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Pre-treatment of adult rats with high doses of erythropoietin induces caspase-9 but prevents light-induced retinal injury

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Abstract

Erythropoietin (Epo) had been shown to have a neuroprotective effect independent from its erythropoietic properties. In this study, we tested whether Epo could protect the retina from damage induced by a long period of moderate light insult and how it protected. First, rats were injected intraperitoneally (i.p.) by human recombinant Epo at 5000 or 30,000 U/kg to assess Epo concentration in plasma and retina. Second, rats were untreated or injected i.p. with Epo at 30,000 U/kg, 1 or 4 h before being placed in constant light (24 h; 2200 lux). Electroretinograms (ERG) were recorded before treatment, 1 day and 15 days (D15) after light exposure. After the last ERG, eyes were taken for histology. In parallel, we tested Epo protection against oxidative stressors on isolated retinas and its effect on caspase-9 activity. Epo injected at 30,000 U/kg body weight, 4 h before exposure to the damaging light, protected retinal function and structure against light damage and induced an increase in caspase-9 activity and expression. Epo had no direct or indirect protective effect against free radicals-induced death on isolated retinas. Epo protected the retina from a long period of moderate light exposure through a mechanism independent from a free radical scavenging property or an antioxidant facilitating activity. The activation of caspase-9, 4 h after Epo injection, corresponding to the start of light exposure, suggests that caspase-9 plays a role in neuroprotection.

Keywords: retina; oxidative stress; neuroprotection; electroretinography; apoptosis; light damage

1. Introduction

Erythropoietin (Epo) is known to be a cytokine produced in the kidney and was thought for several years to be essential only for the development of red blood cells. But recent results have shown that nerve cells also express erythropoietin receptors (EpoR) (Leist et al., 2004) and that Epo is produced in the nervous system (Sasaki et al., 2001) and can function as a neuroprotective agent.

The expression of Epo and EpoR in the central nervous system and the up-regulation of Epo by hypoxia/ischemia *in vitro* and *in vivo* suggested that this cytokine is an important mediator of the brain's response to injury. Consistent with this hypothesis, pre-treatment with exogenous Epo protects cultured neurons from hypoxia (Wen et al., 2002) and glutamate excitotoxicity (Yamasaki et al., 2005). When administered systemically, Epo can cross the blood–brain barrier and reduce neuronal injury, inflammation, kainate toxicity, and spinal cord injury.

In mouse retina, the EpoR is localized mainly in the rod inner segments and pre-synaptic complexes of photoreceptors (Grimm et al., 2002). Epo expression has been shown to be increased after hypoxic exposure which protects mouse photoreceptors from apoptotic cell death induced by damaging light *in vivo* (Grimm et al., 2005). In addition, Epo crossed the retinal blood barrier after systemic administration, and systemic administration of human recombinant Epo (5 000 U/kg) before or after light damage protected photoreceptors in mice. In rats, Epo was detected in the inner normal rat retina and

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decreased after ischemia whereas EpoR increased (Junk et al., 2002). Systemically administrated human recombinant Epo pre-treatment or post-treatment was associated with both histopathological and functional protection of retinal neurons against ischemic injury (Sakanaka et al., 1998).

Further studies have focused on the cellular pathway of Epo. Several serine/threonine kinases have been implicated in the apoptotic process and have been linked intimately to the signal transduction pathway of Epo. Protein kinase B (Akt1), a key target of phosphatidylinositide-3-kinase (PI3K), may be necessary for Epo to prevent apoptosis of erythroid progenitors during proliferation and differentiation (Uddin et al., 2000a; Chong et al., 2003). It was suggested that the phosphorylation of caspase-9 by Akt1 inhibits caspase-9 activation and therefore the induction of apoptosis (Chong et al., 2004). Epo rescued neurons from acute injury at least in part by inhibiting apoptosis via activation of specific protein kinase pathways (Uddin et al., 2000b; Chong et al., 2003) and the recruitment of NF- κB (Bittorf et al., 2001; Digicaylioglu and Lipton, 2001). Besides anti-apoptotic effects, potential protective mechanisms that might be activated by Epo include direct neurotrophic effects (Campana et al., 1998; Chattopadhyay et al., 2000) and activation of angiogenesis (Ribatti et al., 1999).

These findings together with the fact that Epo is an extremely well-tolerated compound, used in millions of patients (Jelkmann, 1994; Bauer, 1995; Nissenson et al., 1995), strongly support assessment of Epo for neuroprotective therapy.

Increasing epidemiologic studies in humans (Taylor et al., 1990; Cruickshanks et al., 1993; Simons, 1993; Cideciyan et al., 1998) and evidence in animal models (Naash et al., 1996; LaVail et al., 1999) have shown that exposure to a high level of light potentiates cellular dysfunction and ultimately, cell death. Therefore, light is suggested to be an important co-factor for development and progression of retinal degeneration. Although the primary function of the retina is to transform the light signal into an electrophysiological signal, excessive visible light can damage the retina. Light exposure results in cumulative oxidative damage caused by reactive oxygen intermediates (ROI). ROI arise as by-products of cellular metabolism or photochemical reactions. The retina is particularly susceptible to oxidative damage because of its high oxygen tension (Adler and Gringle, 1985), its tremendous exposure to irradiation in view of its function, the high proportion of polyunsaturated fatty acids in photoreceptor outer segments (Anderson, 1970; Bazan, 1989), the numerous chromophores in the retina and RPE (rhodopsin, lipofuscin, melanin, cytochrome *c* oxidase) and the generation of ROI by RPE phagocytosis of photoreceptors (Tate et al., 1995). It has been shown that the damaging effects of visible-light depend on the absorption of light by the visual pigment rhodopsin and the regeneration rate of rhodopsin (Grimm et al., 2000; Wenzel et al., 2001). In addition, the involvement of free radicals in the retinal degenerative mechanisms is supported by the high protective effects offered by several anti-oxidants such as Vitamin C (Organisciak et al., 1991), dimethylthiourea (Organisciak et al., 1992; Ranchon et al., 1999), Ginkgo biloba extract (Ranchon et al., 1999), phenyl *N-tert* butylnitrone (Ranchon et al., 2001, 2003). In light-induced retinal degeneration, as in many retinal degenerations, both inherited and induced, loss of function is associated with death of photoreceptors by apoptosis (Doonan and Cotter, 2004). Therefore, light-induced retinal degeneration can be used as a model system to study oxidative stress-induced apoptosis of photoreceptors.

Thus far, the neuroprotective benefits of Epo on the retina have been demonstrated on retinal models induced by a short period of injury (1 h ischemia or 2 h light damage). Because it has been shown that short-term and long-term light exposure differ in the molecular mechanisms leading to photoreceptor degeneration (Hao et al., 2002), the purpose of this study was to test the neuroprotective effect of Epo on a model of light-induced retinal degeneration consisting in exposing the animals for a long period (24 h) to a moderate light intensity (2200 lux). To go further into the neuroprotective pathway of Epo, we tested its free radical scavenging/antioxidant properties and its effect on caspase-9 activity and expression.

2. Materials and methods

2.1. Animals

Albino Wistar rats were raised in dim-cyclic light (12L:12D, <10 lux). They had free access to food and water. The animal care was in agreement with the ARVO Statement for the use of animals in Ophthalmic and Vision Research, and was accepted by the Regional Animal Care Committee.

2.2. Erythropoietin

RhEpo (Epoetin alpha, EPREX®) was graciously donated by Ortho Biotech (Issy Les Moulineaux, France).

2.3. Erythropoietin level in plasma and retina

Rats were injected with erythropoietin at 5000 or 30,000 U/kg body weight. The animals were sacrificed at 1, 4, 8, 16 or 24 h after the injection. Blood samples were obtained by cardiac puncture after anaesthesia. They were centrifuged at $2000 \times g$ in EGTA-containing tubes. Four retinas from two animals were pooled and homogenized in PBS1X and centrifuged at $11{,}000 \times g$ for 20 min at 4 °C. Erythropoietin levels were measured in plasmas and retinas according to EPO-Trac™, ^{125}I RIA Kit (DiaSorin, Stillwater, Minnesota USA).

2.4. Light-induced retinal damage in vivo

2.4.1. Exposure to the damaging light (light damage)

Rats were dark-adapted 16 h before being exposed for 24 h to a white fluorescent light of 2200 lux intensity at the eye level. During exposure they had free access to food and water. After the light exposure they returned to the dark for 24 h.

2.4.2. Electroretinography

Electroretinograms were recorded as described previously (Ranchon et al., 2003) with 10 μs flashes and ERGs were