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PHARMACOLOGIC MODULATION OF ERYTHROPOIETIN PRODUCTION

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INTRODUCTION

Erythropoietin (Ep or Epo), a glycoprotein hormone produced by the kidney, regulates red blood cell production. In 1906 Carnot & DeFlandre (1) gave the name "hemopoietine" to a humoral factor that was thought to control red blood cell production. Later, this hormone was more appropriately named "erythropoietin" by Bonsdorff and Jalavisto (2). Interest in erythropoietin was reawakened by the classical work of Reissmann (3), who provided more definitive proof for the humoral control of red cell production; he found that erythropoietic stimulation of the bone marrow was seen in both hypoxic and nonhypoxic partners even though only one of the parabionts was exposed to hypoxia. Further documentation was provided by Erslev (4) when he noted a reticulocytosis in recipient rabbits infused with donor rabbit plasma from bled animals. The site of erythropoietin production was very controversial until 1957, when Jacobson et al (5) demonstrated that bilateral nephrectomy abolished the erythropoietic response of rats to bleeding. In 1961 Kuratowska et al (6) and Fisher & Birdwell (7) demonstrated that erythropoietin could be produced by the isolated perfused kidney. The mechanism of the control of both renal and extrarenal erythropoietin production is thought to be associated with a renal oxygen sensor mechanism. When the level of oxygen in this sensor cell is reduced below physiologic levels, increased renal biosynthesis of erythropoietin occurs. In this review I discuss the physiologic and pharmacologic mechanisms that regulate renal and extrarenal control of erythropoietin secretion and/or production.

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Physicochemical Characterization of Erythropoietin

Erythropoietin was purified to apparent homogeneity by Miyake et al (8), and its molecular weight is estimated to be 36,000. Based on studies of human erythropoietin using hydroxyl appatite chromatography, Ep was postulated to exist as an alpha form (31% carbohydrate) and a beta form (24% carbohydrate) (9). The gene for erythropoietin has recently been cloned (10–12), and specific activities ranging from 70,000 units per milligram up to 160,000 units per milligram have been reported. The amino acid sequence of mouse, human, and monkey erythropoietin have been determined, and Ep from each of these species contains 166 amino acids (Figure 1) (12). There are four cysteines in the 166 amino acid residues of native erythropoietin, and at least two of these residues contain a disulphide bond (13). The polypeptide chain is susceptible to digestion by trypsin, chymotrypsin, V8-protease, and endoproteinase lysine-C (14). Lysine appears to be required for biologic activity (15), and the molecule contains two internal disulphide bridges (14). Enzymatically deglycosylated Ep is inactive in vivo but retains in vitro biologic activity in marrow cell cultures or radioimmunoassay (9). Using site specific antibodies to Ep, Sytkowski & Donahue (16) reported a high degree of binding with antibodies to the 99–118 and 111–129 amino acid regions of the Ep molecule, which suggests that these two sites are associated with a functional role in the hormone's action. When erythropoietin is labelled with I^{125}, a loss of biologic activity occurs, but immunoreactivity is retained (8, 17). This loss in biological activity in vivo suggests that the carbohydrate moiety protects the erythropoietin molecule from clearance by the liver (9).

Assay and Standardization

Over the past several years, in vivo and in vitro bioassays, hemagglutination inhibition assays, and radioimmunoassays have been used to assay erythropoietin (18, 19). The exhypoxic (20) or hypertransfusion polycythemic mouse assay is presently the international reference standard assay for erythropoietin, and all in vitro and in vivo assays for this hormone should be standardized against this assay utilizing the international reference preparation (IRP) erythropoietin (21).

The IRP for Ep is human urinary erythropoietin, and one IRP unit contains 0.4 mg protein (21). IRP can be obtained from the Division of Biological Standards, National Institute of Medical Research, Mill Hill, London, England. The polycythemic mouse assay requires highly skilled and experienced technical personnel; it is very time consuming, expensive, highly variable, and is not sensitive enough to detect normal human serum levels of erythropoietin. Therefore, several in vitro assays have been used. Following the recent purification of erythropoietin, several investigators have developed radioimmunoassays (RIA) for erythropoietin (17, 22–27). A good correlation has been reported between the exhypoxic polycythemic mouse bioassay and

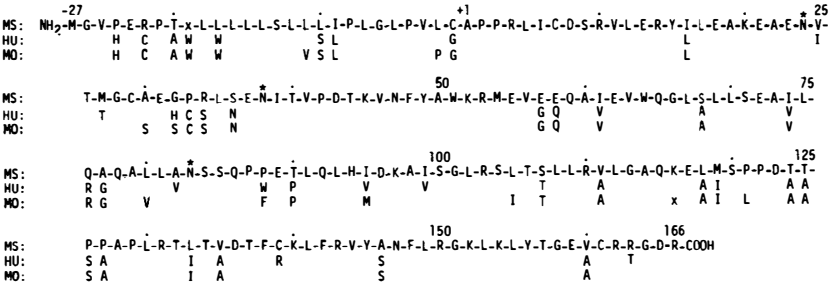

Figure 1 Amino acid sequence of three mammalian erythropoietins. The sequence of the mouse (MS) protein is presented along with differences from this sequence in the human (HU) and monkey (MO) proteins on the first and second lines below the mouse sequence, respectively. Numbering is from the amino terminus of the human protein. The asterisks indicate the sites of potential N-linked glycosylation (12).

the RIA for erythropoietin (26). Normal human serum levels of erythropoietin range between 4 and 36 milliunits per milliliter with a mean of 14.9 milliunits per milliliter reported in 175 hematologically normal human male and female subjects (26). These recent advances in radioimmunoassay technology, determination of the amino acid sequence, and gene cloning of erythropoietin should enable investigators to study the normal physiology of erythropoietin production and the effects of pharmacologic agents in modulating erythropoietin secretion and/or biosynthesis.

Model for Kidney Production of Erythropoietin

Our model for kidney production of erythropoietin postulates that hypoxia (hypobaric, anemic, or ischemic) produces oxygen deprivation in a critical renal sensor cell, thereby initiating a cascade of events leading to increased biosynthesis and/or secretion of erythropoietin (Figure 2) (28). Neither the physiologic nor the pathophysiologic control of kidney production of erythropoietin is clearly understood, but hypoxia is considered to be the fundamental stimulus for the secretion of both renal and extrarenal erythropoietin. The primary O_2-sensing reaction of the kidney is initiated by a decrease in ambient PO_2 (high altitude, hypobaria); interrupted gas exchange in the lung (obstructive lung diseases); diminished O_2-carrying capacity of hemoglobin (anemia); molecular deprivation of oxygen (cobalt); and a decrease in renal blood flow (ischemia due to atherosclerosis, thrombosis, or renal artery constriction) (29).

Adenosine production by the kidney is significantly increased very early following ischemic hypoxia (30). The primary O_2-sensing reaction continues by triggering secondary biochemical changes such as a decrease in cellular ATP, increases in ADP and NADH, or stimulation of adenosine and hypoxanthine (29). Two subclasses of cell membrane adenosine receptors have been

proposed (31, 32). These receptors have been characterized physiologically and pharmacologically. A_1 adenosine receptors exhibit high affinity in binding studies (nanomolar) and are coupled to, and inhibit, adenylate cyclase. On the other hand, A_2 adenosine receptors exhibit lower affinity (micromolar) and are coupled to, but stimulate, adenylate cyclase. Adenosine could stimulate erythropoietin production at high concentrations through adenosine A_2 receptor activation and could inhibit erythropoietin production at lower concentrations through adenosine A_1 receptor activation. A_1 receptor stimulation may lead to the production of an inhibitory G protein that reduces the activity of adenylate cyclase, whereas adenosine A_2 activation may stimulate a G protein that increases adenylate cyclase. An increase in adenylate cyclase activity leads to the generation of cyclic AMP, the activation of protein kinase A, and the phosphorylation of important nuclear proteins that may be important in the transcriptional and/or the translational stage of erythropoietin biosynthesis in the kidney. These phosphoproteins may also be important in the release of Ep from the cell. Dibutyryl cAMP in vitro increases erythropoietin secretion in erythropoietin-producing renal carcinoma cells in culture (40, 41) and produces an increase in red cell mass when injected into mice (42). An increase in renal cortical cyclic AMP levels following cobalt administration in rats showed a temporal relationship with increases in plasma levels of erythropoietin (43). It seems most likely that the external transducers activate adenylate cyclase to generate cyclic AMP, which activates protein kinase A. The latter leads to the production of a phosphoprotein which is involved in transcription and/or translation of the final 166 amino acid Ep molecule. Other stimuli of erythropoietin secretion that may act through the stimulation of adenylate cyclase are the prostanoids PGE_2, PGI_2, 6-keto-PGE_1 (44); H_2O_2, and superoxide ($^{-}O_2$), which are generated during hypoxia as oxygen-free radicals (45–47); and beta adrenergic agonists (48, 49). We have observed previously that the increased Ep production in response to hypoxia cannot be completely blocked by cyclooxygenase inhibitors (44) or beta adrenergic blockers (49). Therefore, we postulate that increased Ep production in response to hypoxia is most likely due to the release of several transducer molecules which may act in concert to increase Ep production, depending upon the severity of the hypoxic stimulus.

There are several possible negative feedback mechanisms in Ep production and/or secretion. Inositol triphosphate (IP_3) increases the mobilization of intracellular calcium from the endoplasmic reticulum, thus providing the calcium for the activation of calcium calmodulin (Ca CAM) kinase. Activation of Ca CAM kinase may increase the level of an inhibitory phosphoprotein, thus resulting in a decrease in Ep secretion. It is quite possible that the reduction in IP_3 that was reported following ischemic hypoxia (33) could lead to a decrease in the mobilization of calcium from the endoplasmic reticulum, a decrease in the intracellular calcium pool, and therefore a decrease in the

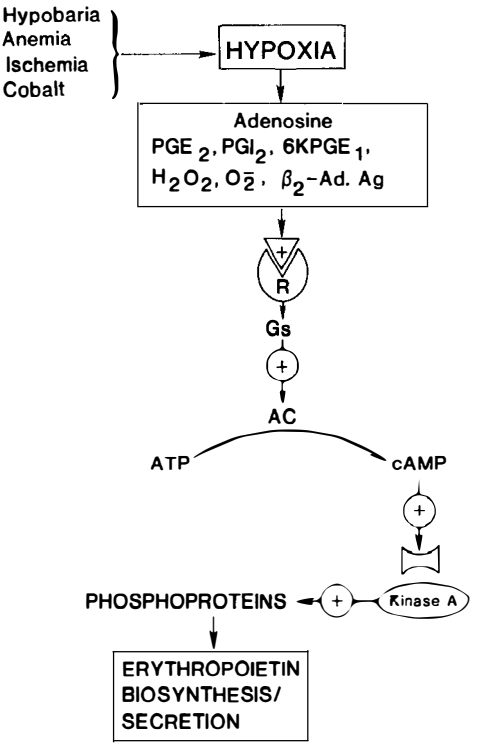

Figure 2 Schematic model for the role of second messengers and hypoxia in the regulation of kidney production of erythropoietin. Erythropoietin biosynthesis/secretion can be switched on by hypoxia through the release of several chemical agents that activate receptors in the cell membrane to increase stimulatory G proteins (Gs): prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), 6-ketoprostaglandin E_1 ($6KPGE_1$), hydrogen peroxide (H_2O_2), superoxide (O_2^-), and β_2-adrenergic agonists (β_2-Ad. Ag). Gs activates adenylate cyclase (AC), which increases cyclic 3',5'- adenosine monophosphate (cAMP); cAMP activates kinase A to phosphorylate proteins (phosphoproteins), which are important in the transcriptional and/or translational stages of Ep biosynthesis and/or secretion.

negative feedback on Ep secretion. Diacylglycerol levels are markedly increased in brain membranes following ischemic hypoxia (39) and may also play a regulatory role by providing a negative feedback for Ep biosynthesis and/or secretion by activating renal kinase C to increase the phosphorylation of inhibitory proteins that decrease Ep biosynthesis (36). Diacylglycerol and the phorbol ester TPA have been shown to inhibit Ep secretion in an Ep-producing human renal carcinoma cell line (50). Diacylglycerol may be generated from a specific renal phosphodiesterase in response to hypoxia, and diacylglycerol lipase may increase the production of arachidonic acid. A rapid and specific biphasic liberation of arachidonic acid and stearic acid has been reported following cerebral ischemic hypoxia, which coincided with the time

course for the decrease in brain ATP (35). Even though this arachidonic acid may be available for eicosanoid synthesis, most of the arachidonic acid produced is due to the action of phospholipase A_2 on membrane phospholipids. The eicosanoids that are produced play a secondary role in Ep production (28). Subcellular distribution studies (38) as well as inhibitors of lysosomal hydrolytic and proteolytic enzymes (38) indicate that an increase in these proteases in the lysosomal granules of the kidney is correlated with an increase in plasma levels of Ep during hypoxia or following cobalt injections. Both cobalt and hypoxia have been reported to provoke a labilization of lysosomal membranes in vivo as indicated by the discharge of lysosomal marker enzymes (38). Injections of cobalt into rats produce significant increases in the activity of the renal proteases cathepsins A and B and an increase in plasma proteases (38). The role of these lysosomal proteases in the cascade of events leading to increased biosynthesis of erythropoietin is not known. Earlier studies indicate that guanylate cyclase activity increases very early following cobalt administration (34) and generates cyclic GMP, which activates protein kinase G to increase the levels of a phosphorylated protein. The function of these cGMP-generated phosphoproteins in Ep production is not clear.

Calcium and Ep Secretion/Production

The various stimuli of Ep production and their relationship to intracellular calcium are shown in Table 1. Calcium levels in both kidney and liver cells may be very important in the regulation of erythropoietin biosynthesis and/or secretion by these cells. Low calcium levels in culture medium (51) and calcium entry blockers such as verapamil and diltiazem (52, 53) were found to enhance erythropoietin secretion and/or production in both an erythropoietin-producing renal carcinoma cell line (51) and in vivo in rats exposed to hypoxia (52). Some of the changes in cytosolic calcium that occur during renin secretion in the kidney (54) and the calcium entry blockers that increase

Table 1 Calcium and erythropoietin (Ep) secretion

Agent	Intracellular calcium	Ep secretion
Cobalt (66, 67, 69, 70)	Decrease	Increase
Adenosine A_2 Ag (NECA) (55, 61)	Decrease	Increase
Verapamil, diltiazem, D600 (52–54, 57, 63)	Decrease	Increase
Low Ca^{2+} medium (51)	Decrease	Increase
Trifluoperazine[a] (TFP) (50)	Decrease	Increase
Adenosine A_1 Ag (CHA) (55, 61)	Increase	Decrease
Calcium ionophore A23187 (60, 63)	Increase	Decrease

[a]Inhibits the effects of calcium on calcium calmodulin kinase (50).

renin release from the kidney (54, 55) may be very similar to the mechanisms by which calcium regulates erythropoietin biosynthesis and/or secretion.

The stimuli that are known to increase intracellular calcium and to decrease renin secretion are adenosine A_1 agonists (55), potassium (56, 57), calcium channel agonists (58), ouabain (59), and the calcium ionophore A23187 (60). Increases in medium levels of the calcium ionophore A23187 and the adenosine A_1 agonist N^6-cyclohexyladenosine (CHA) decrease erythropoietin secretion in erythropoietin-producing renal carcinoma cells in vitro (61–63) (Table 1). On the other hand, the calcium entry blockers (53, 57, 65) and the adenosine A_2 agonist 5'-N-ethylcarboxamide adenosine (NECA) (55, 62) are all known to decrease intracellular calcium and increase both renin and Ep secretion (Table 1). Cobalt is also known to decrease calcium entry into cells and to increase Ep production (7, 66–71) (Table 1). The increase in Ep secretion produced by the calcium entry blocker D600 is probably due to the decreased entry of calcium into the cell (57, 63, 64). The adenosine A_2 agonist NECA has been shown to enhance erythropoietin production in mice exposed to hypoxia (62). The mechanism by which adenosine A_2 receptor agonists decrease cytosolic calcium is not understood (55). Cobalt in a myocardial cell preparation decreases the entry of calcium into the cell (66). It is well known that cobalt stimulates erythropoietin production both in vivo (69) and in the isolated perfused kidney (7, 70). It is not clear whether cobalt's effect on erythropoietin production is due to a decrease in cytosolic calcium levels (66) or whether cobalt acts on an enzyme system in the kidney and through some molecular event deprives the renal cell of oxygen (67, 70) to stimulate Ep production. The adenosine A1 agonist CHA, which inhibits adenylate cyclase and increases intracellular calcium, produced an inhibition of erythropoietin secretion in renal carcinoma cells in culture (61). The calcium entry blocker D600 also produced an enhanced secretion of erythropoietin in an Ep-producing renal carcinoma cell culture system (65). Cerebral ischemic hypoxia results in significant decreases in the labeling of inositol triphosphate (Ins1,4,5P_3) following intracerebral injection of [^{3}H] inositol (33). It is quite possible that hypoxia results in a decrease in inositol Ins1,4,5P_3 (33, 36) in the kidney, which decreases a pool of intracellular calcium (33), whereas hyperoxia may increase IP_3 to increase the mobilization of endoplasmic reticulum calcium, thus causing a decrease in Ep secretion. This endoplasmic reticulum calcium may be in a form that activates a calcium calmodulin kinase, resulting in an increase in the production of phosphoproteins that inhibit Ep generation. The decrease in this negative feedback system, as seen in hypoxia, may permit a more pronounced increase in Ep production/secretion through the activation of kinase A by cAMP.

Diacylglycerol, which is produced by a phosphodiesterase in the cell, may also activate kinase C to produce a phosphoprotein that is inhibitory to

Ep production. This may be a secondary event after prolonged hypoxia, when high levels of diacylglycerol may result in the production of a phosphoprotein that acts as a negative feedback to decrease Ep biosynthesis/secretion. It seems clear that cyclic AMP plays the key role in kidney production of erythropoietin (42, 68, 71–74). Cyclic AMP probably activates renal kinase A to produce a phosphoprotein in the renal cell that is important in the biosynthesis of Ep and possibly in the release of erythropoietin from the cell into the blood.

The precise steps by which calcium and these lysosomal proteases act in the production of erythropoietin in the kidney and the liver have not been elucidated. Fyhrquist et al (75–77) have suggested that a renin substrate with a molecular weight of 56,800 is a likely precursor of Ep. However, Jacobs et al (10) could find no region of homology with the amino acid sequence of human erythropoietin and rat angiotensinogen (renin substrate) and thus argue against any relationship between these two polypetides. Further work is necessary to determine (*a*) whether a proerythropoietin is present in the cell that is cleaved by proteases to produce an intermediate molecular weight substance and/or the final 36,000 dalton erythropoietin within the kidneys or in plasma or (*b*) whether de novo synthesis of erythropoietin occurs within the kidney and liver and does not involve the cleavage of a larger molecular weight proerythropoietin or any other intermediate steps. Several years ago Gordon and co-workers (78–80) developed an hypothesis that erythropoietin was produced in plasma following the release of either a proerythropoietin or an enzyme, called erythrogenin, from the kidney. Unfortunately, there has been no definitive proof for the existence of a distinct chemical entity in plasma that produces this cleavage step nor was there any work to support a proerythropoietin or an enzyme within the kidney with these properties. Recent work on gene cloning of erythropoietin (10–12, 81, 82) indicates that the 36,000 dalton, 166 amino acid glycoprotein Ep molecule is present in the kidney itself. Even though it seems most likely that the kidney produces the final 166 amino acid Ep molecule de novo without an intervening proerythropoietin step, it is still possible that a proerythropoietin could be produced by both kidney and liver (75–77) that is cleaved in these organs into the final 166 amino acid glycoprotein.

RENAL SITE OF PRODUCTION OF ERYTHROPOIETIN

It seems clear from recent studies that erythropoietin can be extracted from the kidney itself (84–87), and there is evidence that de novo synthesis of erythropoietin must occur in the kidney in response to hypoxia, which does not require an interaction of a renal factor with a plasma substrate to produce erythropoietin as previously postulated (78–80, 84).

The cells within the kidney that are known to produce erythropoietin are probably in the kidney cortex (86, 87). The glomerular tuft (89–95), the renal tubules (83), and peritubular capillary endothelial cells (97, 187) have been postulated as sites of production of erythropoietin. Mori et al (93) and Nagakura et al (94) using site specific antibodies to erythropoietin reported the localization of erythropoietin in the glomerular epithelial cells in the human kidney (93) and the rat kidney (94). No staining was found in the juxtaglomerular cells, renal tubules, or any other cells in the kidney (93, 94). In addition, Nagakura et al (94) have localized Ep in the glomerular epithelial cells of both normal and hypoxic rat kidneys using a highly specific antibody to a peptide corresponding to the 111–129 amino acid portion of the Ep molecule. This immunocytochemical staining was not present in any other portion of the kidney and was completely blocked by absorption of the antibody with a purified recombinant Ep. It is difficult to determine whether the Ep localized in the glomerular tuft is trapped or stored by these epithelial cells or whether de novo synthesis of Ep has occurred in these cells. Recent reports on in situ hybridization have demonstrated mRNA for Ep in the kidney and the liver (96). In addition, preliminary in situ hybridization studies, using a ^{32}P-labelled probe encompassing the second exon of the murine Ep gene, have reported Ep mRNA in the renal peritubular capillary endothelial cells (97, 187) and in the proximal tubules using cDNA (98) and SP6 derived RNA (99) probes. Further work with in situ hybridization technology is necessary to clearly establish whether messenger RNA for erythropoietin is present in the glomerular, tubular, or peritubular endothelial cells of the kidney.

EXTRARENAL ERYTHROPOIETIN PRODUCTION

Extrarenal erythropoietin from the liver is the most important source of erythropoietin to maintain erythropoiesis in patients that are anephric or suffer from severe functional degeneration of the kidney. It is well known that after bilateral nephrectomy erythropoiesis is markedly reduced but at least 10% of erythropoiesis is maintained in the anephric animal (5, 100–102). Fried et al (102) have extensively reviewed extra-renal erythropoietin. The small amount of erythropoietin produced at extra-renal sites is apparently very similar to the Ep produced by the kidney (102), in that the immunological properties of extrarenal Ep seem to be similar to those of kidney erythropoietin.

Patients with renal disease who develop hepatitis (103, 104) or suffer from other conditions that injure the liver (105), display hematocrits that are much higher than those expected for the degree of anemia. Hepatectomy prevents the rise in Ep levels in plasma seen after bilateral nephrectomy in rats exposed to intense hypoxia (106), and Ep has been produced in the isolated hypoxemic

perfused livers (107). The liver is also the primary source of Ep in the fetus (108–111). This Ep function apparently shifts from the liver to the kidney shortly after birth (111). The Kupffer cells in the liver have been the most studied and are postulated to be the hepatic site of extrarenal Ep production (112–116). Liver Kupffer cell production of Ep has been reported using a sensitive RIA for Ep (114). Ep has also been localized in the Kupffer cells of the liver by a sensitive immunofluorescence technique (116). Recent preliminary in situ hybridization studies using cRNA for Ep have detected the presence of messenger RNA for Ep in the livers of fetal and neonatal rats exposed to phenylhydrazine anemia (96). No messenger RNA for Ep was noted in the normal fetal or neonatal livers (96). Further studies are needed to determine whether Ep production in the liver is only produced following a severe stimulus when increased amounts of Ep are needed in the anephric animal or whether Ep is produced in the normal adult liver.

SITES OF ACTION OF ERYTHROPOIETIN

The target cell for Ep is the early erythroid colony forming unit erythroid (CFU-E) (117–119). The mechanism of action of Ep in the erythroid cell compartment has recently been reviewed by Spivak (120). In general, Ep acts on the primitive CFU-E and to some extent on the late burst forming unit erythroid colony cell (BFU-E) (121). This process involves a sequence of proliferation, differentiation, and maturation events. Schofield & Lajtha (122) postulated that the cells in the erythropoietic pathway undergo 12 replications from the most primitive stem cell to the reticulocyte. Erythroid progenitors depend upon several growth factors, of which the most important are burst promoting activity (BPA) and Ep. BPA is apparently required for an early (8 day) and a mature (3 day) erythroid precursor known as a "burst forming unit erythroid" (BFU-E), which is a large multiclustered burst of hemoglobinized cells seen in bone marrow and fetal liver cultures when high doses of Ep are used (118, 122–124). The growth of the BFU-E is dependent upon both BPA and Ep and several other glycoprotein regulatory factors (120). The primitive (8 day) BFU-E is relatively insensitive to Ep, whereas the more mature 3 day BFU-E requires Ep for maturation and proliferation. The primary BFU-E requires, for the most part, BPA (121). An erythroid potentiating factor has been purified, apparently derived from T-lymphocytes, which stimulates both BFU-E and CFU-E growth (125). However, it seems clear that other specific glycoprotein regulatory factors are needed for the commitment, proliferation, and differentiation of the BFU-E, some of which are monokines such as interlukin 3 (IL-3) (126), granulocyte-CSF (G-CSF) (127), and macrophage-CSF (M-CSF) (128). Thus, Ep interacts with erythroid progenitor cells of varying degrees of maturity, and their degree of

maturation apparently determines whether these cells respond to Ep by proliferating or differentiating. Recent reports indicate that Ep binds to saturable high-affinity sites on the erythroid cell and moves from the cell membrane into the cell interior (129–30).

PHARMACOLOGIC AGENTS THAT INFLUENCE ERYTHROPOIETIN PRODUCTION

Pharmacologic agents that influence Ep production can be divided into those agents that stimulate (increase) and those that inhibit (decrease) Ep production (Table 2). Several pharmacologic agents including a number of hormones are known to trigger renal and extrarenal production of Ep. Thyroxin, which increases oxygen consumption by most cells in the body, is a potent stimulus of Ep production (131, 132). Growth hormone (131–133) increases Ep production probably by increasing body cell growth in general. Prolactin (134), serotonin (135), vasopressin (136), testosterone (137, 138), 5-alpha-androstanes (138), cyclic nucleotides (39, 41–42, 72–74, 139), beta-2 adrenergic agonists (140–143), angiotensin II (144–148), and prostaglandins (149–152) are all known to trigger Ep production. The chemical agent cobalt triggers erythropoiesis (7, 69, 70, 153–56) by increasing the production of Ep, apparently through some histotoxic hypoxic mechanism in which molecular deprivation of oxygen occurs in the cytochrome oxidase system in cells (70, 156). It was reported several years ago by Fisher et al (70) that cobalt decreased oxygen consumption in the isolated kidney and that this decrease parallels the elevation in Ep titers in the perfusate of the isolated perfused kidney. Nickel is an interesting agent that will increase Ep production by the kidney when injected directly into the kidney (157–159). On the other hand, when nickel is injected subcutaneously it is not effective in triggering production of erythropoietin (158). The anabolic androgenic steroids (137, 138, 160) and prostaglandins (161,162) may offer an important therapeutic intervention, in combination with Ep, for the treatment of anemia of renal failure when sufficient amounts of Ep for therapeutic use are provided through the recently developed gene cloning techniques. Androgens have been used extensively for the treatment of refractory anemias and are apparently useful as an adjuvant in the treatment of anemic patients with end stage renal disease and on dialysis (163–165) and in aplastic anemia (166). The 5 α -androstanes probably exert their erythropoietic effects primarily through enhanced kidney Ep production (138), while 5 β-androstanes probably exert most of their erythropoietic effects directly to increase the sensitivity of the bone marrow erythroid cells to Ep (167, 168).

Agents that inhibit Ep production include mercurial diuretics (169), alkylating agents (170), estrogens (171), and beta-adrenergic blockers (46, 49, 172).

Table 2 Pharmacologic agents that increase and decrease erythropoietin (Ep) production

Increase	Decrease
Cobalt (7, 69, 70)	Mercurial diuretics (169)
Thyroxine (131, 132)	Alkylating agents (170)
Growth hormone (132, 133)	Estrogens (171)
Prolactin (134)	β 2-adrenergic blockers (dl-propranolol-nonselective, butoxamine-selective) (140)
Serotonin (5-HT) (135)	Adenosine A_1 Ag, (CHA) (61, 62)
Vasopressin (136)	Calcium ionophores (A23187) (63)
Testosterone (137, 138, 160)	
5 α-androstanes (5 α-DHT) (138)	Calcium channel blockers (high dose, chronic) (65)
dB 3,5-adenosine cyclic monosphate (cAMP) (40–43, 139)	Phorbol esters (TPA) (50)
Prostacyclin (PGI_2) (43)	Diacylglycerol (50)
Prostaglandin E_2 (PGE_2) (150)	
Prostaglandin E_1 (PGE_1) (43, 152)	
6-Keto PGE_1 (44)	
Angiotensin II (145–147)	
Nickel[a] (158)	
Albuterol (Ad B_2 Ag) (141, 142)	
Terbutalene (Ad B2 Ag) (143)	
Isoproterenol (Ad B_1, B_2 Ag) (48)	
Adenosine A_2 Ag, (NECA) (61, 62)	
Calcium channel blockers (53) (low dose, acute)	

[a]Must be injected directly into the kidney (158).

The mechanism for this suppressed Ep production is not clear. It is of interest that beta-2-adrenergic blocking drugs such as DL-propranolol B_1, B_2, (non-selective) and butoxamine (B_2, selective) probably involve blockade of adrenergic receptor–mediated Ep production by the kidney (46, 49).

PHARMACOKINETICS OF ERYTHROPOIETIN

Ep is metabolized primarily in the liver (173). The elimination half-life of Ep has been reported to be approximately 1.5–3.4 hr in the rat (174–77), 8–10 hr in the rabbit (178), 11 hr in the sheep (179), and approximately 9 hr in the dog (180). Kidney clearance of Ep apparently contributes only minimally to the clearance of Ep in the sheep (179). Recent studies of the clearance of human Ep in rats report an alpha half-life of 54 min and a beta half life of 3.4 hr (174). On the other hand, reports of the pharmacokinetics of human recombinant purified Ep in the intact dog reveal an alpha half-life of 24 min and a beta half-life of approximately 9 hr (181). The beta-half life of human recombinant Ep in the nephrectomized dog (13.8 hr) was significantly (PL<

0.01) more prolonged than in the intact dog (9.0 hr) (181). Studies of the pharmacokinetics of Ep therefore suggest that the kinetics conform to a two compartment model with significant interspecies variation. Studies have yet to be carried out on the pharmacokinetics of purified human Ep in the normal human subject. These studies are important in order to establish the pharmacokinetic parameters for the use of purified Ep in the treatment of patients with anemia of renal failure. Purified recombinant Ep recently provided through genetic engineering technology now seems to be available in sufficient amounts to treat patients with anemia of end stage renal disease.

THERAPEUTIC USES OF ERYTHROPOIETIN

The disease entity for which Ep should be used is the anemia of chronic renal failure. In fact, injections of Ep into peritoneally dialyzed anephric rats partially corrected the anemia (182), and with high doses such injections completely corrected the anemia in uremic rats (183) and subtotally nephrectomized sheep (184, 185). There are approximately 85,000 people in the United States with end stage renal failure who are on dialysis, and most of these patients suffer from varying degrees of anemia. Some of the patients require frequent transfusions that are expensive and only short lasting. The availability of purified recombinant Ep from gene cloning technology should provide this badly needed Ep. Recent studies indicate that rather high doses of purified recombinant Ep will correct the anemia of end stage renal disease in patients on dialysis (186). Improvements in dialysis procedures, such as continuous ambulatory peritoneal dialysis (CAPD), which may more effectively remove uremic toxins that either inactivate Ep or reduce the responsiveness of erythroid cells to Ep, together with the use of pharmacologic dosages of purified recombinant human Ep to treat the anemia of end stage renal disease, constitute important advances in the clinical management of this disease.

It is also possible that the use of agents that trigger the CFU-E compartment in the bone marrow, such as the beta-androstanes, eicosanoids, and beta-2 adrenergic agonists, may increase the number of CFU-E and render the bone marrow more responsive to smaller dosages of Ep. In addition, agents such as 6-keto PGE_1, 15-methyl PGE_2 and 16,16-dimethyl PGE_2 (43, 165), used together with Ep in the treatment of anemia of renal failure, may enhance renal and extra-renal Ep production, provided that there is sufficient residual renal mass to secrete Ep. Other refractory anemias may be expected to respond to Ep, but when these patients have normal renal function, the kidney probably produces sufficient amounts of Ep, but the target cells (CFU-E) in the bone marrow may be refractory to Ep. Another possible clinical use of

recombinant Ep is to raise the red cell mass in patients being prepared for major surgery so that a sufficient amount of blood can be removed for use in transfusions needed during the surgical procedure. Ep measurements using the radioimmunoassay are also expected to enhance research on the physiology and pathophysiology of Ep production. The monitoring of plasma levels of Ep in patients receiving therapeutic doses of Ep in renal failure will also require a sensitive radioimmunoassay for Ep.

SUMMARY

A model for the regulation of erythropoietin production has been presented. This model proposes that a primary O_2-sensing reaction in the kidney is initiated by a decrease in ambient PO_2, a rapid decrease in gas exchange in the lung, a diminished oxygen-carrying capacity of hemoglobin, a molecular deprivation of oxygen, or a decrease in renal blood flow. It is proposed that the primary oxygen-sensing reaction may trigger the release of several mediators that stimulate adenylate cyclase through a receptor-activated stimulation of a G protein in the renal cell membrane. Some of the agents that are thought to be released during hypoxia, which may trigger this cascade, are adenosine (A_2 activation), eicosanoids (PGE_2, PGI_2, and 6-keto PGE_1), oxygen-free radicals (superoxide and H_2O_2), and catecholamines with beta-2 adrenergic receptor agonist properties. The activation of adenylate cyclase generates cyclic AMP, which activates protein kinase A, leading to the production of a phosphoprotein that, in turn, activates a nuclear protein involved in transcription and/or translation for erythropoietin biosynthesis and/or secretion. A second part of this model concerns the effect of hypoxia on a renal cell membrane phosphodiesterase and the generation of inositol triphosphate and diacylglycerol. Diacylglycerol may interact with diacylglycerol lipase to generate arachidonic acid, which, together with arachidonic acid generated by the interaction of phospholipase A_2 on membrane phospholipids, produces eicosanoids. Eicosanoids may play a secondary role in Ep production/secretion.

The model further proposes that calcium levels in both renal and liver cells may be important in regulating erythropoietin biosynthesis and/or secretion. It is proposed that an increase in intracellular calcium leads to the inhibition of erythropoietin biosynthesis and/or secretion and a decrease in intracellular calcium increases erythropoietin production. The specific mechanism by which calcium regulates erythropoietin biosynthesis and secretion is not well understood. However, a good correlation is seen with several agents that decrease intracellular calcium and increase erythropoietin production as well as with other agents that increase intracellular calcium and decrease erythropoietin production. When inositol triphosphate levels are increased, an in-

crease in the mobilization of intracellular calcium from the endoplasmic reticulum or another intracellular pool occurs. This increased intracellular calcium probably activates a calcium calmodulin kinase and produces a phosphoprotein that inhibits erythropoietin production/secretion. High levels of diacylglycerol may activate kinase C and generate a phosphoprotein that may also inhibit erythropoietin production/secretion. Adenosine is released in the kidney following hypoxia, and it is possible that low levels of adenosine bind to the high affinity A_1 receptors that inhibit adenylate cyclase and result in a decrease in erythropoietin production; whereas, higher levels of adenosine enable the lower binding affinity A_2 receptors to activate adenylate cyclase and increase cyclic AMP to enhance erythropoietin production/secretion.

This review has also included recent advances in the physiochemical characterization of erythropoietin; developments in assay and standardization; studies of renal and extrarenal sites of production of erythropoietin; research on the target cell (CFU-E) in the bone marrow for Ep; on pharmacologic agents that are known to increase and decrease erythropoietin production; on the pharmacokinetics of erythropoietin; and consideration of the potential therapeutic uses of erythropoietin.

It seems clear from recent reports that the anemia of end stage renal disease responds to high doses of purified recombinant erythropoietin. Further work is necessary to determine whether other refractory anemias will respond to erythropoietin and to assess the clinical value of erythropoietin in elevating red cell mass in patients prior to major surgery. Recent developments in the radioimmunoassay of erythropoietin are also providing a very useful clinical tool for studying patients with hematopoietic disorders and for basic science research on erythropoietin.

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