal of the terminal arginine and its precise mechanism are not known. Two disulfide bridges are formed between cysteine 7 and 161 and between cysteine 29 and 33, and reduction of the disulfide bridges results in complete loss of the bioactivity. The molecular mass of the mature Epo peptide is 18 kDa. However, Epo is a heavily glycosylated protein and the structures of carbohydrates attached to urinary Epo and recombinant Epo were analyzed. Human Epo has three N-linked sugars at positions 24, 38 and 83 (complex-type sugars not high mannose-type) and one O-linked sugar at position 126 (Fig. 2). Consequently, human Epo has a molecular mass of 30 kDa by the sedimentation equilibrium method and migrates with a size of 35–37 kDa in SDS polyacrylamide gel. The functions of the carbohydrates in Epo were investigated with respect to bioactivity including stability in the circulation, and biosynthesis. O-linked sugar is present in human Epo but not in rodent Epo and therefore O-linked sugar does not have an important function. N-glycosylation is not required for the in vitro activity but is essential for the exhibition of the in vivo activity. Full sialylation of galactose residues in N-linked sugars is very important for stability in the circulation. Production of mutant Epos that have lost N-glycosylation sites indicated that N-linked sugars are required for proper biosynthesis and/or secretion of Epo. The tetraantennary N-linked sugar-containing Epo exhibits a 7-fold greater specific activity in vivo than biantennary sugar-containing Epo, suggesting that the N-linked sugars may influence the bioavailability of Epo in vivo in such a way that is not detected in the in vitro assay. N-linked sugars play a critical role in the apical secretion of Epo produced in polarized epithelial cells transfected with Epo cDNA, although the physiological meaning is unclear.

(2) Biomimetic ligands

Successful clinical applications of recombinant Epo to a number of anemic patients including those with end-stage renal disease have encouraged an attempt to find low-molecular weight ligands that mimic Epo, because the production of Epo by mammalian cells is costly. Investigation on the action mechanism of such ligands has also contributed to our understanding of the interaction between EpoR and Epo that leads to the activation of the Epo signal transduction pathway. By the use of random phage display peptide libraries and affinity selective methods, Wrighton et al. in 1996 described 14-amino acid disulfide-bonded cyclic peptides that have Epo-like activity in vitro and in vivo, although their amino acid sequences are not related to the primary sequence of Epo. Subsequently, analysis of the crystal structure of a complex of the agonist peptide with the extracellular domain of EpoR, capable of binding with Epo, has revealed that a noncovalent dimer of the peptide induces receptor dimerization that is an essential process for activation of the Epo signaling pathway. Covalent dimerization increased the potency of an Epo mimetic peptide by increasing its affinity to EpoR. The crystal structure of a complex of the extracellular ligand-binding domain of EpoR and Epo shows that Epo imposes a unique receptor orientation that is responsible for optimal signaling, suggesting an important parameter in the design of Epo mimetics with higher bioactivity.

**Signal Transduction Pathways**

Erythroid progenitor cells that are concentrated at the CFU-E stage are markedly accumulated in spleen when the Friend virus that produces anemia is administered to mice or when thiampenicol is given to anemic mice and these cells have allowed studies of the biochemical sequence of events after Epo stimulation. Addition of Epo to these cells increases calcium uptake. At 3–4 hr after the addition, total RNA synthesis and glucose uptake increase. By 6 hr, the number of transferrin receptor and the transcription rate of α- and β-globin genes increase. Synthesis of hemoglobin and erythroid-specific membrane pro-
teins (Band 3 and Band 4.1) commences at 12 hr. CFU-E differentiate into erythroblasts and enucleate reticulocytes by 48 to 60 hr of culture.

The above events are induced upon binding of Epo to its receptor. The murine EpoR mRNA encodes a protein of 507 amino acids that contains a single hydrophobic membrane spanning domain (Fig. 2). Sequence analyses of other hematopoietic growth factors revealed a family of receptors that have two distinctive features in their extracellular domain; a set of four conserved cysteine residues and a motif located close to the transmembrane domain, Trp-Ser-X-Trp-Ser (WSXWS), where X represents any amino acid. Receptors for Epo, interleukin-2, granulocyte macrophage colony-stimulating factor, granulocyte colony-stimulating factor, thrombopoietin, leukemia inhibitory factor, growth hormone, prolactin, oncostatin M and ciliary neurotrophic factor belong to this family. Their cytoplasmic portions do not contain a tyrosine kinase domain. The primary function of the ligand is to initiate receptor aggregation; homodimers are formed in the receptors for Epo, granulocyte colony-stimulating factor, thrombopoietin, prolactin and growth hormone, while other ligands form hetero-oligomeric receptors. 

(1) Jak-Stat activation

Ligand-induced oligomerization of the cytokine receptors recruits and activates members of the Janus family of nonreceptor-type protein tyrosine kinases (Jaks). Jak family has no SH2/SH3 (src homology) domain and so far four members (Jak1, Jak2, Jak3 and Tyk2) have been identified. They have a relative molecular mass in the range of 120–130 kDa with a carboxy-terminal kinase domain. Ligand-induced activation of Jaks is due to autophosphorylation. The transcription factor family termed signal transducers and activators of transcription (Stat) are well known substrates of the activated Jaks. The Stat family consists of seven members, which contain a carboxy-terminal SH2 domain, a SH3-like domain, and the middle region responsible for DNA binding. Tyrosine phosphorylation of the carboxy-terminal site in Stats by the activated Jaks results in dimerization of Stats through the SH domains, translocation to the nucleus and binding to the target genes, eventually regulating the expression of many genes.

Binding of Epo induces receptor homodimerization, which results in activation of Jak2 that associates with the membrane-proximal domain (box 1/box 2) of the receptor (Fig. 2). In response to Epo, tyrosine phosphorylation of the receptor also occurs at sites in the membrane-distal region (Fig. 2). Recently, Epo has been shown to stimulate proliferation of the cells that express EpoR lacking the carboxy-terminal region containing box 2, suggesting that the membrane-proximal domain including box 1 is sufficient for mitogenesis. It is noted, however, that this result does not exclude the possibility that the carboxy-terminal region containing box 2 is necessary for the maximal activation of cell proliferation. The activated Jak2 phosphorylates Stat5. There is no doubt on the importance of Jak2 in the Epo signaling pathway; mutant Jak2−/− mouse embryos lack red blood cells, while the significance of Stat5 has not been established. The results of Stat5 activation in the mitogenic action of Epo are contradictory. Similarly, both stimulatory and suppressive effects of Stat5 on Epo-induced differentiation of erythroid cells have been reported. Mice express two isoforms, Stat5a and Stat5b, which are approximately 95% identical in amino acid sequence. These two genes colocalize to murine chromosome 1, indicating that the two genes resulted from a gene duplication. Since both Stat5 genes are expressed in a variety of cell types, it has been thought that they would be functionally redundant and disruption of both genes would be necessary to find an abnormal erythropoiesis. As expected, no abnormality in hematopoiesis including erythropoiesis has been found in mice deficient individually in the isoforms. Unexpectedly, however, the mutant adult mice carrying null mutations of both Stat5a/b genes showed that the responses to a variety of cytokines, including Epo, are largely unaffected. Thus Stat5 may not play important roles in Epo signaling for proliferation and differentiation of CFU-E or they may have evolved backup.

Epo supports the survival of erythroid cells by preventing their apoptotic death without any effect on their cell cycle status. Addition of Epo to the culture of Epo-dependent cells increases Bcl-X, a Bcl-2 family member that acts as an anti-apoptotic protein. An interesting result recently reported is that Epo induces the expression of Bcl-X in Epo-dependent cell lines and this induction is achieved by binding of the Epo-activated Stat5 to a Stat binding element in the Bcl-X promoter. Nevertheless, the phenotypes of the adult mice deficient in both Stat5 genes again cast doubt on the function of Stat5 in Epo-induced survival of the cells. More recently, however, Stat5a−/−5b−/− embryos were found to be severely anemic. Fetal liver erythroid progenitors from the mutant embryos give rise to fewer erythroid colonies in vitro and show a marked increase in their rate of apoptosis. The Stat5 consensus binding sites have been identified in a regulatory region within the first intron of both the human and murine Bcl-X genes. In the developing mouse embryos, a high rate of erythropoiesis is required for sustaining rapid growth, although erythropoietic “reserve capacity” that probably reflects CFU-E population is very small in fetuses in comparison with that in adults. Taken together, the results of mutant embryos suggest that the Epo/Stat5/Bcl-X pathway is very important for the fetal erythropoiesis. Approximately one-third of
the Stat5a/b mutant mice die within 48 hr after birth from unknown causes. Compensatory mechanisms may work in surviving adult mice deficient in Stat5a and 5b. It is of interest to know what may happen in erythropoiesis of the mutant adult mice when they are exposed to hypoxic stress under which rapid erythropoiesis should take place.

(2) Other pathways
Binding of Epo to its receptor induces tyrosine phosphorylation of the proto-oncogene product Vav. The membrane-proximal of the receptor is sufficient for Vav phosphorylation. Tyrosine-phosphorylated Vav, but not the non-phosphorylated protein, has been shown to catalyze GDP/GTP exchange on Rac-1, a protein implicated in cell proliferation and cytoskeletal organization. Epo-induced phosphorylation of Vav was well correlated with growth signaling from EpoR including Jak2 activation. Also phosphorylated Vav has been shown to interact with two phosphotyrosine proteins, EpoR and p85, a regulatory subunit of phosphatidylinositol 3-kinase (PI3-kinase). In response to Epo, tyrosine residues in the membrane-distal domain of EpoR are phosphorylated (Fig. 2). Phosphorylated tyrosines recruit a number of signaling molecules through their SH2 domains and the recruited molecules are activated by subsequent phosphorylation of their tyrosines. Such signaling molecules include an adaptor protein SHC, p85 regulatory subunit of PI3-kinase, phospholipase C-γ1 and phosphatase Syp (also termed SH-PTP2 or PTP-1D) that is thought to stimulate signaling in contrast to HCP described below. Many cytokines activate Ras by inducing phosphorylation of SHC. Other kinases/signal transducers that are tyrosine-phosphorylated in response to Epo include GAP, c-fps/fes, Lyn and c-Cbl. The role of all these signaling molecules in the mitogenic action of Epo is still unclear. Little is known with respect to Epo signal pathway(s) for differentiation of erythroid progenitor cells.

(3) Feedback regulation of Epo signaling
The carboxy region of EpoR has been shown to negatively affect the response to Epo. Tyrosine phosphorylation of the distal region of EpoR creates binding sites for hematopoietic cell phosphatase (HCP, also termed PTP-1C or SH-PTP1). Recruitment of HCP to EpoR is associated with dephosphorylation of Jak2, suggesting that Jak2 is a target of HCP. The motheaten mice that genetically lack HCP suffer from hematopoietic abnormalities due to cell overproduction. Thus HCP may be important as a feedback inhibitor to terminate the ligand-induced mitogenic signals.

Using the yeast two-hybrid system, a new SH2-domain-containing protein, Jak binding protein (JAB), which interacts with the tyrosine kinase domains in Jaks, has been identified. Interaction of JAB with Jak3 markedly reduces their tyrosine kinase activity and suppress activation of Stats. JAB is structurally related to a cytokine-inducible SH2 protein (CIS) family. Thus, JAB and CIS appear to function as negative-feedback regulators in the Jak-Stat pathways activated by cytokines including Epo. SOCS-1 and SSI-1 reported from other laboratories are identical to JAB.

(4) Erythropoietin-induced calcium influx
Sawyer and Krantz showed that Epo stimulates 45Ca2+ uptake into Friend virus-infected erythroid cells. Several subsequent studies have suggested a role of calcium in the mediation of Epo action. The Epo-induced increase of the intracellular free calcium concentration in erythroid precursor cells appears to be mediated through a voltage-independent ion channel and to depend on tyrosine phosphorylation. More recently, the EpoR domain including tyrosine residue at a specific site required for calcium channel activation has been identified. A paper inconsistent with these results has also been published and further studies are clearly needed to understand the role of the calcium influx in Epo-responsive cells. In addition to erythroid cells, the Epo-induced increase of the intracellular free calcium concentration has been found in neuronal cells expressing EpoR. The increase is due to an elevated influx of calcium that is mediated through plasma membrane calcium channels. Epo induced membrane depolarization, supported cell survival, stimulated production of monoamines and nitric oxide, and elevated tyrosine hydroxylase activity, suggesting that Epo is involved in neuronal activity. As described later, Epo protects neurons from ischemic damage.

Cells Producing Erythropoietin for Erythropoiesis
In adults, the kidney is the major site of Epo production. Anemic hypoxia due to loss of red blood cells and hypoxic hypoxia due to ambient low oxygen or CO poisoning elevates dramatically the serum Epo concentration; it is induced by more than 50-fold under severe hypoxia. Carbon monoxide reduces oxygen delivery by decreasing oxygen loading of hemoglobin and also by increasing the affinity of oxygen to hemoglobin. Cobalt is believed to mimic hypoxia by fixing a putative oxygen-sensing heme protein in a deoxygenated form through replacement with iron in the heme (see below). The cloning of Epo cDNA provided a technique to measure Epo mRNA. Northern blot analysis showed that hypoxia- or cobalt-induced increases of serum Epo correlate with those of renal Epo mRNA, which indicates that
Epo production is regulated by its mRNA level. By the in situ hybridization technique, mouse Epo mRNA was detected mainly in the inner cortex of the kidney and the cells accumulating Epo mRNA under hypoxia appeared to be interstitial cells in the peritubular capillary bed. This observation was supported by analyzing the expression of a transgene that contains the kidney-inducible element of the 5′-flanking region and hypoxia-inducible element of 3′-flanking region. Epo mRNA in these interstitial cells has been shown to co-localize with ecto-5′-nucleotidase, indicating that they are fibroblasts. Epo-producing cells in the monkey kidney were also assigned to be peritubular interstitial cells. Thus these cells may sense the balance between the oxygen supply from capillaries and oxygen consumption by tubular cells, thereby Epo gene transcription is regulated. It may be worthwhile to add the claims that tubular cells not interstitial cells are responsible for renal Epo production.

Hypoxic Regulation of Erythropoietin Production

Hypoxia or cobalt activates Epo production by increasing its mRNA level. This increase of Epo mRNA is mainly due to activation of Epo gene transcription but the posttranscriptional events partly contribute, which invited several studies on the stabilization of Epo mRNA.

1. cis-acting sequences required for tissue-specific expression in regulated manner; analyses of transgenes

To find cis-acting sequences that allow Epo gene to be expressed with tissue specificity and hypoxia inducibility, the expression of transgenes has been analyzed using human and mouse sequences. Semenza et al. showed that expression of the human Epo gene in liver and kidney is controlled by different cis-acting DNA sequences. The 4 kb human Epo transgene encompassing the Epo gene, 0.4 kb of 5′-flanking sequences, and 0.7 kb of 3′-flanking sequences was expressed in the liver, kidney and other tissues that did not express Epo gene normally. Hypoxia-induced expression, however, was found only in the liver and not in any other tissues, indicating that a liver-inducible element exists within the 4 kb. Expression of the 10 kb transgene containing the Epo gene, 6 kb of 5′-flanking sequences, and 0.7 kb of 3′-flanking sequences was detected in the liver of anemic or cobalt-treated mice but not either the liver or kidney of unstimulated mice. Thus a negative regulatory element between 0.4 and 6 kb of 5′-flanking sequences has been proposed. The transgene consisting of the Epo gene, 14 kb of 5′-flanking sequences, and 0.7 kb of 3′-flanking sequences was inducibly expressed in the kidney as well as the liver. Expression of Epo in the kidney, therefore, is dependent on sequences between 6 and 14 kb 5′ to the Epo gene. More recently, Madan et al. reported that sequences required for induction in the kidney are located more than 9.5 kb 5′ to the human Epo gene.

Maxwell et al. showed using mouse Epo gene that cis-acting sequences which regulate the expression of renal Epo are within the transgene boundaries with 9 kb 5′- and 3.5 kb 3′-flanking sequences. Using chimeric Epo-LacZ gene constructs as transgenes, Haidar et al. indicated the importance of a sequence within a 1.2 kb 3′ region for potent induction in adult kidney but not in the adult liver. This sequence is located downstream of the well characterized 3′ hypoxia-responsive enhancer element (see below). No information of the sequences that allow expression of the Epo gene in brain and uterus with tissue-specific regulatory manners is so far available.

2. Cell lines producing Epo in an oxygen-regulated manner, cis-acting sequence and transactivator

To enable a molecular approach to the mechanism underlying the hypoxic activation of Epo gene expression, suitable cell lines are necessary. There are renal cell lines that produce Epo but the production is always oxygen-independent. An important finding was made by Goldberg et al., who demonstrated oxygen-regulated expression of Epo gene and production of Epo by human hepatoma cells lines (Hep3B and HepG2). As observed in vivo, production of Epo by these cell lines was also increased by transition metal ions (Co²⁺, Ni²⁺ and Mn²⁺). These cell lines made it possible to determine the cis-elements responsible for hypoxic induction of Epo gene expression, to identify transactivators bound to these elements, and to propose a model of oxygen sensing.

The DNA sequences which mediate hypoxia-induced expression of Epo gene were identified by a reporter assay after transfection of Hep3B or HepG2 cells. These studies identified a powerful 50 bp
regulatory element locating 120 bp 3' to the poly(A)-addition site (Fig. 3). Detailed studies of this 3' enhancer defined three important sites, at one of which was bound a protein complex termed hypoxia-inducible factor (HIF-1).132 This site, 5'-TACGTCG-3', is highly conserved between human and mouse gene, and locates near the 5' end of the enhancer. Affinity purification of HIF-1 revealed that HIF-1 is a heterodimer of two basic-helix-loop-helix proteins (HIF-1α and HIF-1β) of the PAS family (Fig. 3).133,134 PAS is an acronym derived from Per, AHR, ARNT and Sim which are members of the PAS gene family. The feature of the PAS domain is an imperfectly duplicated sequence of approximately 50 amino acids containing the characteristic motif His-X-X-Asp.135 HIF-1α was a new 120 kDa protein, while HIF-1β with 91–94 kDa had previously been identified as ARNT (aryl hydrocarbon receptor nuclear translocator) which is a dimerization partner of AHR (aryl hydrocarbon receptor). Levels of both mRNAs are relatively constant irrespective of the oxygen concentration.136-139 HIF-1β (ARNT) remains abundant irrespective of oxygen concentration, while HIF-1α can not be detected in oxygenated cells.137 Under normoxia, HIF-1α is rapidly degraded via a ubiquitin-proteasome system.138,139 Under hypoxia, HIF-1α is somehow stabilized by a mechanism yet to be studied, resulting in an increase of HIF-1 and thereby transcription of the Epo gene is activated (Fig. 3).

Although the middle segment of the enhancer containing CA repeats in the human gene is not well conserved in mice and humans, the mutation of this region abolishes the function of both human and mouse enhancers.132 Thus far a specific protein that binds to this region has not been demonstrated.

The third element is the 3' segment of the enhancer, which is again highly conserved as a direct repeat of a hexanucleotide consensus nuclear receptor binding half site (5'-YGACCY-3' where Y = C or T) separated by a 2-bp spacer (DR-2) (Fig. 3).132,140,141 Expression of the Epo gene appears to be modulated by binding of a variety of proteins to the DR-2. Binding of hepatocyte nuclear factor-4 (HNF-4) increases the hypoxic inducibility of the Epo gene in Hep3B cells dramatically, while binding of the EAR/COUPI family and TR-2 decreases the inducibility by antagonizing the binding of HNF-4.141 Like Epo, HNF-4 is expressed in the kidney, liver, and Hep3B cells. In most, if not all, of the hypoxia-inducible genes, HIF-1 binding sites are found near the promoters and the presence of HIF-1 site alone is sufficient for their hypoxic activation. Thus the middle and third DR-2 segments in the 3' enhancer are characteristics of the Epo gene.

As shown in Fig. 3, the C-terminal portion of HIF-1α binds to p300,142,143 a general transcriptional activator that is involved in expression of a large variety of genes. This protein is highly homologous to CREB binding protein (CBP); CREB is the cyclic-AMP responsive element-binding protein. p300/CBP does not bind to DNA but does interact with a variety of transcriptional factors by which gene expression is regulated. It is likely that p300 also interacts with HNF-4.144

Transfection assay of the reporter gene linked to Epo 3' enhancer has shown that an oxygen sensing system found in Hep3B and HepG2 is widespread in mammalian cells.145 The HIF-1 DNA binding activity (HIF-1 stabilization) is also induced by hypoxia in a variety of cells.146 In addition to Epo, hypoxia-inducible genes containing HIF-1 binding site are glycolytic enzymes including phosphofructokinase, aldolase, phosphoglycerate kinase, enolase and lactate dehydrogenase, glucose transporter 1, vascular endothelial growth factor (VEGF), transferrin, nitric oxide synthase, hem oxygenase, and tyrosine hydroxylase.27,147 Hypoxia-induction of these genes plays a significant role in the adaptation of mammalian organisms to hypoxia. The glycolytic enzymes and glucose transporter are involved in ATP production through anaerobic glycolysis and glucose uptake, respectively. VEGF activates formation of blood vessels (angiogenesis). The increased iron demand by erythropoiesis induced by Epo will be fulfilled by the induction of transferrin. The genes en-
coding inducible nitric oxide synthase and heme oxygenase produce the vasodilators nitric oxide and carbon monoxide, respectively. The tyrosine hydroxylase gene encodes a key enzyme in the synthesis of dopamine that accelerates breathing. Thus, HIF-1 controls multiple hypoxic responses including adaptation to anaerobic metabolism, erythropoiesis, angiogenesis, vasodilation, and breathing. Hif1α−/− embryos die by E11, showing neural tube defects, cardiovascular arrest and marked cell death within the cephalic mesenchyme.148

(3) Oxygen sensing and signal transduction
The possibility that the oxygen-sensing mechanism leading to the stimulated Epo production is related to a nonspecific blockade in oxidative phosphorylation has been excluded, because inhibitors such as cyanide of the oxidative phosphorylation do not affect Epo production. By using Hep3B cells, Goldberg et al. have presented results suggesting that the oxygen sensor is a heme protein (Fig. 3).105 Hypoxia, Co2+ and Ni2+ stimulated Epo production. Stimulation of Epo production by hypoxia but not by Co2+, was inhibited when the cells were exposed to carbon monoxide. Carbon monoxide, like when it binds to hemoglobin, may stabilize the conformation of the oxygen-sensing heme protein in an oxygenated form, inhibiting a hypoxia-induced signal transduction to activate Epo gene transcription. Based on the fact that cobalt protoporphyrin does not bind oxygen, they proposed that cobalt could substitute for iron in a heme of the sensor protein, yielding a constitutive deoxy form which would stimulate expression of Epo gene despite normoxia. Desferrioxamine, a potent iron chelator and inhibitor of heme synthesis, reduced hypoxia-induced Epo production, supporting their hypothesis. The soil bacterium, Rhizobium melliloti, fixes dinitrogen into ammonia when it is in symbiotic association with its plant host, alfalfa. More than 23 genes that are involved in nitrogen fixation are expressed in response to low oxygen concentration through the two component system. Oxygen concentration is thought to be sensed by the first transmembrane component, an oxygen-binding heme protein termed FixL with kinase and phosphatase activities. This signal is transmitted to the second component, FixJ, via phosphorylation-dephosphorylation reactions.149 Search for an analogous oxygen sensor in mammalian cells has been so far unsuccessful. Other oxygen-sensing mechanisms including molecules that may be involved in the signal transduction have been presented but no compelling observation has been made.5,26,31,144

More recently, a critical role for the von Hippel-Lindau (VHL) tumor suppressor gene product pVHL in HIF-1 regulation has been proposed.150 In VHL-defective cells, HIF-1α is constitutively stabilized and HIF-1 is activated even under normoxia. The interaction between HIF-1 and pVHL is necessary for the oxygen-dependent degradation of HIF-1α. Thus VHL plays a role as a tumor suppressor by repressing angiogenesis required for progression of tumors through suppression of VEGF gene expression, and further studies of VHL may provide a new clue to understand oxygen sensing.

Other Factors Regulating Erythropoietin Production
In addition to oxygen, other factors may be involved in the regulation of Epo production. Thyroid hormones have been shown to enhance hypoxia-induced Epo production in isolated perfused rat kidneys and HepG2 cells.151 Experimental results of the effects of nitric oxide on Epo production are contradictory. Nitric oxide was found to stimulate Epo production in the isolated perfused rat kidney,152 while others showed that endogenous nitric oxide attenuates Epo gene expression in vivo.153 Mouse embryonic cells and embryonal carcinoma cell lines produce Epo. Interestingly, the expression of Epo gene in these cells is not induced by hypoxia, although hypoxia induces other hypoxia-inducible genes.154 Survey of factors that may regulate Epo production revealed that retinoic acid (RA) stimulates Epo production in the embryonal carcinoma cell lines, P19 and F9.155 RA induced Epo production through the transcriptional activation of the Epo gene in an oxygen-independent manner. The DR-2 in the 3' enhancer (see Fig. 3) has been demonstrated to act as a binding site of RA receptor. Although further studies are clearly needed to understand the physiological significance of RA-induced transcriptional activation of the Epo gene, RA may play a role in the maintenance of Epo level appropriate to the day-to-day production of red blood cells under normal, steady-state conditions, because RA increased the serum Epo level in vitamin A-depleted rats.156 A finding that Epo production in mouse yolk sacs is stimulated by RA has made it plausible that RA is also involved in the regulation of Epo production in an early stage of animal development.157 It thus appears that DR-2 is critical for the Epo gene to select the signals to respond in a tissue- and/or developmental stage-specific manner.

Neurotrophic Function of Erythropoietin
Neuronal cell lines such as PC12 and SN6 express EpoR.99 Using radioiodinated Epo, the specific Epo binding sites have been found in some defined areas of the adult mouse brain including hippocampus and cerebral cortex, the areas containing neurons vulnerable to ischemic insult.158 Expression of EpoR mRNA has also been demonstrated in primary cultures of rat hippocampal and cerebral cortical neu-
rons and also in the adult mouse brain. Binding of Epo to PC12 cells causes a rapid and transient influx of calcium from outside of the cells, and also increases the intracellular concentration of monoamines. Epo augments choline acetyltransferase in mouse embryonic primary septal neurons and supports the survival of septal cholinergic neurons in the adult rats that have been subjected to fimbria-fornix transection. More recently, Epo has been shown to affect Ca\(^{2+}\)-homeostasis of neuronal cells through an increase in Ca\(^{2+}\) influx via plasma membrane Ca\(^{2+}\) channels.

Since the blood-brain barrier does not allow the passage of the kidney-derived circulating Epo to the central nervous system (CNS), Epo production site(s) must exist in CNS if the above effects of Epo on neuronal cells are to be physiologically meaningful. Astrocytes have been found to produce brain Epo and the low oxygen tension stimulates the Epo production through an increase of its mRNA. Epo mRNA is expressed in adult rat brain and the expression is increased by hypoxia. Immunoreactive Epo is present in human ventricular cerebrospinal fluid. Thus, the brain has a paracrine Epo/EpoR system that is independent of the erythropoietic system.

Both Epo and EpoR are expressed in the fetal brain. EpoR was detected in neural plate of mouse embryos (around day 7). EpoR mRNA in brain of mouse embryos at day 10.5 is expressed in abundance comparable to that in adult hematopoietic tissues and its level is dramatically reduced during development; the level of EpoR mRNA in the adult brain is 2 orders of magnitude lower than that observed in the embryonic brain at day 10.5. Epo and mRNA of EpoR were also detected in CNS of human fetuses. In addition to the neurotrophic function of Epo in the adult brain, these results suggest that Epo plays a role in neuronal development in fetuses, which remains to be studied.

(1) In vivo evidence that Epo protects neurons from ischemic damage

Epo gene expression in the brain is induced by hypoxia. Accordingly, whether or not Epo protects neurons from ischemic death has been examined. Infusion of Epo into the lateral ventricles of gerbils prevented ischemia-induced learning disability and rescued hippocampal CA1 neurons from lethal ischemic damage (Fig. 4, IA and IIA-H). The neuroprotective action of exogenous Epo was also confirmed by counting synapses in the hippocampal CA1 region.

Conclusive evidence that the endogenous brain Epo is really critical for neuronal survival was obtained by the use of soluble EpoR (sEpoR, an extracellular domain capable of binding with the ligand). Infusion of sEpoR into animals given a mild ischemic treatment that did not produce neuronal damage, caused neuronal degeneration and impaired learning ability, whereas infusion of the heat-denatured sEpoR was not detrimental (Fig. 4, IB and IIA-C). Thus Epo prevents the ischemia-induced delayed neuronal death in the hippocampal CA1 field.

EpoR is also expressed in the cerebral cortex. The results of the Morris water maze test indicated that Epo infusion into the cerebroventricles of stroke-prone spontaneously hypertensive rats with permanent occlusion of the left middle cerebral artery alleviated the ischemia-induced place navigation disability. The left (ischemic)-to-right (contralateral non-ischemic) (L/R) ratio of cerebrocortical area in the Epo-infused ischemic group was larger than that in the vehicle-infused ischemic group. The occlusion caused secondary thalamic degeneration but infusion of Epo prevented the decrease in the L/R ratio of thalamic area and supported neuron survival in the ventroposterior thalamic nucleus.

(2) In vitro neurotrophic action of Epo

Glutamate is a principal excitatory amino acid neurotransmitter in the mammalian CNS and also mediates pathological neuronal injury. Normally the increase of glutamate in the extracellular concentration in CNS is limited to a very short period and in the spatially localized region appropriate for synaptic transmission, but a massive, sustained and unlocalized increase has been thought to be mainly responsible for neuronal death associated with reduction in oxygen (hypoxia) or glucose (hypoglycemia), or both (ischemia). At least three types of membrane-spanning ionotropic receptors for glutamate have been found in neurons, each termed according to their agonists: N-methyl-D-aspartate (NMDA), kainate, and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA). A number of pharmacological studies indicate that the NMDA receptor activation by binding with glutamate or agonists is directly involved in most glutamate neurotoxicity. The NMDA receptor activation opens a channel permeable to both Na\(^{+}\) and Ca\(^{2+}\), while activation of two other receptors opens Na\(^{+}\) channels. A lasting and massive increase of intracellular Ca\(^{2+}\) concentration evoked by glutamate-induced NMDA receptor activation plays a critical role in triggering intracellular events that elicit the cell destruction. Dissociated and cultured neurons of the hippocampus and cerebral cortex have been used to investigate glutamate neurotoxicity in vitro, because both areas contain neurons vulnerable to ischemia-induced degeneration.

Hippocampal and cortical neurons were cultured to examine whether Epo protects the cultured neurons from glutamate toxicity. Neurons cultured for 7–10 days were exposed to glutamate for 15 min. After culture for a further 24 h in the absence of
glutamate, the neurons were examined for survival. More than 60% of the neurons treated with glutamate died. MK-801, an antagonist of the NMDA receptor and EGTA completely protected neurons, which indicates that this glutamate-induced neuron death is due to an increase in intracellular Ca\(^{2+}\) concentration mediated through a glutamate-activated NMDA receptor. Significant protection was observed with Epo in a dose-dependent manner from 3 pm (approx. 100 pg/ml) and 30 pm Epo caused complete survival of neurons. The protection was completely reversed by coapplication of sEpoR. For exhibition of the neuroprotective effect, exposure of neurons to Epo approximately 8 h prior to exposure to glutamate was required. Experiments with the inhibitors indicated that RNA and protein syntheses were necessary for the protection. However, exposure to Epo for a short period (5 min or less) was sufficient to elicit the protective effect. The protective effect of Epo was blocked by the simultaneous addition of EGTA, suggesting that a small increase of intracellular Ca\(^{2+}\) concentration is required for Epo-induced neuroprotection. This increase may be related to Epo-induced Ca\(^{2+}\) influx found in the neuronal cell lines.\(^{99,101}\) It is noted that the Epo-induced Ca\(^{2+}\) influx is neuroprotective, while the NMDA receptor-mediated Ca\(^{2+}\) influx is neurotoxic. The NMDA-mediated influx is far greater than the Epo-induced influx.

A glutamate-mediated increase in the intracellular Ca\(^{2+}\) concentration activates neuronal nitric oxide synthase that requires the Ca\(^{2+}\)-calmodulin complex,\(^{178}\) and the increased nitric oxide mediates glutamate neurotoxicity by being rapidly converted to highly toxic compounds such as peroxynitrite.\(^{179}\) Consequently, possible steps on which Epo acts to rescue neurons from NMDA receptor-mediated death would be that (1) Epo may inhibit glutamate-induced activation of NMDA receptor, thereby Ca\(^{2+}\) influx is prevented and (2) Epo may prevent neuronal death by mitigating the toxicity of NO-derived compounds. The presence of Epo in neuron cultures did not repress the NMDA receptor-mediated increase in intracellular Ca\(^{2+}\), excluding the first possibility. Addition of nitroprusside, a nitric oxide generator, to neuron cultures caused neuron death and Epo rescued the neurons from nitroprusside-induced death, indicating that the second possibility is the case.
Taken together Epo appears to exert its neuroprotective effect by reducing the nitric oxide-mediated formation of free radicals or antagonizing their toxicity. Epo supports the survival of erythroid precursor cells by inducing the expression of Bcl-X, a Bcl-2 family member that acts as an anti-apoptotic protein.\textsuperscript{75} Epo-induced expression of Bcl-2 family member in neurons is not known.

(3) Regulation of Epo production in the brain

In addition to hypoxic stimulation of brain Epo production,\textsuperscript{163-165} Epo production by cultured astrocytes is stimulated by insulin and insulin-like growth factors (IGFs) in a dose-dependent manner; IGF-I is the most potent activator.\textsuperscript{180} Half maximal activation was obtained with 3 nm IGF-I, 10 nm IGF-II, and 100 nm insulin. The stimulatory effect of IGFs and insulin was not affected by the oxygen concentration of astrocyte culture. Insulin and IGFs did not increase the total protein synthesis of astrocytes but increased Epo mRNA levels, indicating that Epo production is stimulated at the mRNA level. The growth factor-induced accumulation of Epo mRNA in astrocytes appears to be caused by activation of the tyrosine kinase signal transduction pathway, because tyrosine phosphorylation of receptors for IGF-I and insulin was activated when astrocytes were stimulated by these growth factors. The stimulatory effect of insulin and IGFs on Epo production is specific for the Epo production in the brain, because these substances do not influence Epo production either by Hep3B cells or the uterine tissue cultured as described later. Insulin and IGFs are abundantly expressed in CNS\textsuperscript{181} but the physiological significance of their stimulatory effect on Epo production by astrocytes remains to be studied.

Angiogenic Function of Erythropoietin in the Uterus

Epo has been shown to have \textit{in vitro} angiogenic activity by the use of cultured endothelial cells. EpoR mRNA is expressed in endothelial cells from human umbilical vein, bovine adrenal capillary, and rat brain capillary.\textsuperscript{182,183} Epo stimulates proliferation and migration of human and bovine endothelial cells,\textsuperscript{184} and also \textit{in vivo} angiogenesis of the rat thoracic aorta.\textsuperscript{185} Epo depresses nitric oxide synthase expression by human endothelial cells\textsuperscript{186} and induces a potent angiogenic response in the chick embryo chorioallantoic membrane.\textsuperscript{187} Recent studies using human umbilical vein endothelial cells indicate that Epo signaling in endothelial cells is conducted \textit{via} tyrosine phosphorylation of proteins including phosphorylation of transcription factor Stat5, which is similar to that in erythroid cells.\textsuperscript{188} However, it is unknown whether endothelial EpoR is physiologically functional or is only a vestige reflecting a common developmental lineage between endothelial cells and hematopoietic cells.\textsuperscript{189}

Angiogenesis is the formation of new blood vessels by the extension of preexisting vessels into avascular areas and involves the proteolytic degradation of the vascular basal membrane, proliferation and migration of endothelial cells, and alignment of the migrating cells for tubular formation. Angiogenesis occurs very actively in embryogenesis, but it is repressed in the healthy adult. Active neovascularization in adults takes place in certain pathological conditions such as arthritis, diabetic retinopathy, wound healing, and tumor growth.\textsuperscript{190} An exception in adults is the female reproductive organ, where active angiogenesis is demanded to support the cyclic remodeling of tissues. In every estrus cycle, capillary networks in the ovaries are formed for supporting the development of follicles and corpora lutea. In the uterus, cyclic formation of blood vessels in the functional endometrium occurs to compensate for the lost vessels. Cyclic development of the uterine endometrium is under the control of 17-\(\beta\) estradiol (E2), an ovarian hormone.\textsuperscript{191} This endometrial development can be mimicked by the administration of E2 to the ovariectomized (OVX) immature or adult animals.\textsuperscript{192-194} A number of growth factors including fibroblast growth factor, tumor growth factor, and vascular endothelial growth factor (VEGF) have been implicated in angiogenesis.\textsuperscript{189,195,196} One of the early events caused by the administration of E2 to the OVX rats is the increased vascular permeability in the endometrium.\textsuperscript{197} Based on the temporal pattern of mRNA expression after the E2 administration\textsuperscript{198} and capability of increasing vascular permeability as well as the mitogenic activity for vascular endothelial cells,\textsuperscript{199-203} VEGF has been proposed to be a critical factor in the early phase of E2-induced angiogenesis.\textsuperscript{198,202} The uterus where the active angiogenesis takes place in an E2-dependent manner may be a target pertinent to examine the physiological significance of Epo in angiogenesis.

(1) Estrogen-dependent production of Epo by the uterus and its implication in uterine angiogenesis

Assuming that Epo plays an important role in E2-dependent angiogenesis in the uterine endometrium, two possibilities exist for Epo production. One is that uterine target cells become responsive to Epo by the action of E2 and the serum Epo derived from the kidney acts on the E2-sensitive cells. The other would be that a local site for Epo production exists in the uterine tissues and the production is induced by E2, resulting in an E2-dependent increase of Epo concentration in uterine tissues sufficient for activating angiogenesis. In the \textit{in vitro} cultured uterus from OVX mouse, Epo protein and its mRNA were produced in an E2-dependent manner.\textsuperscript{170} The de novo protein synthesis was not needed for the E2-induced increase of
Epo mRNA. Administration of E2 to the OVX mouse induced a rapid and transient increase in Epo mRNA in the uterus. Injection of Epo into the OVX-mouse uterine cavity promoted blood vessel formation in the endometrium. Furthermore, injection of sEpoR capable of binding with Epo into the uterine cavity of non-OVX mouse in diestrus stage inhibited the endometrial transition to proestrus stage, whereas heat-inactivated sEpoR allowed the transition to occur. These results, combined with the finding that the endothelial cells in the uterine endometrium express EpoR, strongly suggest that Epo is an important factor for the E2-dependent cyclical angiogenesis in the uterus. Interestingly, the uterine Epo mRNA in OVX mice was hypoxia-inducible only in the presence of E2. Furthermore, the hypoxia (7% oxygen)-induced increase of Epo mRNA is far smaller in the uterus (3-fold induction) than in the kidney or brain (30-fold induction) (see below).

(2) Erythropoietin in other reproductive organs

The above findings prompted us to examine other female reproductive organs with respect to Epo mRNA expression and its stimuli (E2 and hypoxia)-induced changes. Epo mRNA expression was detected in the ovary and oviduct of mice but the E2-induced stimulation of Epo mRNA was found only in the oviduct. Epo mRNA expression in the oviduct was hypoxia-inducible both in the presence and absence of E2. E2-dependent production of Epo and its mRNA expression were also found by the use of cultured oviducts. The oviduct provides the appropriate environment for fertilization of the ovum released from the ovary and embryonic development, and transports the embryo to the uterus. The oviduct can be divided into three segments, infundibulum, ampulla and isthmus. The infundibulum secretes oocytes extruded from the ovary. The ampulla is the site of fertilization. Ciliated cells in the isthmus propel embryos towards the uterus. The isthmus is located near the uterus and is thought to be an important region for the capacitation of spermatozoa. It is speculated that secretory cells play a role in the capacitation. The isthmus appears to be a major site of the E2-dependent production of Epo in the oviduct. Further studies are clearly needed to elucidate the physiological function of Epo in the oviduct.

Epo may play a physiological role in the reproductive organ not only in the female but also in the male. Rat Leydig cells express EpoR and the binding of Epo stimulates testosterone production. Epo stimulates epididymal sperm maturation and sperm fertilizing activity in rats with chronic renal failure. Injection of human recombinant Epo to men stimulates testosterone production. Epo mRNA was detected in the rat testis under normoxia and interestingly its level was increased 3-fold when the animal was exposed to hypoxia. Hypoxic inducibility of the Epo production appears to be much lower in the reproductive organs in both sexes than in the kidney (~30-fold).

### Tissue (kidney, brain, uterus and oviduct)-specific regulation of Epo production

Manifold physiological functions of Epo, multiple production sites and the presence of two potent stimuli (hypoxia and E2) suggest that Epo production is regulated in a tissue-specific manner. To characterize the tissue-specific regulation of Epo gene expression, OVX mice were given E2 and/or exposed to hypoxia (7% oxygen), and the temporal patterns of stimuli-induced changes in Epo mRNA level in kidney, brain (cerebrum and cerebellum), uterus, and oviduct were examined. The results are summarized in Table 1. The Epo mRNA level in the kidney and brain markedly (30-fold) elevated at 2-4 hr after exposure to hypoxia. While the renal mRNA level was rapidly (at 8 hr) lowered before an increase of red blood cells despite continuous hypoxia, the high Epo mRNA level was sustained in the brain (at least for 24 hr), indicating that a negative-feedback loop operates in the kidney but not in the brain. Operation of such a negative-feedback loop in the hypoxic induction of renal Epo mRNA and the serum Epo has been well documented. Although the molecular mechanism of this down-regulation is not known, the striking difference in temporal patterns of hypoxia-induced Epo mRNA level in the kidney and brain provide physiologically important implications. In the brain, Epo supports neuronal survival under hypoxia and therefore a high Epo expression needs to be sustained as far as hypoxia continues. In the erythropoietic system, the continuous activation of Epo gene expression overproduces erythrocytes, causing a variety of trouble.

E2 did not show any significant effect on EPO
mRNA level in the kidney and brain under either normoxia or hypoxia. Administration of E2 induced a 20-fold increase in the uterine mRNA level under normoxia. The E2-induced increase was rapidly down-regulated due to desensitization of the uterine Epo-producing cells to E2. This short-lived stimulatory effect of E2 may be critical for ensuring the function of uterine Epo without significant perturbation of erythropoiesis. The uterine Epo production was hypoxia-inducible only in the presence of E2. Expression of the reporter gene flanked by a 5' flanking region of Epo gene was activated by E2 and this activation required estrogen receptor-α.

The physiological functions of Epo in the cerebellum and oviduct are not known. The stimuli-induced temporal pattern of Epo mRNA in the cerebellum was almost identical to that in the cerebrum. The temporal pattern in the oviduct was similar to that in the uterus except that Epo mRNA in the oviduct is hypoxia-inducible even without E2 but E2 is required for hypoxic induction of uterine Epo mRNA.

Acknowledgments

This work was supported by Grants-in-aid from the Ministry of Education, Science and Culture of Japan, and from the “Research for the Future” program in The Japan Society for the Promotion of Science. We also thank supports from Snow Brand Milk Products Co., Ltd.

References


76) Silva, M., Benito, A., Sanz, C., Prosper, F.,
Erythropoietin


100) Koshimura, K., Murakami, Y., Sohmiya, M.,


