Oxygen-dependent and tissue-specific regulation of erythropoietin gene expression

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Fandrey, Joachim. Oxygen-dependent and tissue-specific regulation of erythropoietin gene expression. Am J Physiol Regul Integr Comp Physiol 286: R977–R988, 2004; 10.1152/ajpregu.00577.2003.—Hypoxia-inducible expression of the gene encoding for the glycoprotein hormone erythropoietin (EPO) is the paradigm of oxygen-regulated gene expression. EPO is the main regulator of red blood cell production and more than 100 years of research on the regulation of EPO production have led to the identification of a widespread cellular oxygen sensing mechanism. Central to this signaling cascade is the transcription factor complex hypoxia-inducible factor-1 (HIF-1). Meanwhile, it is known that HIF-1 controls more than 50 oxygen-dependent genes and is now recognized as the main regulator of oxygen homeostasis in the body. In addition to hypoxic induction, expression of the EPO gene is tightly regulated in a tissue-specific manner. During ontogeny, production of EPO required for erythropoiesis is switched from the fetal liver to the kidneys. Here EPO is mainly synthesized in adulthood. Production of EPO has also been found in organs where it has nonerythropoietic functions: EPO is important for development of the brain and is neuroprotective, whereas it stimulates angiogenesis in the reproductive tract and possibly in other organs. Understanding oxygen and tissue-specific regulation of EPO production is of high relevance for physiology. Moreover, this knowledge might be useful for new therapies to treat human diseases.

hypoxia-inducible factor-1; hypoxia; oxygen sensing; prolyl hydroxylases

THE HORMONE ERYTHROPOIETIN (EPO) is the principle regulator of red blood cell production (68). Attempts to understand the increased synthesis of EPO by hypoxia have enabled a deeper insight into the mechanisms of oxygen sensing in general, which is the prerequisite for oxygen-regulated gene expression. Whereas the electrophysiology of oxygen sensing in the carotid body had already been investigated for many years (1), the studies on the regulation of EPO as the paradigm of a hypoxia-inducible gene brought molecular biology into this field. Unraveling why EPO expression is increased under hypoxia when general protein synthesis may be reduced by two-thirds to save energy has paved the way to recognize altered gene expression as a cellular response to hypoxia. The identification of the transcription factor complex hypoxia-inducible factor-1 (HIF-1) has enabled a deeper understanding not only of the physiology but also of the pathophysiology and tumor biology of O2 deficiency (132–134). It is just fitting that this better knowledge of basic physiology is about to enter clinical medicine and once more exemplifies an extremely rapid transfer of knowledge from bench to bedside. Still, nothing is lost from the fascination of the question that drove Claude Bernard more than 150 years ago, namely, how changes in oxygen pressure are sensed and transduced into altered organ and cell function.

HISTORY OF EPO

One of Claude Bernard’s pupils, Paul Bert, known as the pioneer of aviation medicine, was the first to recognize an increase in the number of red blood cells at reduced barometric pressure and thus decreased PO2 (8). He had studied animals living at high altitude and suspected a genetic reason for their elevated O2 capacity of the blood. Viault (149) recognized that acute exposure to high altitude was stimulus enough to increase the number of erythrocytes during a 3-wk expedition to the Peruvian mountains. Almost 100 years ago, it was Paul Carnot and his research assistant Mlle. C. Deflandre (15) who first proposed humoral regulation of erythropoiesis by a “hemopoietin.” Using serum from anemic rabbits, they reported an increase of red blood cell counts on injection into nonanemic recipient animals (15). However, due to the experimental conditions—their donor animals were only moderately challenged by mild anemia—their results were difficult to reproduce; details on that controversy in the beginning of erythropoietin research were very recently highlighted (39). In 1948, Bonsdorff and Jalavisto (11) coined the name “erythropoietin” for the humoral mediator of which the existence was proven by the classical parabiotic animal experiments performed by Rhenstroth-Bauer (124) and Reissmann (121). In a landmark publication 50 years ago, Alan Erslev (31) unambiguously elicited reticulocytosis on injection of serum from anemic donor animals to prove the existence of EPO. In his publication, Erslev already envisioned the potential therapeutic value of EPO to treat anemia. Although it took another few decades
to prove the successful treatment of an anemia caused by EPO deficiency with recombinant EPO (32, 154), many of the effects observed by Erslev foretold the later story of the successful use of EPO as a therapeutic agent. To obtain recombinant EPO, however, the tedious work of Miyake and colleagues (111) was a prerequisite. They purified human EPO from 2,550 liters of urine from severely anemic patients to allow the identification of initial parts of the amino acid sequence from tryptic fragments (111). From there, DNA probes were generated and the successful cloning of the EPO gene was reported by Lin et al. (93) and Jacobs et al. (66).

**OXYGEN-DEPENDENT PRODUCTION OF EPO**

Although steady-state production of EPO is necessary to maintain the physiological daily renewal of red blood cells, it is the stimulation by hypoxia on exposure to high altitude that promoted research on EPO from very early on (8, 68). It was necessary to understand that it is not the number of red blood cells itself that is sensed, but the blood O2 content that in turn determines the oxygenation of EPO-producing tissues and thus EPO production. In general, plasma EPO protein concentrations show an inverse log/linear relationship with O2 capacity of the blood. Thus acute hemorrhage, hemolysis, or insufficient erythropoiesis due to bone marrow failure all result in anemia and cause EPO levels to rise exponentially up to several hundred-fold of the normal concentration (33, 131). This increase is rapid and depends on de novo synthesis of EPO, which is not stored preformed in EPO-producing cells (68).

Likewise, functional anemia caused by CO intoxication and/or other increases in O2 affinity of hemoglobin (71) as well as reduced arterial PO2 values under normobaric or hypobaric (high altitude) hypoxia strongly stimulate EPO production. Taken together, a reduced O2 content of the blood results in tissue hypoxia, which is the proximate stimulus for expression of the EPO gene primarily in the adult kidneys and the fetal liver. Thus hypoxic activation of DNA sequences flanking the EPO gene control temporal-spatial regulation of EPO expression.

**REGULATORY DNA ELEMENTS IN THE EPO GENE**

To identify these regulatory DNA sequences, Semenza and colleagues (135–137) generated a series of transgenic mice by introducing DNA fragments encompassing the human EPO gene into a mouse germ line by pronuclear microinjection. The shortest DNA fragment they injected was a 4-kb fragment (tgEPO4) that included all five exons and four introns of the human EPO gene as well as 0.4 kb of DNA upstream (5′/H11032 to the transcription initiation site and 0.7 kb of DNA downstream (3′/H11032) to the polyadenylation site (137). The fact that three noncoding regions of the EPO gene are highly conserved between the human and the mouse gene sequence suggested that they might contain regulatory DNA elements: the promoter, the first intron and a 120-bp region 3′ to the polyadenylation site (43). Those sequences were included in the tgEPO4 construct and, indeed, expression in the liver of the transgenic mice was induced by anemia (137). Therefore, it was concluded that the transgene...
contained a liver inducible element (LIE) in Fig. 1. However, these mice failed to show induction of EPO expression in the kidneys and had promiscuous expression of the transgene in organs where the mouse gene was not expressed physiologically. This indicated that the construct lacked some elements that restrict expression to the physiologically appropriate sites. Moreover, the majority of the mice were moderately polycythemic due to elevated serum EPO levels when compared with control nontransgenic animals (137). When the DNA constructs for injection were extended to 10 kb of human DNA by including additional 6 kb of 5' DNA (tgEPO10), liver inducibility was retained, but promiscuous expression was suppressed by a negative regulatory element (NRE in Fig. 1) (135). Within this 6-kb region, which shows a high degree of conservation compared with the mouse sequence, lie a considerable number of binding sites for transcription factors that may be involved in negative regulation of EPO expression (92). Extending the DNA fragment by another 8 kb to an 18-kb construct (tgEPO18) resulted in transgenic mice in which hypoxia and anemia induced the transgene in liver and kidney (136). Expression of the transgenes in the kidneys was localized to the correct physiological site, the interstitial peritubular cell population (136).

TgEPO18 mice, however, were even more polycythemic than tgEPO4 animals, reaching hematocrit values of up to 87%. Particularly, expression of the transgene in the liver was much higher than that of the endogenous gene, contrasting with the normal ratio between kidney to liver expression in the adult of 9:1 (136). This was not different in lines carrying 16.5 kb 5' and 2.2 kb 3' of the EPO gene (tgEPO22), but a remarkably different expression pattern was observed when Madan et al. (94) extended the constructs to 7 kb of sequence 3' to the gene (EPO22 and EPO33). These transgenic mice were no longer polycythemic and showed a markedly reduced EPO mRNA expression in the liver, resulting in normal kidney-to-liver expression ratios. Because hypoxic inducibility was preserved, additional transcription factors binding to this region between 2.2 and 7 kb 3' to the gene repress steady-state hepatic expression in the adult but do not interfere with oxygen-dependent expression (94). Thus a negative regulatory liver element (NRLE in Fig. 1) lies within this fragment. This finding also indicates that the adult liver maintains the potential for high-level expression of the EPO gene but production at this site is physiologically repressed.

In addition, the conclusion may be drawn that kidney and liver hypoxia-responsive elements (HRE) of the EPO gene are different. For the kidney, the HRE is most likely located between 9.5 and 14 kb 5' of the gene (KIE). Description of a DNase I hypersensitivity site between 14 and 9.5 kb 5' to the gene suggested a potential transcriptional factor binding site for renal expression (82). Although DNase I hypersensitivity was increased by anemia this was not specific to nuclei from renal cells but also found in DNA from liver nuclei (82). Interestingly, part of the sequence hypersensitive to DNase I resembled that of the consensus-binding site for HIF-1 similar to that lying in the 3' enhancer identified from liver cells (see below). Possibly, other tissue-specific factors bind to this DNA region and are required for hypoxia inducibility in the kidney.

Collectively, transgenic animal studies revealed the following. 1) Hypoxia inducibility elements for the liver (LIE, within 0.7 kb 3' to the gene) and the kidney (KIE, between 9.5 and 14 kb 5' to the gene) are located in flanking sequences at opposite ends of the gene. 2) Generally active repressive elements lie within 6 kb 5' to the gene (NRE). 3) A specific negative regulatory liver element (NRLE) is located between 2.2 and 7 kb 3' to the gene (Fig. 1).

IN VITRO DISSECTION OF CIS-ACTING ELEMENTS OF THE EPO GENE

Further analyses of the molecular mechanisms of hypoxia-induced EPO expression were hampered by the lack of an appropriate renal cell culture system resembling the regulation of EPO in vitro. However, in 1987 Nielsen et al. (115) and Goldberg et al. (47) identified two human liver tumor cell lines, HepG2 and Hep3B, that showed regulated expression of the EPO gene that was dependent of the pericellular P0 (33, 155). Even today, no renal cell lines with O2-regulated EPO expression are available. Recently, two neuroblastoma cell lines, SH-SY5Y and Kelly, have been found to serve equally as cellular models for studying the molecular mechanisms of hypoxia-induced EPO expression in nonhepatic cells (143).

With the help of the hepatoma lines, putative hypoxia-activated regulatory DNA sequences of the EPO gene were tested in vitro. Nuclei isolated from tgEPO10 mice that had high hepatic expression under hypoxia revealed a DNase I hypersensitivity site 3' of the EPO gene only in extracts from the liver but not from brain, kidney, or spleen (136). A 256-bp fragment spanning the DNase I hypersensitivity site controlling a reporter gene was transfected into Hep3B cells. Detailed analyses of this fragment have led to the identification of a hypoxically inducible enhancer lying 120 bp 3' to the EPO polyadenylation site in a region highly conserved between human and mouse sequence (5, 120, 136). This cis-acting DNA element showed all typical features of a eukaryotic enhancer and was responsive to hypoxia, cobalt chloride, and iron chelation like the EPO gene in vivo but not to cyanide or 2-deoxyglucose (120).

Within this EPO enhancer three defined sites are important for the response to hypoxia (Fig. 1). At the 5'-end, the sequence CACGTGCT was the first to be identified as an HIF-1 binding site (HBS). Double-stranded oligonucleotides were used to demonstrate proteins from nuclear extracts from Hep3B cells that specifically bound only when the cells were challenged with hypoxia. Two protein complexes were identified, one bound constitutively and the other (HIF-1) was present only in hypoxic cells (138). Components of the constitutive complex are ATF-1 and CREB-1, but the role for hypoxia-regulated EPO expression has not yet been settled for these factors (87). In contrast, an intact HBS and HIF-1 binding are indispensable for hypoxia-induced EPO expression.

Seven base pairs further 3' of the HBS, a CACA repeat is found in the human EPO gene. Mutation of this site abrogates hypoxia-inducible activity of the enhancer, but so far no protein bound to this repeat has been identified. Interestingly, however, close to the HBS of the lactate dehydrogenase A gene, a similar site has been found in close proximity to the HIF-1 site (38).

The third site in the EPO enhancer is a direct repeat of two steroid receptor half sites separated by two base pairs, termed a DR-2 site (10). This DR-2 site is absolutely required for hypoxic regulation of the EPO gene and thus deserved detailed
analysis. Direct repeats of steroid receptor half sites are known to bind nuclear receptors for hormones, but none of the known ligands significantly affected EPO expression through the DR-2 site (10). Screening for orphan receptors, however, revealed specific binding of hepatocyte nuclear factor-4α (HNF4α). The functional importance of HNF4 binding was demonstrated by use of a dominant negative mutant of HNF4 that abolished EPO expression in Hep3B cells (44). It was hypothesized that HNF4 may play an important role for tissue-specific expression of EPO. During mouse development the retinoic acid receptor RXRα, another member of the nuclear receptor family, binds to the DR-2 site, whereas fetal erythropoiesis is located in the liver (embryonic day E9.5-E11.5). From E11.5, HNF4-activity becomes dominant, competes out binding of RXRα, and may initiate the switch from retinoic acid control to hypoxic control of EPO expression (97). In P1 embryonic carcinoma cells that produce EPO but not in an O2-regulated manner, retinoic acid has also been found to bind to the DR-2 site and to increase expression of a reporter gene driven by the EPO enhancer as well as the endogenous EPO gene (78). More recently, oxygen-regulated expression of EPO was found in neuroblastoma cell lines that lack HNF4α or other HNF4 isoforms (143). Which factors substitute for HNF4 in these cells is still under active investigation. Because neuroblastoma cells may serve as a model for EPO expression in neuronal cells, the role for tissue-specific factors binding to the DR-2 site deserves further study.

Although the EPO promoter by itself is a weak promoter, it shows hypoxic inducibility and, moreover, acts synergistically with the enhancer to achieve a 50- to 100-fold stimulation in vitro and several hundred to even 1,000-fold stimulation in vivo (10, 68). The minimal EPO promoter encompasses 117 bp 5′ to the transcription start site and has no consensus HBS. Proteins interacting with a 17 bp (nt −61 to −45) sequence designated EP17 synergize with transcription factors binding to the 3′ enhancer of the EPO gene. Recently, this protein termed hypoxia-associated factor (HAF) was cloned and partially characterized (52). HAF is the murine homologue of Sart-1, a 125-kDa human protein recognized by cytotoxic T lymphocytes. HAF antisense treatment of Hep3B cells reduced hypoxia-induced EPO expression but did not abrogate basal expression, suggesting that HAF acts in conjunction with other transcription factors to modulate O2-dependent EPO expression (52).

There appear to be some negative cis-acting elements in the promoter that repress EPO expression as well (Fig. 1). Whereas a ribonucleoprotein negatively regulating EPO expression has already been described several years ago but not further characterized (9), two GATA-sites have been extensively studied with respect to oxygen sensitivity, tissue specificity, and also their potential role during ontogeny (25, 62). Increased expression and binding of GATA-2 were found at high PO2 in Hep3B cells, and overexpression of GATA-2 and -3 suppressed EPO expression (62). Hydrogen peroxide (H2O2), a potent inhibitor of EPO expression (34), was found to increase GATA-2 binding to the EPO promoter and may thus convert redox sensitivity to the EPO gene (Ref. 144 and see below). In contrast, it has just been reported that GATA-4 also binds to the promoter but increased EPO expression and may be required for hepatic expression during fetal development (26).

One further regulating mechanism of EPO expression that has not been fully elucidated in vivo is the role of methylation of CpG sites in the EPO promoter (152, 161). Methylation of CpGs in the promoter represses EPO expression by recruiting methyl CpG-binding proteins that can interfere with other transcription factors or recruit histone deacetylases and corepressors (161). In EPO-producing cells, these CpGs are less methylated that in non-EPO-producing cells (152, 161) and, at least for a CpG near the transcription start site, the methylation status appears to be partly influenced by hypoxia (161). Thus methylation of CpGs may effect tissue-specific EPO expression, but factors controlling the methylation state of the CpGs in the EPO promoter still need to be defined.

**EPO mRNA STABILITY**

About 50% of the hypoxic increase of EPO mRNA can probably be attributed to increased transcription, leaving a significant contribution of mRNA stabilization to the total amount of cellular EPO mRNA. Studies on EPO mRNA half-life were always hampered by the fact that inhibitors of transcription markedly prolong the half-life of the EPO mRNA (57). By use of a marked EPO gene, however, a sevenfold increase of the half-life of EPO mRNA on hypoxia was found that depended on the lack of binding of destabilizing EPO mRNA binding proteins (ERBP) under normoxia to a pyrimidine-rich region in the 3′-untranslated region (UTR) of the EPO mRNA (57). Activity of the sequence appears to depend on p38α MAP kinase activity (145). The importance of this stress kinase as well as the stabilization of EPO mRNA for hypoxia-induced EPO synthesis is underlined by the finding that p38α−/− mice showed a phenotype that was comparable to EPO−/− embryos (158). In fact, p38α−/− embryos die in midgestation due to anemia (145).

**HIF-1**

Thorough studies on the 3′ enhancer of the EPO gene have probably led to the most interesting and important finding that originates from basic research on understanding the regulation of EPO production: the evidence that hypoxia-induced EPO gene expression is under control of a widespread oxygen-sensing mechanism (107). Transfection of the EPO enhancer into non-EPO-producing cells clearly showed that the enhancer could fully operate and respond to hypoxia irrespective of whether the cells produced EPO (107). Moreover, mimics of hypoxia, cobalt chloride and iron chelators, were fully active but not chemical hypoxia caused by agents such as cyanide.

Analysis of the proteins bound to the 3′ EPO enhancer revealed a protein complex termed HIF-1 of which the binding was essential for hypoxic induction of the EPO gene (138). Affinity purification using DNA binding of this complex and subsequent cloning resulted in the identification of a protein heterodimer consisting of the two basic-helix-loop-helix PAS proteins HIF-1α and HIF-1β (150). Although the 120-kDa α-subunit was entirely new in sequence, the 91- to 94-kDa β-subunit turned out to be identical with aryl hydrocarbon receptor nuclear translocator (ARNT), the dimerization partner of dioxin receptor (AHR, aryl hydrocarbon receptor) (150). Both the α- and β-subunit are members of the PAS protein family where the acronym PAS stands for the first three
members of this family of transcription factors Per/ARNT/Sim (for a review on PAS proteins, see Ref. 50).

Steady-state levels of HIF-1α and HIF-1β mRNA are not significantly affected by hypoxia compared with the great increase in cellular HIF-1α protein content under hypoxia (156). In fact, hypoxia inducibility of the HIF-1 complex is solely achieved by hypoxic accumulation of the α-protein subunit, whereas the β-subunit is constitutively expressed. In normoxic cells, HIF-1α is barely detectable due to rapid degradation via the ubiquitin proteasome pathway (60, 127). HIF-1α is continuously synthesized irrespective of the oxygenation status of the cells. This provides a very rapid response of HIF-1α accumulation when hypoxia occurs, simply by blocking the degradation of the protein, and allows an almost instantaneous response to hypoxia by accumulation of HIF-1 without activating the transcriptional/translational machinery (72). Therefore, the search for the oxygen-sensing mechanism was focused on the rapid degradation of HIF-1α under normoxia. Maxwell and colleagues (108) first described the critical role of the tumor suppressor protein von Hippel-Lindau (VHL) for this degradation process. VHL protein was found to bind to the two oxygen-dependent degradation domains (ODD) of HIF-1α that had previously been described to confer oxygen-dependent instability on the HIF-1α protein and also on other proteins fused with the ODD (60, 73, 118). In addition, it turned out that HIF-1α protein abundance—regulated via the ODD and normoxic degradation—as well as its trans-activity via an NH2-terminal and a COOH-terminal transactivating domain (N-TAD and C-TAD, respectively) was independently regulated by oxygen (28, 116). Whereas this already indicated regulation of HIF-1α at multiple molecular levels, cellular localization of HIF-1α also appeared to be O2 dependent, because translocation from the cytosol to the nucleus was increased under hypoxia (77).

OXYGEN SENSING THROUGH POSTTRANSLATIONAL MODIFICATION OF HIF-α

Study of the role of VHL for HIF-1α degradation provided the new focus for analysis of oxygen-sensitive pathways. Cells deficient in functional VHL accumulate large amounts of HIF-1α irrespective of their oxygenation status (108). Each of the two ODDs contains the amino acid proline embedded into the common LxxLxP motif for hydroxylation (101). Different peptides comprising HIF-1α sequences were used to study the interaction with VHL and revealed that enzymatic hydroxylation of HIF-1α at proline residues 564 and 402 (in human HIF-1α) was necessary for recognition by VHL (63, 65, 101). VHL bound to HIF-1α then recruits other components of the E3 multicomponent ubiquitin ligase that tags HIF-1α for subsequent proteasomal degradation (142). On the basis of Cae.

norhabditis elegans genetics, Epstein et al. (30) succeeded in the cloning of the mammalian HIF prolyl hydroxylases that were designated “prolyl hydroxylase domain” containing (PHD) 1, 2, and 3 (30). Independently, Bruick and McKnight (13) identified three HIF-prolyl hydroxylases (termed HPH3, 2, and 1, respectively) based on their close relationship to collagen-modifying prolyl hydroxylases.

The activity of PHDs depends on the availability of oxygen and thus qualifies these enzymes as O2 sensors. Recently, the O2 affinity has been determined and suggests appropriate sensitivity in the physiological relevant PO2 range (56). In addition, these dioxygenases require 2-oxoglutarate as co-substrate and vitamin C to keep their central non-heme iron in the ferrous state. O2 binds to this central moiety and is split, with one oxygen atom being incorporated into the proline residue of HIF-1α and the second reacting with 2-oxoglutarate to yield succinate and CO2. Under hypoxia, activity of the enzymes is greatly reduced, the specific proline residues in HIF-α are no longer hydroxylated, and HIF-α subunits can accumulate to activate HIF-1-dependent genes like EPO. Hypoxia-mimicking induction of EPO by cobalt chloride or iron chelators may also be explained by effects on the PHDs, because these chemicals may either substitute for (cobalt chloride) or chelate the central iron and inactivate the prolyl hydroxylases like hypoxia (151).

Interestingly, the C-TAD is also modulated by an O2-sensitive hydroxylation step, this time, however, on the asparagine residue 803 [in human HIF-1α (90)]. Here, hydroxylation of asparagine under high oxygen pressure inhibits recruitment of the transcriptional coactivator CBP/p300 (3, 128) necessary for hypoxic HIF-1 activity. This asparagine hydroxylation had been identified earlier as factor inhibiting HIF-1 (FIH-1), because its interaction, hydroxylation of asparagine N803, inhibited the trans-activity of HIF-1 (96). FIH-1-like PHDs turned out to be a 2-oxoglutarate-dioxygenase requiring O2 and iron for its function (55, 89). The recent localization of PHDs (59) and FIH-1 (109) in different cellular compartments and the inducibility of the enzymes themselves by hypoxia (30, 109) as well as the differences in O2 affinities between PHDs and FIH-1 (56, 83) are currently promoting studies on the regulation of the activity of these potential cellular O2 sensors. In addition to the O2-dependent hydroxylation, phosphorylation by p42/p44 (Erk2/Erk1) mitogen-activated protein kinases (MAPKs) of HIF-1α (and HIF-2α; see below) is required for transcriptional activity (20, 122). Although phosphorylation does not appear to be O2 regulated and the exact phosphorylation sites have not yet been defined, this posttranslational modification may allow to modulate HIF-1 activity by other cellular signaling pathways (151). Likewise, receptor tyrosine kinase-PI3-kinase-PTEN-Akt kinase signaling is critically involved in HIF-1 regulation but rather by increasing HIF1α translation than protein stability (see Refs. 12, 102, 151 for further references).

Taken together, O2-dependent regulation of HIF-α (these mechanisms also regulate the other two known members of the HIF family, HIF-2α and HIF-3α; see below) resembles many if not all the features characteristic for physiological regulation of EPO by hypoxia. A defect in the degradation of HIF-α caused by a mutation in VHL leads to polycythemia due to increased expression of the HIF-1 target EPO. Hypomorphic VHL alleles have recently been detected in congenital Chuvash polycythemia, underlining the importance of proper HIFα regulation for the control of EPO production (2).

Today, more than two dozen oxygen-regulated HIF-1 target genes with critical importance for physiology and pathophysiology of ischemic disease and tumor biology are known. Thus HIF-1 has been recognized as the master regulator of O2 homeostasis (132). For further details on HIF, the reader is referred to some recent reviews (12, 119, 139, 151).
WHICH HIF-α IS REGULATING EXPRESSION OF THE EPO GENE?

HIF-1α is ubiquitously expressed but is not the only member of this subfamily of PAS proteins. HIF-2α (29, 41, 58, 147) and HIF-3α (51) partly overlap with HIF-1α in their expression pattern, but assignment of specific functions to the different HIF-α's has not yet been achieved. Knockout mice for HIF-1α and HIF-2α dramatically differ in their phenotype. Whereas HIF-1α−/− mice die around midgestation due to severe cardiovascular and central nervous system (CNS) malformations (64, 125), three different phenotypes for HIF-2α−/− mice (perhaps depending on the genetic background) were observed (18, 117, 148). Although one HIF-2α−/− mouse evidently died of bradycardia because the organ of Zuckerkandl responsible for catecholamine synthesis during development was not productive, one other HIF-2α−/− mouse showed gross deficits in remodeling of the primary vascular network (117, 148). A third phenotype was observed where the mice died after birth due to a respiratory distress syndrome caused by insufficient surfactant production from type II alveolar cells (18). From all those animals no clear conclusions with respect to defects in EPO expression could be drawn. In contrast, mice containing only one mutant HIF-1α allele (HIF-1α−/+− mice) developed normally but, among other impaired physiological responses to hypoxia, these mice had delayed erythrocytosis on exposure to hypoxia (163). Although HIF-2α and HIF-3α may interact with the HIF-1-binding site and could also compete for dimerization partners, the data from Yu et al. (163) suggest a dominant role for HIF-1α for EPO regulation.

Recently Rosenberger et al. (123) studied the expression pattern of HIF-1α and HIF-2α in hypoxic rat kidneys. Because only HIF-2α was localized in the peritubular fibroblasts that produce EPO, the authors proposed a role for HIF-2α in hypoxic rat kidneys in the regulation of the EPO gene (123). This hypothesis is supported by a study on the retinopathy of prematurity to which expression of EPO in the retina significantly contributes (112). In this study, HIF-2α knockdown, i.e., reduction of HIF-2α expression to about one-fifth of the normal level, lowered EPO expression induced by relative hypoxia that was not compensated by unchanged HIF-1α accumulation (112). In contrast, light-induced retinal degeneration was reported to be prevented by hypoxia-induced EPO expression in the retina that coincided with increased HIF-1α levels (49).

Thus in vivo studies do not provide firm evidence which HIF-α controls EPO mRNA expression. Although HIF-1α was cloned from binding to the HBS of the EPO gene in human hepatoma cells, these cells also express HIF-2α. Therefore, there may be cell- and organ-specific differences and/or different affinities of HIF-1α and -2α for the HBS in the EPO gene that depend on other transcription factors that help to form the HIF-1 complex (see above). At least in vitro, overexpression and activation of HIF-1α and -2α can activate the EPO enhancer by forming an active HIF-1 complex (28). A definite answer as to which HIF-α controls EPO expression still awaits experimental evidence.

TISSUE-SPECIFIC EPO EXPRESSION

Two further questions with respect to tissue-specific expression of EPO await answers: the developmental switch from expression of EPO in the fetal liver to renal expression in the adult and the expression in organs where synthesized EPO does not play a role as an erythropoietic hormone but more that of a paracrine protective factor.

Adult expression of EPO in mammals is primarily localized in the kidney as first demonstrated by the classical organ ablation studies by Jacobson et al. (67). Before birth, however, the fetal liver is the main production site (165). Although species differences in the timing of the switch and the degree of contribution of the liver to total body EPO mRNA content exist (for review, see Ref. 113), the switch is undoubtedly found in humans as well (23). The mechanism and the main factors determining the switch still remain to be identified, but recent data indicate that GATA-4 may be one of the factors that specifically promotes EPO expression in the liver (26). Fetal expression of EPO is present in all lobes of the human liver (23) and EPO-expressing cells in the liver—hepatocytes and Ito cells (86, 105)—display an expression pattern that follows the PO2 distribution (74): high expression around central veins at lower PO2 values and expression extending to the perportal field on anemia or hypoxia (86). Because the hepatic expression appears so strictly regulated by the PO2 gradient, a major role for changes of this gradient around birth has been proposed to control the switch (for review, see Ref. 113). However, as pointed out above, specific sequences in the regulatory DNA elements that are different for the kidney and the liver regulate expression presumably through the binding of tissue and developmental specific transcription factors (44, 97).

Moreover, although changes in the oxygenation around birth are similar in different species, the switches differ significantly with respect to birth date (113). Finally, surgical procedures aimed at reducing hepatic or renal oxygenation do not significantly affect the switch (40). It is, however, of interest that liver cells retain their capacity for high EPO expression in an adult environment as documented by the transgenic mice studies performed by Semenza et al. (137). Thus it is very likely that tissue factors in the adult kidney may well arise around birth that are indispensable for EPO expression and repressive factors are expressed in the liver of the adult.

This is underlined by the fact that, so far, the isolation of renal EPO-producing cells and the setup of renal cell lines for studies of the regulated expression of EPO have not been successful. EPO-expressing cells in the kidney have been identified as fibroblast-like type 1 interstitial cells filling the peritubular space in the cortex and outer medulla (4, 106). There has been some debate about the identity of the cell expressing EPO in the kidney that has recently been highlighted extensively (39). Particularly interesting is a major difference between liver and kidney after challenge with hypoxia. Whereas the amount of EPO mRNA per hepatocyte appears to rise on hypoxia, in the kidney an increasing number of cells with fixed EPO mRNA content is recruited (85, 86). Whether this is caused by tissue-specific factors or potentially different HIF-α isoforms (see above) or oxygen gradients needs to be determined. Although this hepatocyte characteristic response is reflected by the hepatoma cells available for in vitro studies (33), the isolation of renal EPO-producing cells remains an important aim.

Much recent attention has been paid to EPO expression in other organs, particularly in the brain (22, 33, 98, 146) and the reproductive tract (81, 104, 146, 160).
EPO expression in the CNS has recently been proposed to be central for normal brain development (164), although in an earlier study mice with an EPO gene knockdown were neurologically normal (105). EPO has been found to be neuroprotective (7, 103, 126, 140) and has opened a rapidly developing field that deserves much more attention than can be paid here due to space limitations. The reader is referred to some very recent reviews (24, 99 and references therein). EPO expression in the CNS is also found in the human fetus early in gestation (22, 75). Interestingly, although the expression is stimulated by hypoxia, such as in the kidneys or the liver, the temporal pattern is significantly different (17). Whereas EPO expression in kidneys and liver on hypoxia is transient, peaking \( \sim 6-24 \) h in vivo (depending on the severity of hypoxia; see Ref. 68 for references) and at 6 h in vitro (33), EPO mRNA levels remain elevated in the brain for the duration of the hypoxic stimulus (17). Even relative hypoxia keeps EPO mRNA levels up at least for 12 h (112), and it has been argued that the paracrine neuroprotective effect of EPO should require expression for the duration of the hypoxic stress to be effective (129). In vitro studies revealed that expression in hepatic and neuronal cells similarly shows a high degree of redox sensitivity of the EPO gene (6, 34). The recent availability of neuronal-like cell lines with regulated expression of EPO suggests significant differences (143). Whereas HNF-4 was indispensable for hepatic expression (Ref. 44 and see above), neuroblastoma cells express the EPO gene in an oxygen-dependent manner in the absence of HNF-4 (143). EPO expression is under control of the HIF-1 complex, but ARNT-2, an isoform of HIF-1\( \beta \) (= ARNT-1) that is specifically expressed in the kidney and brain (27) contributes to the HIF-1 complex in these neuroblastoma cells (143). Moreover, preliminary data from experiments with these cells suggest a role for HIF-1\( \alpha \) but not HIF-2\( \alpha \) in hypoxic regulation (U. Berchner-Pfannschmidt and J. Fandrey, unpublished). That would indicate important differences compared with kidney EPO expression (123).

An interesting feature that is paralleled by EPO expression in the brain and the testis (81, 95, 146) is the fact that EPO production at these sites may be separated from the systemic circulation by the blood-brain or blood-testis barrier, respectively. This fact would suggest a more paracrine than endocrine mode of action of EPO produced at these locations (21). However, EPO is also supposed to cross the blood-brain barrier mode of action of EPO produced at these locations (21). The importance of paracrine EPO is certainly significantly lower than that of systemic EPO levels for erythropoiesis as evident from the renal anemia in terminal kidney disease. For the heart, EPO appears to be necessary for proper cardiac morphogenesis (157), although expression of EPO in the heart appears to be very low. It is still questioned whether EPO from the heart itself can protect the myocardium from ischemia reperfusion injury (14), which can be achieved by systemic application of EPO (14). Taken together, many questions regarding tissue-specific expression, particularly different isoforms of HIF-1\( \alpha \) or additional transcription factors that modulate hypoxia-induced expression, remain unanswered. Once more, studies beyond its erythropoietic function make EPO an attractive field of clinical research.

**FACTORS NEGATIVELY REGULATING EPO GENE EXPRESSION**

The finding that \( \text{H}_2\text{O}_2 \) inhibits EPO expression has fueled the discussion on oxygen-sensing and \( \text{O}_2 \)-dependent gene expression for some time (34). On the basis of the work of Goldberg and colleagues (46) suggesting that the oxygen sensor might be a heme protein, a role for \( \text{b-type cytochromes} \) in \( \text{O}_2 \)-dependent EPO expression was investigated (35). Subsequent studies revealed a nonmitochondrial heme protein in HepG2 cells of which the spectral properties changed on oxygenation (48). The spectral properties were similar to the \( \text{O}_2 \)-generating NADPH oxidases, and, indeed, an inverse relationship of \( \text{H}_2\text{O}_2 \) (dissimulated from \( \text{O}_2 \)) and EPO expression was found (34). Because exogenous \( \text{H}_2\text{O}_2 \) inhibited EPO expression mimicking high \( \text{PO}_2 \), and scavenging of high \( \text{H}_2\text{O}_2 \) increased EPO expression despite high \( \text{PO}_2 \), a role for \( \text{H}_2\text{O}_2 \) as a signaling molecule in \( \text{O}_2 \) sensing was proposed. In subsequent work by Huang et al. (61), \( \text{H}_2\text{O}_2 \) reduced HIF-1\( \alpha \) and thus reduced the hypoxic induction. Although redox sensitivity of the HIF-1 complex has been confirmed (91) changes in reactive \( \text{O}_2 \) species (ROS) levels in response to changes in \( \text{PO}_2 \) have been controversial (16). In part, conflicting results may be
the result of cell-specific effects or be explained by different culture conditions and detection systems for ROS (79). Rapid progress in the work on prolyl hydroxylase-dependent modification of HIF-α that depends on the P02 instead of ROS (see above) has been made since. Undoubtedly, PHDs appear to sense O2 directly. The inhibition of EPO expression by ROS has been shown to occur through activation of GATA-2, which acts as a repressor of the EPO gene (62). Still redox chemistry may be involved in an O2-sensing process indicated by the fact that the central iron atom of PHDs and FIH-1 undergoes redox cycling and critically needs vitamin C for proper function (56, 102).

Affecting the redox state may indeed play a role in the recent report of direct inhibition of PHDs by NO (110). Interestingly, however, the increase in HIF-1 levels and activity by NO is apparently in conflict with the inhibition of EPO expression and decrease of HIF-1α by NO in some studies (130, 141). Although NO affects ROS levels and EPO expression in HepG2 and Hep3B cells, the exact mechanisms of inhibition of EPO production in the face of an increase of HIF-1 activity have not been resolved (45). Very recent work indicates that inhibition of mitochondrial respiration by NO might cause a redistribution of O2 from the mitochondria to the cytosol and could thus interfere with HIF-1α regulation (53). Likewise, the inhibition of EPO expression by proinflammatory cytokines (36, 70), which contributes to the anemia of chronic inflammatory and malignant disease (69), is still not well understood. Although proinflammatory cytokines potently inhibit EPO expression in vitro and in vivo (42, 70), they induce HIF-1 and some HIF-1-dependent genes (54). Again, other transacting factors such as nuclear factor-κB may be responsible for mediating the repressive effect on the EPO gene that overcomes hypoxia-induced and HIF-dependent induction (37, 88). In general, elucidation of the inhibition of EPO expression was intended to improve the understanding of the founding mechanisms that regulate physiological signaling during hypoxia. Instead, by acting on different positive and negative cis-acting elements, these inhibitory factors add another level of complexity for the understanding of the regulation of EPO gene expression by hypoxia.

CONCLUDING REMARKS

Studying EPO production in the past almost 100 years has enabled physiologists to get an understanding of O2 sensing with respect to oxygen-dependent gene expression. HIF-1 is today acknowledged as the master regulator of oxygen homeostasis (132). HIF-1 was discovered as a trans-acting factor complex bound to cis-acting DNA elements of the EPO gene. Unraveling the activation back from HIF-1 has led to the result of cell-specific effects or be explained by different culture conditions and detection systems for ROS (79). Rapid progress in the work on prolyl hydroxylase-dependent modification of HIF-α that depends on the P02 instead of ROS (see above) has been made since. Undoubtedly, PHDs appear to sense O2 directly. The inhibition of EPO expression by ROS has been shown to occur through activation of GATA-2, which acts as a repressor of the EPO gene (62). Still redox chemistry may be involved in an O2-sensing process indicated by the fact that the central iron atom of PHDs and FIH-1 undergoes redox cycling and critically needs vitamin C for proper function (56, 102).

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