Erythropoietin Selectively Attenuates Cytokine Production and Inflammation in Cerebral Ischemia by Targeting Neuronal Apoptosis

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Abstract

Ischemic brain injury resulting from stroke arises from primary neuronal losses and by inflammatory responses. Previous studies suggest that erythropoietin (EPO) attenuates both processes. Although EPO is clearly antiapoptotic for neurons after experimental stroke, it is unknown whether EPO also directly modulates EPO receptor (EPO-R)–expressing glia, microglia, and other inflammatory cells. In these experiments, we show that recombinant human EPO (rhEPO; 5,000 U/kg body weight, i.p.) markedly reduces astrocyte activation and the recruitment of leukocytes and microglia into an infarction produced by middle cerebral artery occlusion in rats. In addition, ischemia-induced production of the proinflammatory cytokines tumor necrosis factor, interleukin 6, and monocyte chemoattractant protein 1 concentration is reduced by >50% after rhEPO administration. Similar results were also observed in mixed neuronal–glial cocultures exposed to the neuronal-selective toxin trimethyl tin. In contrast, rhEPO did not inhibit cytokine production by astrocyte cultures exposed to neuronal homogenates or modulate the response of human peripheral blood mononuclear cells, rat glial cells, or the brain to lipopolysaccharide. These findings suggest that rhEPO attenuates ischemia-induced inflammation by reducing neuronal death rather than by direct effects upon EPO-R–expressing inflammatory cells.

Key words: stroke • erythropoietin • inflammation • apoptosis • ischemia

Introduction

Inflammatory responses within the central nervous system are triggered by widely diverse etiologies and can provide either beneficial or harmful results. For example, brain or spinal cord injuries are characterized by a central necrotic core surrounded by a variably sized region populated by injured cells (the penumbra) at risk for further degeneration over hours to days. If uncontrolled, self-perpetuating inflammatory reactions within the penumbra significantly expand the extent of infarcted tissue.

Recent interest has focused upon the role of local protective mechanisms reducing the penumbral volume. One promising endogenous neuroprotective is the cytokine erythropoietin (EPO) and its receptor (EPO-R). These proteins are abundant in brain and spinal cord (1) and are significantly up-regulated by metabolic stress (2, 3). In this role, a local system independent of the renal–bone marrow axis represents a biological substrate for "preconditioning," a phenomenon in which mild hypoxic stress increases tissue resistance to subsequent severe hypoxia (4).

We have previously shown that exogenously administered recombinant human EPO (rhEPO) effectively crosses the blood-brain barrier and provides neuroprotection in rodent models of cerebral ischemia and brain traumatic injury (5). Mechanistically, rhEPO rescues neurons within the penumbra from apoptosis (6), which is also observed in vitro for primary neuronal cultures and neuronal cell lines

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(6, 7) exposed to hypoxic stress. However, inflammatory mechanisms contribute importantly to the brain damage of cerebral ischemia, such as by the production of neurotoxins, including nitric oxide, and reactive oxygen species by immune cells (for review see reference 8). It is notable that a number of cells participating in inflammatory responses to ischemia (e.g., astrocytes and microglia) express the EPO-R (9, 10). However, it is currently unclear how extensively an endogenous neuroprotective system can modulate ischemia–induced inflammation and if so, whether this results from a direct antagonism of inflammatory processes and/or indirectly via reduced neuronal death. Specifically, does EPO-reduced injury depend upon a dual activation of both antiapoptotic and antiinflammatory activity?

In this study, we evaluated the effect of rhEPO administration on the inflammatory component in a three vessel rat model of middle cerebral artery (MCA) ischemia reperfusion. Specifically, rhEPO was administered at the time of MCA occlusion (MCAO) and the brains were evaluated histologically for inflammatory responses 24 h after reperfusion by immunostaining using markers of glial and leukocyte/microglial activation. We also determined the tissue concentrations of cytokines known to play important roles in brain inflammation (i.e., TNF, IL-6, and monocyte chemoattractant protein 1 [MCP-1]). These results were compared with those obtained using an in vivo model of inflammation where TNF production is not related to neurodegeneration, but is rather induced in the brain by exposure to LPS, an endotoxin associated with Gram-negative bacteria. Parallel in vitro studies assessed the effects of rhEPO on TNF production by peripheral blood mononuclear cells (PBMCs) or glial cells directly stimulated by LPS, as well as in a neuronal-glial coculture model of ischemic injury. The results indicate that rhEPO is a selective antiinflammatory cytokine, active only in the setting of neuronal degeneration, by limiting neuronal apoptosis.

Materials and Methods

Materials. Trimethyltin (TMT) chloride was purchased from Società Italiana Chimici. rhEPO (epoetin alfa, Procrit) was from Ortho Biotech. All other reagents were from Sigma-Aldrich.

Procedures involving animals were conducted in conformity with institutional guidelines in compliance with national and international laws and policies.

MCAO. Surgery was performed on male Crl:CD(SD)BR rats weighing 250–280 g (Charles River Laboratories) as previously described (5). The core temperature was maintained at 36.5–37.5°C by use of a homeothermic heating blanket connected to a rectal probe. PBS or rhEPO (5,000 U/kg body weight, i.p.) was administered at the time of occlusion of the contralateral carotid artery. TNF and IL-6 levels were quantified in brain cortex homogenates (11) and MCP-1 was measured using a commercial ELISA (Biosource International). All cytokine levels were measured at their respective peak time of production (8 h for TNF and 24 h for IL-6 and MCP-1).

Immunohistochemistry. 24 h after MCAO, perfused brains were sectioned (30 μm) in the transverse plane on a cryostat. Free floating sections were processed for immunoreactivity using anti–

glial fibrillary acidic protein (GFAP) mouse monoclonal (1:250; Boehringer) and anti–cd11b (MRC OX-42) mouse monoclonal antibodies (1:50; Serotec), according to the protocols of Houser et al. (12) and the manufacturer, respectively.

Intracerebroventricular (icv) Injection of LPS. Male CD-1 mice (22–25 g; Charles River Laboratories) were injected icv with 2.5 ug LPS via a 28-gauge needle (13) and TNF was measured in brain homogenates 90 min later (11). In additional experiments, rhEPO was administered either systemically (100 U/mouse, i.p., 24 h and 30 min before LPS) or centrally (100 U/mouse, icv, at the same time of LPS injection).

PBMCs. PBMCs were prepared from buffy coats obtained from healthy donors using standard Ficoll-Hypaque gradients. Cells were resuspended at 2.5×10^6/ml in RPMI 1640 medium with 10% FCS and cultured for 4 h with 1 ug/ml LPS. TNF was assayed in the supernatants as described above.

Primary Cultures of Glial Cells and Hippocampal Neurons. Primary cultures of glia and neurons were prepared as previously described (14). All experiments were performed on 2–3-wk-old glial cell cultures with 97% astrocytes and 3% microglia, as assessed by immunocytochemistry for GFAP and *Griffonia simplicifolia* isolectin B_4. Hippocampal neuronal cultures were established from 18-d rat fetuses and 160,000 cells were plated onto individual polyornithine-coated coverslips. After 24 h, coverslips were transferred to dishes containing a glial monolayer in neuron maintenance medium (Dulbecco's modified Eagle's medium and Ham's nutrient mix F12 supplemented with 5 μg/ml insulin, 100 μg/ml transferrin, 100 μg/ml putrescin, 30 nM Na selenite, 20 nM progesterone, and 100 U/ml penicillin) supplemented with 5 μM cytosine arabinoside. Coverslips were inverted and arranged such that the hippocampal neurons faced the glia monolayer with paraffin dots on the coverslips creating a narrow gap that allowed the diffusion of soluble substances between the cell layers. These conditions allowed for the maintenance of differentiated neuronal cultures with >98% homogeneity. Cells were then treated for 24 h with 1 μM TMT with or without 10 U/ml rhEPO. Viability was assessed by the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (15) and the supernatants were assayed for TNF.

To obtain homogenates from differentiated hippocampal neurons, glass coverslips with 6–8-d-old cultured hippocampal cells were scraped off in their culture medium and the resultant suspension was sonicated in ice twice for 15 s. Glial cells were then exposed to homogenates or to neuronal culture medium in the presence or absence of 10 U/ml rhEPO for 24 h at 37°C before TNF determination. Each sample of glial cell was exposed to the homogenate obtained by ~300,000 cells. The neuronal homogenate itself contained no appreciable TNF (3.4 ± 0.4 pg/ml).

Results

EPO Reduces Inflammatory Cell Infiltration after Ischemia. Consistent with our previous report (5), 24 h after of the induction of MCAO, the ipsalateral cerebral cortex exhibited extensive tissue injury within the territory of the MCA in saline-treated ischemic rats. The volume of injury was reduced by ~75% after the administration of rhEPO (5,000 U/kg body weight, i.p.). Within the region of the frontal cortex supplied by the MCA, brain sections obtained from saline-treated ischemic rats were abundantly populated by activated glia, as assessed by GFAP immunoreactivity (Fig. 1). In contrast, reactive glia were markedly reduced in either rhEPO-
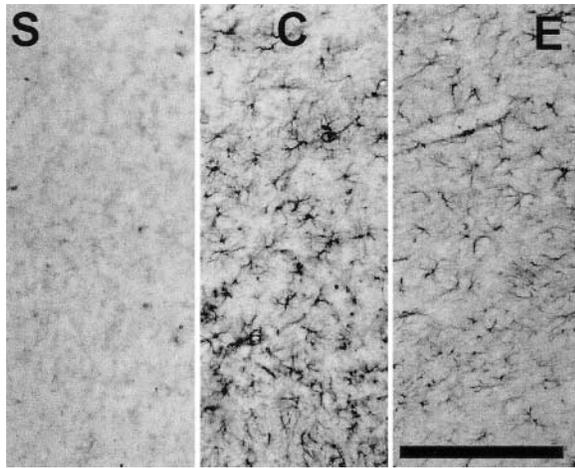
Figure 1. rhEPO dramatically attenuates glial activation in rat MCAO model. Frontal cortical sections adjacent to the region of infarction stained by GFAP antibody. S, Sham operated rat; C, ischemic rat treated with PBS; E, ischemic rat treated with rhEPO. Activated astrocytes are visualized by their GFAP⁺ processes (C). Note the marked reduction in number as well as staining intensity in a representative rhEPO-treated animal (E). Bar, 200 μm.

Figure 3. rhEPO decreases inflammatory cytokine levels in ischemic brain. TNF (A) was measured 8 h after MCAO. MCP-1 (B) and IL-6 (C) were measured 24 h after MCAO. Open bars, ischemia plus PBS; solid bars, ischemia plus rhEPO. No cytokines were detectable in control, nonischemic, and brains (not depicted). Data are mean ± SE. $n = 9$. *, $P < 0.05$; **, $P < 0.01$ versus ischemia plus PBS by Student's t test. Cytokine levels in the contralateral cortex were below the limit of detection (TNF < 1 U/g tissue; IL-6 < 150 U/g; MCP-1 < 24 pg/g).

treated ischemic or sham-operated rats (Fig. 1). Microglia within these brain regions were identified by staining with anti-CD11b (OX-42) and found to be numerous and hypertrophic within and around the infarction core, extending far into the ischemic penumbra of saline-treated ischemic rats (Fig. 2). In contrast, reactive microglia were observed rarely and only within a limited cortical volume immediately adjacent to the necrotic core in rhEPO-treated rats. Within the central core, the same antibody also identified a small number of cells morphologically consistent with monocytes and macrophages (not depicted). These observations are consistent with an ischemia–induced reactive astrocytosis and recruitment of mononuclear/microglial cells, which were all markedly reduced in rhEPO-treated ischemic rats.

rhEPO Attenuates the Production of Inflammatory Cytokines after MCAO. The appearance of inflammatory cells within the penumbra after MCAO could depend in part upon the production of proinflammatory cytokines. When measured at their respective peak time of production, TNF (Fig. 3 A), MCP-1 (Fig. 3 B), and IL-6 (Fig. 3 C) were all detected at high concentrations within the ischemic brain cortex. In contrast, systemic administration of rhEPO (5,000 U/kg body weight, i.p. immediately after MCAO) was associated with a significant reduction of the production of all three cytokines, whereas their levels in the contralateral cortex of the same animals were below the limit of detection (Fig. 3).

rhEPO Does Not Inhibit LPS-induced TNF Production. To evaluate the possibility that rhEPO might directly affect TNF production, we investigated whether rhEPO would modify LPS-induced TNF release in vitro or in vivo. As shown in Fig. 4 A, 10 U/ml rhEPO did not attenuate LPS-induced TNF production in cultured glial cells, nor did it

Figure 2. rhEPO inhibits leukocyte infiltration and microglia activation in rat MCAO. Brain sections stained by OX-42 antibody. Representative equivalent areas of the penumbra from an ischemic rat treated with PBS (C), or with rhEPO (E). In the ischemic cerebral hemisphere, the cellular staining is especially prominent around the infarcted tissue in both treatment groups, but is much denser and extends further in the saline-treated group. Bar, 100 μm.

Figure 4. rhEPO does not affect LPS-induced TNF production in vitro or in vivo. (A) TNF production by glial cells cultured with 0.1 μg/ml LPS for 24 h. (B) Brain TNF levels in mice injected icv with 2.5 μg/mouse of LPS. 100 U/mouse rhEPO was given either icv at the same time of LPS (time 0) or as a systemic pretreatment (i.p. 24 h and 30 min before the icv administration of LPS). Open bars, LPS plus PBS; solid bars, LPS plus EPO.

modify TNF production induced in human PBMCs by 1 μg/ml LPS (for 4 h) for rhEPO concentrations up to 100 U/ml (three donors studied; unpublished data). Similarly, administration of rhEPO either systemically (100 U/mouse, i.p., 24 h and 30 min before LPS) or centrally (100 U/mouse, icv, at the same time of LPS injection) did not effect brain TNF levels stimulated in vivo by direct icv injection of 2.5 μg LPS in mice (Fig. 4 B).

rhEPO Inhibits TNF Production by Glial Cells after Neuronal Death in Neuronal-Glial Cocultures. We have previously demonstrated that the selective neurotoxin TMT increases glial TNF production and release as a strict function of neuronal death from apoptosis (16). To evaluate whether the rhEPO-mediated differences in cytokine levels in MCAO could be explained by reduction of neuronal death rather than a primary effect on the inflammatory cells, mixed cultures of neurons and glia were exposed to TMT in the presence or absence of rhEPO. As shown in Fig. 5, 10 U/ml rhEPO significantly protected neurons from the toxicity of 1 μm TMT evaluated 24 h later, reducing cell death by ∼50% (Fig. 5 A). In this model in which the cell types are separated, neuronal cell death stimulates the production of TNF by glial cells. The addition of rhEPO significantly reduced the amount of TNF produced (Fig. 5 B). In contrast, similar treatment of neuron-depleted glial cultures by TMT failed to stimulate TNF production. Finally, rhEPO did not affect the astrocyte-derived TNF produced by exposure to neuronal homogenates in the absence of cocultured neurons (Fig. 5 C).

Discussion

Collectively, these data show that administration of rhEPO in a rat model of cerebral focal ischemia markedly reduces the influx of inflammatory cells into the region of injury. By this mechanism, rhEPO attenuates the production of proinflammatory cytokines, which in turn, results in a much smaller volume of injury. This concept is supported
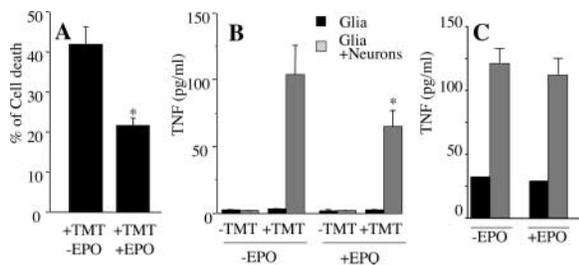
Figure 5. rhEPO prevents neuronal death–induced TNF production in mixed neuron-glia cocultures. (A) Percentage of neural cell death induced by 1 μM TMT without or with treatment with 10 U/ml rhEPO. (B) Release of TNF from glial cells exposed to 1 μM TMT in the presence (gray bars) or absence (black bars) of neurons, with or without 10 U/ml rhEPO. (C) Cytokine production by glial cells exposed to the products of neuronal homogenates with or without rhEPO. Neuronal death, $P < 0.05$ (Student's *t* test) versus TMT alone; TNF production, *, $P < 0.05$ versus neurons plus glia (ANOVA plus Tukey's multiple comparison test). The same experimental conditions were used for the three panels of this experiment.

by the strong protective effect of anti-TNF antibodies in reducing the volume of injury in this model (17).

However, unlike "classical" antiinflammatory cytokines (e.g., IL-10 and IL-13) that inhibit TNF production directly in vitro and in vivo (18, 19), rhEPO does not appear to directly affect TNF release in vitro (glial cells or PBMCs) or in vivo (either administered centrally or systemically). Significantly, a number of molecules have been identified that do inhibit cytokine production after LPS exposure (e.g., ciliary neurotrophic factor [20], interleukin-10 [18], or neurosteroids [21]). Therefore, rhEPO exhibits selective activity only in the setting of ischemic injury. That is, the inflammation-attenuating effects of rhEPO do not result from a direct action upon inflammatory cells known to express EPO-R, by blocking the release of cytokines.

Alternatively, rhEPO might exert its antiinflammatory effects by preventing the generation of molecular signals by injured neurons (e.g., the appearance of phosphatidylserine within the cell membrane, known to be a critical signal for the attraction and activation of microglia and astrocytes; 22, 23). The in vitro experiments using cocultures of glial and neuronal cells, in which neuronal death is associated with the release of factors that induce TNF release by glial cells (16), provide convincing evidence that the antiinflammatory action of rhEPO is, in fact, secondary to its neuroprotective activity. In this experimental system, neural death was achieved by exposing the cells to TMT, a selective neurotoxicant and a useful tool to induce glial activation through neural degeneration. Although there are common signaling pathways for inflammation and neuronal programmed cell death (e.g., caspase I is important in neuronal apoptosis as well as processes inflammatory cytokines IL-1 and IL-18) the observations reported here suggest that such a common mediator cannot be a target for rhEPO, as rhEPO has no direct effect upon the release of cytokines by inflammatory cells.

Even though rhEPO has no direct effect on proinflammatory cytokine production, it does provide increased resistance of cellular targets, e.g., neurons, to the effects of inflammation. It is notable that inflammation increases the ultimate volume of cerebral injury by several mechanisms, including a direct inhibition of local EPO production. In fact, although EPO is endogenously produced in the brain after ischemia (2, 3), its expression is markedly inhibited by inflammatory cytokines (e.g., TNF; 10), IL-1, and IFN-γ (24, 25), as well as by reactive oxygen and nitrogen species (26, 27). On the other hand, inflammatory cytokines such as TNF greatly increase the level of expression of EPO-R in astrocytes and neurons (10). In this way, the inhibition of endogenous EPO by inflammatory cytokines and reactive oxygen species might contribute to the role of these two classes of mediators in the pathogenesis of ischemia and explain why exogenously administered rhEPO is especially beneficial.

In summary, exogenously administered rhEPO confers a strong antiinflammatory activity within the setting of experimental brain injury. In contrast, inflammation produced by nonneurological mechanisms, e.g., endotoxin, are not affected by exposure to rhEPO. Our data support a

model wherein rhEPO attenuates neuronal injury inflammation by reducing neuronal apoptosis and increasing resistance to inflammatory injury rather than by a direct inhibition of cytokine release.

References

1. Juul, S.E., D.K. Anderson, Y. Li, and R.D. Christensen. 1998. Erythropoietin and erythropoietin receptor in the developing human central nervous system. *Pediatr. Res.* 43:40–49.

2. Bernaudin, M., H.H. Marti, S. Roussel, D. Divoux, A. Nouvelot, E.T. MacKenzie, and E. Petit. 1999. A potential role for erythropoietin in focal permanent cerebral ischemia in mice. *J. Cereb. Blood Flow Metab.* 19:643–651.

3. Siren, A.L., F. Knerlich, W. Poser, C.H. Gleiter, W. Bruck, and H. Ehrenreich. 2001. Erythropoietin and erythropoietin receptor in human ischemic/hypoxic brain. *Acta Neuropathol. (Berl.).* 101:271–276.

4. Ruscher, K., D. Freyer, M. Karsch, N. Isaev, D. Megow, B. Sawitzki, J. Priller, U. Dirnagl, and A. Meisel. 2002. Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an in vitro model. *J. Neurosci.* 22:10291–10301.

5. Brines, M.L., P. Ghezzi, S. Keenan, D. Agnello, N.C. de Lanerolle, C. Cerami, L.M. Itri, and A. Cerami. 2000. Erythropoietin crosses the blood-brain barrier to protect against experimental brain injury. *Proc. Natl. Acad. Sci. USA.* 97:10526–10531.

6. Siren, A., M. Fratelli, M.L. Brines, C. Goemans, S. Casagrande, P. Lewczuk, S. Keenan, C. Gleiter, C. Pasquali, A. Capobianco, et al. 2001. Erythropoietin prevents neuronal apoptosis after cerebral ischemia and in metabolically stressed neurons. *Proc. Natl. Acad. Sci. USA.* 98:4044–4049.

7. Digicaylioglu, M., and S.A. Lipton. 2001. Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. *Nature.* 412:641–647.

8. Becker, K.J. 2001. Targeting the central nervous system inflammatory response in ischemic stroke. *Curr. Opin. Neurol.* 14:349–353.

9. Sugawa, M., Y. Sakurai, Y. Ishikawa-Ieda, H. Suzuki, and H. Asou. 2002. Effects of erythropoietin on glial cell development; oligodendrocyte maturation and astrocyte proliferation. *Neurosci. Res.* 44:391–403.

10. Nagai, A., E. Nakagawa, H.B. Choi, K. Hatori, S. Kobayashi, and S.U. Kim. 2001. Erythropoietin and erythropoietin receptors in human CNS neurons, astrocytes, microglia, and oligodendrocytes grown in culture. *J. Neuropathol. Exp. Neurol.* 60:386–392.

11. Agnello, D., L. Carvelli, V. Muzio, P. Villa, B. Bottazzi, N. Polentarutti, T. Mennini, A. Mantovani, and P. Ghezzi. 2000. Increased peripheral benzodiazepine binding sites and pentraxin 3 expression in the spinal cord during EAE: rela-

12. Houser, C.R., R.P. Barber, G.D. Crawford, D.A. Matthews, P.E. Phelps, P.M. Salvaterra, and J.E. Vaughn. 1984. Species-specific second antibodies reduce spurious staining in immunocytochemistry. *J. Histochem. Cytochem.* 32:395–402.

13. Sacco, S., D. Agnello, M. Sottocorno, G. Lozza, A. Monopoli, P. Villa, and P. Ghezzi. 1998. Non-steroidal antiinflammatory drugs (NSAID) increase TNF production in the periphery but not in the central nervous system. *J. Neurochem.* 71:2063–2070.

14. Viviani, B., E. Corsini, C.L. Galli, and M. Marinovich. 1998. Glia increase degeneration of hippocampal neurons through release of tumor necrosis factor-alpha. *Toxicol. Appl. Pharmacol.* 150:271–276.

15. Denizot, F., and R. Lang. 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods.* 89:271–277.

16. Viviani, B., E. Corsini, C.L. Galli, A. Padovani, E. Ciusani, and M. Marinovich. 2000. Dying neural cells activate glia through the release of a protease product. *Glia.* 32:84–90.

17. Meistrell, M.E., III, K.M. Cockroft, G.I. Botchkina, E. Di Santo, O. Bloom, J. Murthy, P. Ulrich, P. Ghezzi, and K.J. Tracey. 1997. TNF is a brain damaging cytokine in stroke. *Shock.* 8:341–348.

18. Di Santo, E., M. Adami, R. Bertorelli, and P. Ghezzi. 1997. Systemic interleukin 10 administration inhibits brain tumor necrosis factor production in mice. *Eur. J. Pharmacol.* 336:197–202.

19. Di Santo, E., M. Sironi, P. Fruscella, A. Mantovani, J.D. Sipe, and P. Ghezzi. 1997. Interleukin-13 inhibits TNF production but potentiates that of IL-6 in mice suggesting a differential cellular origin for TNF and IL-6. *J. Immunol.* 159:379–382.

20. Benigni, F., P. Villa, M.T. Demitri, S. Sacco, J.D. Sipe, L. Lagunowich, N. Panayotatos, and P. Ghezzi. 1995. Ciliary neurotrophic factor (CNTF) inhibits brain and peripheral TNF production and, in association with its soluble receptor, protects mice against LPS toxicity. *Mol. Med.* 1:568–575.

21. Di Santo, E., M.C. Foddi, P. Ricciardi-Castagnoli, T. Mennini, and P. Ghezzi. 1996. DHEA inhibits TNF production in monocytic, astrocitic and microglial cells. *Neuroimmunomodulation.* 3:285–288.

22. Henson, P.M., D.L. Bratton, and V.A. Fadok. 2001. The phosphatidylserine receptor: a crucial molecular switch? *Nat. Rev. Mol. Cell Biol.* 2:627–633.

23. Savill, J., V. Fadok, P. Henson, and C. Haslett. 1993. Phagocyte recognition of cells undergoing apoptosis. *Immunol. Today.* 14:131–136.

24. Fandrey, J., and W.E. Jelkmann. 1991. Interleukin-1 and tumor necrosis factor-alpha inhibit erythropoietin production in vitro. *Ann. NY Acad. Sci.* 628:250–255.

25. Vannucchi, A.M., A. Grossi, D. Rafanelli, M. Statello, S. Cinotti, and P. Rossi-Ferrini. 1994. Inhibition of erythropoietin production in vitro by human interferon gamma. *Br. J. Haematol.* 87:18–23.

26. Tabata, M., T. Tarumoto, K. Ohmine, Y. Furukawa, K. Hatake, K. Ozawa, Y. Hasegawa, H. Mukai, M. Yamamoto, and S. Imagawa. 2001. Stimulation of GATA-2 as a mechanism of hydrogen peroxide suppression in hypoxia-induced erythropoietin gene expression. *J. Cell. Physiol.* 186:260–267.

27. Schobersberger, W., G. Hoffmann, and J. Fandrey. 1996. Nitric oxide donors suppress erythropoietin production in vitro. *Pflugers Arch.* 432:980–985.