Erythropoietin Is a Novel Vascular Protectant Through Activation of Akt1 and Mitochondrial Modulation of Cysteine Proteases

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Background—Erythropoietin (EPO) is a critical regulator for the proliferation of immature erythroid precursors, but its role as a potential cytoprotectant in the cerebrovasculature system has not been defined.

Methods and Results—We examined the ability of EPO to regulate a cascade of apoptotic death–related cellular pathways during anoxia-induced vascular injury in endothelial cells (ECs). EC injury was evaluated by trypan blue, DNA fragmentation, membrane phosphatidylserine (PS) exposure, protein kinase B activity, mitochondrial membrane potential, and cysteine protease induction. Exposure to anoxia alone rapidly increased genomic DNA fragmentation from 2% to 40% and membrane PS exposure from 3% to 56% over 24 hours. Administration of a cytoprotective concentration of EPO (10 ng/mL) prevented DNA destruction and PS exposure. Cytoprotection by EPO was completely abolished by cotreatment with anti-EPO neutralizing antibody, which suggests that EPO was necessary and sufficient for the prevention of apoptosis. Protection by EPO was intimately dependent on the activation of protein kinase B (Akt1) and the maintenance of mitochondrial membrane potential. Subsequently, EPO inhibited caspase 8−, caspase 1−, and caspase 3−like activities that were linked to mitochondrial cytochrome c release.

Conclusions—The present work serves to illustrate that EPO can offer novel cytoprotection during ischemic vascular injury through direct modulation of Akt1 phosphorylation, mitochondrial membrane potential, and cysteine protease activity. (Circulation. 2002;106:2973-2979.)

Key Words: apoptosis ■ cytochrome c ■ cysteine endopeptidases ■ proteins, mitochondrial ■ proteins, proto-oncogene

To foster new therapeutic approaches for cerebral vascular disease, it becomes critical to dissect the underlying subcellular pathways used by potential cytoprotectants. In this regard, erythropoietin (EPO) has become especially attractive as a potential vascular protectant. Recent work has extended the traditional role of EPO from a mediator of erythropoiesis to one that offers protection against toxic stimuli in the nervous system1,2. Yet, knowledge concerning the ability of EPO to function as a specific vascular protectant, especially in the nervous system, is virtually nonexistent and requires further definition.

The potential for EPO to be an efficacious cytoprotectant in the nervous system may weigh heavily on its ability to foster the survival of cerebral microvascular endothelial cells (ECs) and prevent programmed cell death. Ischemic injury in ECs can lead to the active destruction of the endothelium and can mediate vascular degeneration that may ultimately impair cortical function.3,4 Cerebral EC injury is composed of 2 independent apoptotic pathways that consist of nuclear DNA degradation and the exposure of membrane phosphatidylserine (PS) residues.5,6 Although DNA degradation in ECs may lead to the acute loss of cerebral microvascular integrity, the exposure of membrane PS residues in ECs may play a more formidable role that involves the precipitation of a procoagulant environment7 and cellular inflammation.8

Several downstream signal transduction pathways may ultimately determine both the scope and capacity of the protective role of EPO. In particular, the serine/threonine kinase protein kinase B (Akt1), a key determinant of cell survival, appears to be necessary for EPO to prevent apoptosis of erythroid progenitors.9 In addition, both the independent preservation of mitochondrial membrane integrity and the modulation of cysteine protease activity through cytochrome c release may influence the protective role of Akt1.10 As a result, mitochondrial membrane depolarization, cytochrome c release, and the subsequent activation of a family of executioner cysteine proteases (caspases) that include caspase 8, caspase 1, and caspase 3 may be relevant for EPO to increase vascular cell survival during injury.11,12 In light of the strong potential of EPO to provide protection against...
cerebral vascular disease, we investigated the underlying cellular mechanisms that may determine protection by EPO to gain greater insight into potential therapeutic targets for EC injury.

**Methods**

**Cerebral Microvascular EC Cultures**

Vascular ECs were isolated from Sprague-Dawley adult rat brain by a modified collagenase/dispase-based digestion protocol. Briefly, ECs were cultured in endothelial growth media consisting of M199E with 20% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 90 μg/mL heparin, and 20 μg/mL EC growth supplement (ICN Biomedicals). Cells from the third passage were identified by positive direct immunocytochemistry for factor VIII-related antigen.

**Experimental Treatments**

To induce anoxia, EC cultures were deprived of oxygen by placing them into an anoxic (95% N₂ and 5% CO₂) chamber system (Sheldon) at 37°C for 12 hours. For treatments applied 1 hour before anoxia, application of EPO, EPO antibody (R&D Systems), or the phosphatidylinositide-3-kinase (PI3K) inhibitors wortmannin or LY294002 (Tocris) was continuous.

**Assessment of Cell Survival and Injury**

EC injury was determined by bright-field microscopy with a 0.4% trypan blue dye exclusion method 24 hours after anoxia per our previous protocols. Mean survival was determined by counting 8 randomly selected nonoverlapping fields with each containing ~10 to 20 cells (viable and nonviable) in each 24-well plate.

**Assessment of DNA Fragmentation**

Genomic DNA fragmentation was determined by the terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay. Briefly, ECs were fixed in 4% paraformaldehyde/0.2% picric acid/0.05% glutaraldehyde, and the 3'-hydroxy ends of cut DNA were labeled with biotinylated dUTP with the enzyme terminal deoxynucleotidyl transferase (Promega) followed by streptavidin-peroxidase and visualization with 3,3'-diaminobenzidine (Vector Laboratories).

**Assessment of Membrane PS Residue Externalization**

Per our prior protocols, a 30 μg/mL stock solution of annexin V conjugated to phycoerythrin (R&D Systems) was diluted to 3 μg/mL in warmed calcium containing binding buffer (10 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 1.8 mmol/L CaCl₂). Plates were incubated with 500 μL of diluted annexin V for 10 minutes. Images were acquired by “blinded” assessment with a Leitz DMRB microscope (Leica) and a Fuji/Nikon Super CCD (6.1 megapixels) that used transmitted light and fluorescence single-excitation light at 490 nm and detected emission at 585 nm.

**Assessment of Mitochondrial Membrane Potential**

The fluorescent probe JC-1 (Molecular Probes), a cationic membrane potential indicator, was used to assess mitochondrial membrane potential. EC monolayers in 35-mm² Petri dishes were incubated with 2 μg/mL JC-1 in growth medium at 37°C for 30 minutes. ECs were then analyzed immediately under a Leitz DMRB microscope with a dual-emission fluorescence filter with 515 to 545 nm for green fluorescence and emission at 585 to 615 nm for red fluorescence.

**Western Blot Analysis for Akt1 Phosphorylation and Cytochrome c Release**

Cells were homogenized, and after protein determination, each sample (50 μg/lane) was then subjected to 7.5% SDS-PAGE. Membranes were incubated with a mouse monoclonal antibody against phospho-Akt1 (p-Akt1; 1:1000; Active-Motif). After being washed, membranes were incubated with a horseradish peroxidase–conjugated secondary antibody (goat anti-mouse IgG, 1:2000; Pierce). The antibody-reactive bands were revealed by chemiluminescence (Amersham Pharmacia Biotech).

**Preparation of Mitochondria for Analysis of Cytochrome c Release**

Briefly, cells were harvested and homogenized, and the harvested supernatants were centrifuged at 10 000g for 10 minutes at 4°C. Mitochondrial proteins were then separated by 12.5% SDS-PAGE. The Western blot for cytochrome c was performed with primary mouse polyclonal antibody against cytochrome c (1:2000; Pharmingen) and revealed by enhanced chemiluminescence.

**Assessment of Caspase Activity**

At 12 hours after anoxia exposure, cysteine protease activities were determined as described previously. Cell suspensions were prepared, and an aliquot of supernatant containing 30 μg protein was incubated with a 250 μmol/L colorimetric substrate for caspase 8 (Ac-IETD-pNA), caspase 1 (Ac-YVAD-pNA), or caspase 3 (Ac-DEVD-pNA; Calbiochem). Absorbance was measured at 405 nm and substrate cleavage reported in micromoles per minute per gram of protein (μmol · min⁻¹ · g⁻¹) against standard p-nitroaniline solutions.

**Modulation of Caspase Activity**

Modulation of cysteine protease activity in ECs was performed with the irreversible and cell-permeable caspase inhibitors (50 μmol/L 1 hour before anoxia) Z-IETD-FMK for caspase 8 (IETD), Z-YVAD-FMK (YVAD) for caspase 1, and Z-DEVD-FMK (DEVD) for caspase 3 obtained from Pharmingen Inc.

**Statistical Analysis**

For each experiment that involved assessment of EC survival, DNA degradation, membrane PS exposure, and caspase activity, the mean and standard error were determined from 4 to 6 replicate experiments. Statistical differences between groups were assessed by means of ANOVA.

**Results**

**EPO Prevents EC Injury During Anoxia**

No significant toxicity during normoxia over a 24-hour period was present in the cultures exposed to EPO alone in the concentrations of 0.01 to 1000 ng/mL compared with EC survival in untreated control cultures (95±3%; data not shown). As shown in Figure 1A, EC survival was significantly reduced to 45±5% after exposure to anoxia alone compared with untreated control cultures (97±1%, P<0.01). In contrast, application of EPO of 10 ng/mL achieved maximal EC survival (86±3%), but concentrations lower than 0.1 ng/mL or higher than 10 ng/mL did not improve EC survival during anoxia.

**EPO Is Necessary and Sufficient for EC Protection During Anoxia**

Administration of EPO antibody in a series of concentrations of 0.01 to 2.00 μg/mL did not significantly alter EC survival compared with untreated control cultures (data not shown). In the presence of anoxia, application of the EPO antibody (0.01 to 1.00 μg/mL) also did not alter EC survival compared with cultures treated with anoxia alone (Figure 1B). In the presence of the EPO antibody, concentrations of EPO antibody of 0.10, 0.50, and 1.00 μg/mL significantly decreased the protective capacity of EPO, yielding EC survival rates of...
EPO is necessary and sufficient for EC protection during anoxia. A, ECs were pretreated with EPO (0.01 to 1000 ng/mL) 1 hour before exposure to anoxia, and cell survival was assessed 24 hours later (*P<0.01 vs anoxia). B, EPO antibody (Ab; 0.01 to 1.00 µg/mL) was applied 1 hour before anoxia, and EC viability was not altered. C, increasing concentrations of EPO Ab (0.01 to 1.00 µg/mL) were applied to ECs in conjunction with EPO (10 ng/mL) for 1 hour before anoxia (*P<0.01 vs anoxia). In all cases, control indicates untreated ECs.

50±4% (P<0.01), 42±3% (P<0.01), and 43±5% (P<0.01), respectively (Figure 1C).

EPO Prevents DNA Fragmentation in ECs During Anoxia
ECs were demonstrated to undergo apoptosis manifested by chromatin condensation and nuclear fragmentation 24 hours after anoxia (Figure 2A). In contrast, ECs pretreated with EPO (10 ng/mL) 1 hour before anoxia were without nuclear fragmentation. As shown in Figure 2A, anoxia resulted in a significant increase in percent DNA fragmentation (40±5%) compared with untreated control cultures (2±1%). DNA fragmentation was reduced to 12±2% in EC cultures during application of EPO over a 24-hour period.

EPO Maintains Membrane PS Asymmetry During Anoxia
In Figure 2B, exposure to anoxia resulted in the marked induction of membrane PS exposure that is present throughout the membrane of ECs. Administration of EPO (10 ng/mL) 1 hour before anoxia prevented the externalization of membrane PS residues in ECs. In Figure 2b, a significant increase in membrane PS residue exposure was observed in EC cultures at 24 hours after anoxia (56±5%) compared with untreated control cultures (3±2%). Application of EPO (10 ng/mL) 1 hour before anoxia significantly inhibited externalization of membrane PS residues to 11±3%.

EC Protection by EPO Is Dependent on Increased Activity of Akt1
Western blot assay was performed for p-Akt1 (the activated form of Akt1) 12 hours after anoxia. In Figure 3A, EPO and anoxia independently (as well as combined) significantly increased the expression of p-Akt1. This increased expression of p-Akt1 was blocked by the agents wortmannin (500 nmol/L) and LY294002 (25 µmol/L), which are inhibitors of Akt1 phosphorylation. In Figure 3B, application of EPO (10 ng/mL) 1 hour before anoxia significantly increased EC survival to 78±2%. Yet, coapplication of wortmannin (500 nmol/L) or LY294002 (25 µmol/L) at concentrations that block activation of p-Akt1 during anoxia (Figure 3A) with EPO (10 ng/mL) significantly reduced the ability of EPO to protect ECs against anoxia, which suggests that EPO required some level of Akt1 activation to offer protection.

EPO Modulates Mitochondrial Membrane Depolarization and Release of Cytochrome c During Anoxia
Compared with untreated control cultures, exposure to anoxia produced a significant decrease in the red/green fluorescence intensity ratio when the cationic membrane potential indicator JC-1 was used within 3 hours (Figures 4A and 4B), which suggests that anoxia results in mitochondrial membrane depolarization. Application of EPO (10 ng/mL) 1 hour before anoxia significantly increased the red/green fluorescence intensity of the ECs, which indicates that mitochondrial permeability transition pore membrane potential was restored to baseline (Figures 4A and 4B). Administration of EPO (10 ng/mL) 1 hour before anoxia maintained mitochondrial permeability transition pore membrane function and prevented mitochondrial cytochrome c release as demonstrated by Western blot analysis (Figure 4C).

EPO Decreases Caspase 8–, Caspase 1–, and Caspase 3–Like Activities During Anoxia
In Figure 5, EPO (10 ng/mL) was applied to the EC cultures 1 hour before anoxia, and data for caspase 8, caspase 1, and caspase 3 activities were obtained 12 hours after anoxia, because this time period represented the peak activities for these cysteine proteases. Administration of EPO significantly decreased caspase 8–like activity to 0.09±0.04 µmol · min⁻¹ · g⁻¹ (P<0.01; Figure 5A). Similarly, EPO pretreatment significantly reduced the activity of caspase 1–like activity (0.12±0.03 µmol · min⁻¹ · g⁻¹) and caspase 3–like activity (0.19±0.07 µmol · min⁻¹ · g⁻¹) compared with cultures treated with anoxia alone (0.42±0.08 and 0.48±0.07 µmol · min⁻¹ · g⁻¹, respectively; Figures 5B and 5C).
EPO Protects ECs From Injury Through Modulation of Caspase 8–, Caspase 1–, and Caspase 3–Like Activities

As shown in Figure 6A, pretreatment of ECs with 50 μmol/L of IETD, YVAD, and DEVD to inhibit caspase 8–, caspase 1–, and caspase 3–like activities significantly increased EC survival to 83%, 80%, and 85%, respectively. In Figure 6B, coapplication of EPO with the caspase 8 inhibitor (IETD, 50 μmol/L), caspase 1 inhibitor (YVAD, 50 μmol/L), or caspase 3 inhibitor (DEVD, 50 μmol/L) did not provide a synergistic level of protection against anoxic injury, which suggests that cytoprotection by EPO requires the modulation of caspase 8–, caspase 1–, and caspase 3–like activities.

Discussion

We identified several key characteristics of the cytoprotectant EPO that were critical for protection against EC injury and apoptosis. First, although administration of EPO was not toxic in relation to EC survival or programmed cell death, protection with EPO was achieved only in a limited concentration range. Concentrations of EPO <0.1 ng/mL or >100 ng/mL did not enhance EC survival during anoxia. Similar to the present work, other studies also illustrated a tight therapeutic concentration range for EPO with corresponding extracellular concentrations of ~10 ng/mL. Continuous infusion of EPO at 5 to 25 U/d for 7 days reduced infarct size in models of cerebral ischemia, but this protection was lost when administration of EPO was increased above 50 U/d. Second, administration of EPO is both necessary and sufficient to protect ECs from anoxia. In the presence of anoxia, application of the EPO antibody alone, which can bind to EPO and block its biological activity, did not alter EC survival. Yet, protection by EPO is prevented only with coapplication of the EPO antibody. These results illustrate that EPO provides necessary and sufficient protection against EC injury.
EPO complement prior work that illustrates a separate biological role for DNA degradation that is distinct from the inversion of membrane PS residues.\textsuperscript{6,12} Intact cellular function is afforded by EPO through the maintenance of genomic DNA in ECs. Long-term protection results through the inhibition of membrane PS residue exposure, because PS externalization marks cells for phagocytic elimination,\textsuperscript{6} leads to the propagation of a procoagulant surface,\textsuperscript{7,8} and can promote cellular inflammation.\textsuperscript{8}

Several cellular signal transduction pathways may mediate the protection of EPO. EPO can increase the activity of Akt1, and inhibition of Akt1 activation can impair the protective capacity of EPO, which suggests that EPO is dependent on the activation of Akt1 for its cellular protection in ECs. Interestingly, blockade of Akt1 activation can account only partially for the loss of protection by EPO in ECs during anoxic injury. The present work suggests that alternate cellular pathways are responsible for the mediation of EC protection by EPO. One particular pathway that is closely associated with Akt1 activation is the modulation of mitochondrial membrane potential.\textsuperscript{10} Mitochondrial mediated apoptosis can result in the cytoplasmic release of cytochrome c.\textsuperscript{13} In the present studies, we demonstrate that anoxia leads to depolarization of the mitochondrial membrane, with subsequent release of cytochrome c. Consistent with earlier clinical studies that demonstrate preserved mitochondrial function as a result of EPO administration,\textsuperscript{19} the present studies illustrate that EPO directly maintains mitochondrial membrane potential and prevents the release of cytochrome c.

In response to mitochondrial membrane depolarization and cytochrome c release, induction of caspase activation occurs.
Anoxia can directly stimulate caspase 1– and caspase 3–like activities after mitochondrial release of cytochrome c to precipitate DNA fragmentation and membrane PS exposure.14,20 Caspase 8 serves as the upstream initiator of executioner caspases, such as caspase 1 and caspase 3, and also leads to the release of cytochrome c.21 Previous studies suggested that prevention of apoptosis in erythroid progenitor cells may be associated with modulation of caspase activity by EPO.22 The present work demonstrates that EPO prevents the induction of caspase 8–, caspase 1–, and caspase 3–like activities during anoxia. In addition, experiments that examined the combined application of cysteine protease inhibitors with EPO during anoxia demonstrated no synergistic increase in EC survival, which suggests that EPO directly inhibits caspase 8–, caspase 1–, and caspase 3–like activities to provide EC protection. The ability of EPO to prevent cysteine protease activity appears to occur at the level of downstream cellular pathways, such as through the prevention of mitochondrial membrane depolarization and the release of cytochrome c.


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