Erythropoietin and Acute Renal Failure

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The hemopoietic growth factor erythropoietin (EPO) has been recognized to be a multifunctional cytokine that plays a key role in ischemic preconditioning in the brain and heart. The EPO receptor is expressed widely in the kidney, and we review the important findings from the use of EPO in experimental models of acute renal failure that show that EPO reduces tubular cell death and hence the dysfunction induced by ischemia reperfusion injury, and we explore how these observations may be translated into the clinical arena.

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A cut renal failure (ARF), characterized by a sudden deterioration of renal function over a period of hours or days, resulting in the failure of the kidney to excrete nitrogenous waste products and to maintain both electrolyte and fluid homeostasis, complicates approximately 7% of all hospital admissions in patients older than 40 years.¹

The frequency of ARF varies greatly depending on the clinical setting, the age of the patient, and, particularly, the definition of renal insufficiency used. In certain scenarios, such as cardiac surgery requiring cardiopulmonary bypass, the frequency of ARF may be as high as 15%.² Hou et al³ showed an incidence of ARF of 5% (109 of 2,216 medical and surgical patients), associated with decreased renal perfusion (42%), major surgery (18%), radiocontrast exposure (12%), and aminoglycoside administration (7%). Predictors of poor prognosis included oliguria and relatively modest degrees of renal dysfunction.

The classification and assessment of severity of ARF can be difficult to determine, and this hinders the design and recruitment to randomized clinical trials using novel agents identified in animal studies. These issues of classification also are hampered by the lack of accurate sensitive biomarkers to allow early detection of alterations in glomerular filtration rate (GFR). The recognition that serum creatinine is a relatively poor marker of acute changes in GFR has led to interest in novel biomarkers, including cystatin c and neutrophil gelatinase-associated lipocalin (NGAL).⁴,⁵

The severity of renal dysfunction may determine the natural history and patient outcome in ARF. Few studies have examined the association between small changes in serum creatinine level and patient outcome. Epidemiologic studies can show only an association between ARF and mortality, especially in (intensive therapy unit) ITU studies, because ARF often is part of the spectrum of multi-organ failure. However, published studies have shown a consistently increased relative risk associated with ARF despite adjustment for comorbid conditions and severity of illness. Levy et al⁶ compared 183 patients with radiocontrast-associated ARF and 174 age-matched patients who received similar radiocontrast loads without developing ARF. The mortality rate was 34% in patients with ARF versus 7% in those patients without ARF. Adjusting for differences in comorbidity, the odds of death were increased 5.5-fold in the ARF group. This high relative risk has been shown for a more heterogenous cohort of patients. Chertow et al⁷ recently showed that, in a study of 19,201 admissions to a large urban hospital, even a 50% increase in serum creatinine level was associated with an increased mortality adjusted odds ratio of 5.8 (confidence limits 4.6-7.5), with similar increases in mortality observed with only a 0.3 mg/dL increase in serum creatinine level.

Improvements in supportive therapy, and increased access to hemofiltration and ITU support, have not led to a reduction in the mortality associated with the development of ARF.⁸ Currently therapy is limited to supportive and, in certain situations such as radiocontrast nephropathy, preventative strategies. There still is, therefore, an urgent need to discover new agents to alter the natural history of ARF. Despite a large number of agents that have proved successful in animal models of renal ischemia, the results of the clinical trials of these agents, such as insulin-like growth factor-1, have been disappointing.⁹,¹⁰

Improved understanding of the pathologic mechanisms of...
experimental acute kidney injury, and how the kidney responds and undergoes repair after an insult, is required to determine which new therapies may be effective when translated into clinical trials. In light of this knowledge, we review the recent literature investigating the potential use of erythropoietin (EPO) as a therapy in ARF.

**A Model of Pathophysiology of Ischemic ARF**

Prolonged renal ischemia initiates epithelial and vascular cell injury, resulting in a rapid decrease in GFR, correlating with the clinical initiation phase of ARF. The extent of cellular injury depends on the severity and duration of the ischemic insult. Sublethal insults disrupt the ability of vascular endothelial cells and tubular epithelial cells to maintain normal homeostatic processes, and initiate an inflammatory milieu that contributes to the extent of injury. In the commonly studied standard small rodent models of renal artery clamping, the S3 segment of the proximal tubule that traverses the outer-medullary segment, and the medullary thick ascending limb are extremely susceptible to ischemic injury. The sensitivity of the outer medulla derives from a combination of its microvascular architecture and a relatively low glycolytic capacity to generate adenosine triphosphate in the setting of rapid adenosine triphosphate (ATP) depletion resulting from impaired oxidative phosphorylation. The medullary thick ascending limb of the loop of Henle, although situated in the same region, does not undergo the same extent of cell death because there is a greater glycolytic capacity to generate adenosine triphosphate under ischemic conditions. The initiation phase is followed immediately by a phase that recently has been termed the *extension* phase. During the extension phase, persistent hypoxia and hypoperfusion lead to worsening of epithelial and endothelial cell injury and cell death, primarily in the corticomedullary region of the kidney. The maintenance phase represents a phase of stabilization of injury, resulting in a rapid decrease in GFR, correlating with the clinical initiation phase of ARF. The extent of cellular injury depends on the severity and duration of the ischemic insult. Sublethal insults disrupt the ability of vascular endothelial cells and tubular epithelial cells to maintain normal homeostatic processes, and initiate an inflammatory milieu that contributes to the extent of injury. In the commonly studied standard small rodent models of renal artery clamping, the S3 segment of the proximal tubule that traverses the outer-medullary segment, and the medullary thick ascending limb are extremely susceptible to ischemic injury. The sensitivity of the outer medulla derives from a combination of its microvascular architecture and a relatively low glycolytic capacity to generate adenosine triphosphate in the setting of rapid adenosine triphosphate (ATP) depletion resulting from impaired oxidative phosphorylation. The medullary thick ascending limb of the loop of Henle, although situated in the same region, does not undergo the same extent of cell death because there is a greater glycolytic capacity to generate adenosine triphosphate under ischemic conditions. The initiation phase is followed immediately by a phase that recently has been termed the *extension* phase. During the extension phase, persistent hypoxia and hypoperfusion lead to worsening of epithelial and endothelial cell injury and cell death, primarily in the corticomedullary region of the kidney. The maintenance phase represents a phase of stabilization of injury, and subsequent correcting events leading to cellular repair, division, and redifferentiation. This sets the stage for improved epithelial and endothelial cell function and recovery of GFR during the recovery phase.

**EPO**

EPO is a glycoprotein hormone with a molecular weight of 30.4 kDa. The gene for EPO, situated on chromosome 7q11 to 22, consists of 5 exons and 4 introns, and encodes a protein precursor of 193 amino acids. During posttranslational modification, which consists of cleavage of a 27 amino acid sequence, glycosylation of 3 N-linked (at Asn-24, Asn-38, and Asn-83) and 1 O-linked (ser-126) amino acids, the removal of arginine residue (Arg-166) from the C-terminal end yields the final circulating EPO molecule comprising 165 amino acids. The tertiary structure of erythropoietin is defined by 4 antiparallel α-helices. EPO was purified successfully by Miyake et al from the urine of a patient with aplastic anemia. From tryptic fragments of this urinary EPO, DNA probes were synthesized for the isolation and cloning of the human EPO gene. Clinical trials of the use of recombinant human EPO in patients with anemia associated with end-stage renal failure commenced shortly after, leading rapidly to universal uptake of EPO as standard therapy.

In adults, EPO secretion is primarily from the kidney, regulated in response to hypoxia, to maintain an appropriate red cell mass to manage normal tissue oxygen demand. Renal production is restricted to a population of cells in the interstitium of the cortex and outer medulla. Immunohistochemical characterization of EPO-producing cells by light and electron microscopy shows that they are fibroblast-like type I interstitial cells. The basal level of EPO secretion in the picomolar range maintains a plasma concentration equivalent to 15 to 25 IU/L. The effects of erythropoietin on the erythroid components of bone marrow are mediated by binding to specific receptors on erythroid precursors (intermediate-stage erythroid burst-forming units and the erythroid colony-forming units), which already have differentiated from pluripotent stem cells. Differentiation to this stage is not dependent on EPO because EPO receptor knock-out mice are incapable of erythropoiesis but have committed erythroid burst-forming units and erythroid colony-forming units in fetal liver tissue. In the absence of EPO, the erythroid progenitors undergo apoptotic cell death. EPO can support efficiently the proliferation of murine erythroid progenitor cells in vitro, and induce entry into the cell cycle in dormant cells.

EPO gene expression is under the control of the oxygen-sensitive transcription factor hypoxia-inducible factor (HIF-1), which consists of the regulatory subunit HIF-1α and the constitutively expressed subunit HIF-1β. Both subunits are members of multiprotein families and belong to the extended family of basic helix-loop-helix Per-Arnt-Sim homology (PAS) domain transcription factors. Low oxygen tension acts enzymatically on prolyl-residue hydroxylation by prolyl-4-hydroxylase, which, in normoxia, serves as a signal for von Hippel-Lindau–dependent polyubiquitination and proteosomal degradation, thereby preventing HIF degradation, leading to nuclear accumulation of HIF-1. The von Hippel-Lindau gene product is the recognition component of a multiprotein E3 ubiquitin-ligase complex that captures HIF 1α chains that have undergone enzymatic prolyl hydroxylation.

HIF activation has a diverse range of effects on the expression of several cytokines that mediate the adaptive response to stress and ischemia, including pro-angiogenesis hormone vascular endothelial growth factor, glucose transporters (GLUT1) and glycolytic enzymes, iron metabolism (transferrin), and a variety of genes involved in cellular proliferation, differentiation, and viability. The prolyl-4-hydroxylase requires iron as a cofactor, and administration of cobalt mimics the effect of hypoxia with upregulation of HIF-1α–dependent genes, including EPO.

There has been interest in the role of the HIF/EPO axis in ischemic preconditioning. Evidence supporting the essential role of EPO in delayed ischemic preconditioning in the heart comes from work in transgenic HIF−/− mice, which express
Erythropoietin and Ischemic Injury in the Brain and Heart

Both EPO and the EPO receptor are expressed functionally in the nervous system of rodents, primates, and human beings. In the mouse, EPO is present in the hippocampus, capsula interna, cortex, and midbrain areas. In human beings, EPO and the EPO receptor are expressed in both astrocytes and neurones, although the level of expression varies according to gestation age, with reduced production after birth.

Oxygen deficiency results in the induction of EPO in brain tissues. Accumulation of EPO messenger RNA and the EPO protein in response to hypoxia has been observed in cultured astrocytes. EPO and the EPO receptor also are inducible in the hippocampal neurones, although in vivo models show a broader upregulation during hypoxia. Initial experiments therefore have examined the potential role of EPO in the nervous system during cerebral ischemia. Infusion of EPO into the lateral ventricle of gerbils subjected to the occlusion of the common carotid arteries prevented ischemia-induced learning disability and rescued hippocampal neurones from degeneration. Several studies have confirmed the beneficial effects of EPO administration in the course of ischemic brain injury in vivo, using systemic administration to overcome the impracticalities of ventricular delivery systems.

Ischemia-related investigations examining the potential of EPO to prevent neuronal injury also have been extended to the spinal cord, peripheral nervous system, and visual system. Systemic administration of EPO before or immediately after retinal insult protects retinal ganglion cells from apoptosis and promotes the recovery of retinal function in mice. These findings led to the experimental use of EPO in models of myocardial infarction. Several studies have shown that EPO reduces infarct size, and reduces the degree of apoptosis, with beneficial effects on left ventricular function and remodeling at later time points.

The discovery by Westenfelder et al in 1999 that the EPO receptor is expressed throughout the kidney, including tubular epithelium, the glomerulus, and in mesangial cells, and that, in culture, proximal tubule epithelial cells proliferated in response to EPO, led a number of groups to examine the effects of EPO on the natural history of ischemia-reperfusion injury to the kidney.

In Vivo Evidence for the Protective Role of EPO in Renal Ischemic Injury

Several studies of the adverse effect of anemia on the course of ARF and the response to EPO were undertaken in the early 1990s. Nemato et al administered a low- and high-dose EPO protocol (500 kg to 3,000 U/kg) to rats subjected to unilateral nephrectomy and ischemia-reperfusion injury. EPO administration was associated with an increased hematocrit level, and there was a trend to increased survival in the high-dose EPO-treated animals. In comparison with latter studies, the rats used in these experiments only weighed 200 g, in a relatively severe injury model with a high animal mortality rate, which may have caused the relative lack of observed effect of EPO on renal dysfunction.

Yang et al administered high-dose EPO (3,000 U/kg) intraperitoneally as a single dose 24 hours before the onset of ischemia to simulate preconditioning before acute renal ischemia-reperfusion injury. EPO preconditioning significantly attenuated the degree of renal dysfunction (serum creatinine) observed by 24 hours, which was associated with a reduced proximal tubular epithelial cell death, measured by terminal deoxynucleotidyl-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining. EPO administration caused an increase in the expression of the antiapoptotic modulators Bcl-2 and heat shock protein-70 in both sham-operated and animals subjected to ischemia reperfusion, with a concurrent reduction in the activation of stress-activated mitogen activation stress kinase c-jun N-terminal kinases (JNK) after renal ischemia.

Vesey et al showed that EPO was effective when administered only 30 minutes before the initiation of ischemia, in a model in which Sprague-Dawley rats underwent nephrectomy and unilateral ischemia (30 min). A single high-dose EPO regimen (3,000 U/kg intraperitoneally) attenuated the increase in creatinine level observed at 24 hours, and the difference between treatment groups was maintained at 72 hours. EPO significantly reduced apoptotic cell death in the outer medullary thick ascending limb and S3 segment of the proximal tubule. The decrease in cell death was associated with evidence of an effect on tubular proliferation, with increased proximal tubule epithelial cell mitosis seen at 24 hours. This enhanced tubular regeneration associated with EPO was confirmed by increased tubular staining for proliferating cell nuclear antigen. This apparent effect of a single preischemia administration on tubular recovery is an important potential mechanism by which EPO may act in the recovery phase of ARF, even when solely administered as a bolus pretreatment.

We have studied the effects of several different EPO protocols on the course of ARF in an established short-term rat model of bilateral ischemia-reperfusion injury. Low-dose EPO was administered as a single intravenous bolus (300 U/kg) to 3 treatment groups: a 30-minute pretreatment, at the time of reperfusion, or 30 minutes after the onset of reperfusion, to determine which protocol offered the best
organ protection. A single dose was studied to determine the efficacy of the timing of treatments. All 3 EPO-treated groups showed significant reduction in the degree of renal dysfunction observed at 6 hours, although administration 30 minutes after reperfusion was associated with lesser, but significant, functional and histologic protection. The improvement in renal function and preservation of normal tubular architecture on histologic examination was associated with a significant reduction in tissue caspase-3 activity, using immunohistochemistry for the active fragment of caspase-3 and quantitative analysis of apoptotic cell death in proximal tubule segments.39 We also have observed that EPO therapy given as late as 2 hours into the reperfusion period still is associated with significant functional protection at 24 hours (Sharples EJ, unpublished data). A beneficial response to late initiation of EPO treatment has been observed in models of myocardial infarction and stroke, although there may be an important effect of dose on these late effects because Moon et al40 showed only a beneficial effect 12 hours after reperfusion in those animals treated with very high EPO dosages.

These findings are encouraging, however, because even administration late in the clinical presentation may influence the subsequent natural history of acute ischemic injury, and allow those patients who present late to benefit from therapy.

**Does EPO Solely Act on the Proximal Tubular Epithelium?**

Although these studies have concentrated on the effects of EPO administration on tubular cell injury, the endothelium plays an important role in the development and maintenance of ischemic renal injury.

A decrease in renal blood flow is of critical importance in initiating and extending the pathophysiology of ischemic ARF. Under physiologic conditions, the oxygen tension of the kidney decreases from the outer cortex to the inner medulla.41 The blood flow to the outer medullary or corticomedullary junction remains approximately 10% of normal during early reperfusion, leading to congestion caused by interstitial edema, red blood cell trapping, leukocyte adherence, and extravasation.42 Permanent damage to the peritubular capillaries occurs in rats subjected to prolonged renal ischemia, and this may be associated with the development of tubulo-interstitial fibrosis and poor urine concentrating ability in the postischemic kidney.43

Interventions designed to reverse endothelial dysfunction have been shown to minimize subsequent renal injury. Transplantation and engraftment of functionally mature endothelial cells into the circulation of postischemic rats protected the kidney from ischemic injury. In the same study, a reduction in renal dysfunction also was observed after transplantation of human embryonic kidney cells (HEK293) stably transfected with human endothelial nitric oxide synthase (eNOS), showing the importance of eNOS-derived NO in the maintenance of endothelial integrity.44

Capillary endothelial cells express the EPO receptor in their intraluminal surfaces. EPO antagonizes apoptosis of endothelial cells subjected to hypoxic stress in vitro,45 and therefore might play a role in maintaining the integrity of the microvasculature. EPO also upregulates the expression of eNOS in endothelial cells, and might rapidly increase eNOS activity via protein kinase B (AKT)-dependent eNOS phosphorylation.46 Through this mechanism, EPO may maintain normal vascular autoregulation, thereby preventing amplification of tubular hypoxia. The effects of EPO on endothelial cell survival and function are likely to be a major component of the mechanism of organ protection. These beneficial effects of EPO on the endothelium may be dose dependent because higher doses of EPO reduce the activity of dimethylarginine dimethylaminohydrolase, the enzyme that degrades asymmetric dimethylarginine, which is an endogenous inhibitor of eNOS and accumulation of asymmetric dimethylarginine is associated with oxidative stress and endothelial dysfunction.47

EPO also has been shown to stimulate endothelial cell mitogenesis and angiogenesis, which improve tissue oxygenation. EPO stimulates revascularization and healing in animal models of ischemic skin flaps.48 Studies in mice and human beings have shown that EPO is a potent stimulator of endothelial progenitor cell mobilization from the bone marrow, increasing the number of circulating endothelial progenitor cells, which may play a role in regeneration of damaged endothelium.49,50 A 3-day subcutaneous preconditioning EPO regimen (1,000 U/kg EPO per day), based on the regimen known to stimulate production of endothelial progenitor cells, was compared with a single subcutaneous administration of EPO (1,000 U/kg) at the time of reperfusion in a murine model of ischemia-reperfusion injury.51 Male C57BL/6j mice weighing 25 to 30 g underwent 30-minute bilateral renal clamping followed by 24 hours of reperfusion. Both protocols conferred significant protection from ischemia-reperfusion injury, although a greater reduction in injury marker intensity was observed in the preconditioned group. Given the half-life of EPO, this is unlikely to be merely a dose effect, and suggests that genomic pathways induced by EPO are crucial to its effects. That mobilization of endothelial progenitor cells and either maintenance or repair of endothelial integrity are involved in the protective effect of EPO in this model is an attractive hypothesis. Further work is required to determine the contribution of endothelial progenitor cell mobilization and preservation of capillary architecture to the minimization of renal dysfunction after ischemia.

**Does EPO Have Similar Effects in Other Models of Experimental Renal Failure?**

Vaziri et al52 previously had used EPO in a model of cisplatin nephrotoxicity (7 mg/kg intraperitoneally) in Sprague-Dawley rats, and then administered EPO 100 U/kg/d for 9 days. EPO significantly increased the calculated creatinine clearance at day 9 when compared with vehicle-treated animals. The early improvement in creatinine clearance in EPO-treated animals was maintained over the course of 6 weeks, at
which point full functional recovery occurred in the vehicle-treated animals. The enhanced functional recovery was accompanied by increased [³H]thymidine incorporation as a marker of increased tubular regeneration.

The long-term benefit of reducing the severity of initial kidney injury also has been shown to reduce progressive dysfunction in a rat remnant kidney model. Sprague-Dawley rats underwent a single-stage 5/6th nephrectomy procedure, and were followed-up for 6 weeks. Darbopoetin (0.1 μg/kg) subcutaneously administered once weekly significantly reduced apoptotic cell death between days 4 and 14 after surgery, associated with AKT phosphorylation, and this reduction in apoptosis led to partial preservation of renal function at 6 weeks when compared with vehicle-treated animals.¹³

The angiogenic effects of EPO and the synergistic interaction with vascular endothelial growth factor on vessel generation observed in vivo also may play an important role in remodeling peritubular capillary networks after ischemic injury.¹⁴ Rarefaction of the networks can be seen in the remnant model, but also occurs after ischemic injury, and contributes to persistent tissue hypoxia and promotion of fibrosis.¹⁵ Bahlmann et al.¹⁵ showed that EPO maintained peritubular capillary density in the remnant kidney by immunohistochemical staining for CD31.

**Mechanisms of Cellular Protection by EPO**

Much of the pathways activated by EPO binding to its membrane-bound homodimeric receptor have been established in hematopoietic cells.¹²

Fishbane et al.¹³ exposed porcine kidney epithelial cells (LLC-PK₁) to several insults with and without darbopoetin (50 ng/mL). Darbopoetin significantly reduced apoptotic cell death in response to 16 hours of hypoxia (1% oxygen). Similar experiments performed with an inactive recombinant EPO molecule did not offer protection. Vesey et al.¹⁷ showed that high doses of EPO reduced hypoxia-induced cell death in primary proximal tubular epithelial cells. These studies did not address the mechanism by which EPO exerts these effects.

We have examined the effects of EPO on immortalized human proximal tubular epithelial cells (HK-2) in a variety of experimental settings. EPO caused a dose-dependent (10-100 U/mL) increase in cell viability in serum-deprived cells, associated with a reduction in DNA fragmentation observed at 24 and 48 hours. EPO increased the expression of several antiapoptotic proteins, including Bcl-X₉ and XIAP, and prevented the activation of caspase-3.¹⁹ There is evidence that the phosphatidylinositol 3-kinase/AKT pathway is involved in EPO-dependent cell survival with the demonstration that the phosphatidylinositol 3-kinase inhibitor LY294002 inhibits the protective effects of EPO in these models. AKT phosphorylates multiple targets that influence apoptotic signaling. These preliminary investigations allow us to develop a paradigm for the multiple effects of EPO that may contribute to the reduction in ischemic injury (Fig 1).

**Can These Experimental Findings Be Translated to Clinical Practice?**

There is now experimental evidence from these rodent models of ARF that EPO can reduce the severity of acute kidney injury induced by ischemia reperfusion, systemic shock, and nephrotoxic insults, and also may contribute to the process of tubular regeneration through direct effects on tubular epithelial cells. It has been established that EPO exerts direct effects on both endothelial and tubular epithelial cells that contrib-
ute to the reduction in organ damage, although the relative importance of the site of action remains to be elucidated. It is still to be determined which of the different treatment strategies used in these models may translate best to human beings, and particularly the timing of intervention and how late in the natural history of ARF can EPO still achieve a significant response are of great interest. The doses used in several early trials (3-5,000 U/kg) would equate to massive doses in human beings, with particular concerns on the adverse effects on polycythemia, endothelial dysfunction, and thrombogenicity, although it seems as though significantly lower doses, such as those used by Vaziri et al. and Bahlmann et al., would have similar therapeutic potential. The success of a proof-of-concept trial in stroke published in 2002 should encourage clinical trials in ARF. The introduction of new derivatives of EPO that do not significantly affect hemopoiesis may make these agents attractive for use in clinical trials, but standard EPO formulations have the advantage of long-term patient safety data, familiarity, and availability.

EPO therapy does suffer from potential side effects. Recently, the observation that a large number of tumors and tumor cell lines express a functional EPO receptor, and the early stoppage of a trial of EPO in patients with head and neck cancers because of increased mortality rates, have led to a great deal of debate over the potential use of EPO in new clinical arenas. As yet, the scientific studies have not come to a conclusion, with some evidence suggesting a synergistic effect of EPO and conventional chemotherapy in some tumors.

The management of the patient with ARF remains a major challenge, with significant morbidity and mortality. The experimental evidence suggests that EPO may open a new direction in the treatment of these patients.

References