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Research Report

Nuclear factor-κB p65 and upregulation of interleukin-6 in retinal ischemia/reperfusion injury in rats

Jiantao Wang[a], *Shaoyun Jiang*[b], *Jacky M.K. Kwong*[c], *Ruben N. Sanchez*[a], *Alfredo A. Sadun*[a], *Tim T. Lam*[a,*]

[a]*Department of Ophthalmology, Doheny Eye Institute, University of Southern California Keck School of Medicine, Los Angeles, CA 90033, USA*
[b]*Center for Craniofacial Molecular Biology, School of Dentistry, University of Southern California, Los Angeles, CA 90033, USA*
[c]*Glaucoma Division, Department of Ophthalmology, Jules Stein Eye Institute, University of California at Los Angeles David Geffen School of Medicine, Los Angeles, CA 90095-7000, USA*

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ABSTRACT

We previously demonstrated that endogenous interleukin-6 (IL-6) is upregulated and may be neuroprotective after retinal ischemia. The purpose of this study is to investigate the role of nuclear factor kappa-B (NF-κB) in regulating IL-6 expression after ischemia. NF-κB p65 mRNA levels were significantly elevated between 2 and 12 h after the insult. A high number of NF-κB p65 positive cells were detected in the inner retina at 12 h after ischemia. Activated nuclear NF-κB p65 and IL-6 were colocalized in cells, which were also marked by a microglial/phagocytic cell marker (ED1) in the inner retina. Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132, a proteasome inhibitor, which inhibits IκB degradation and hence prevents the activation and translocation of NF-κB into the nucleus) abolished the increase in NF-κB p65 mRNA levels after the insult, while there was no effect by helenalin (an inhibitor which inhibits NF-κB activity by alkylation of the p65 subunit, thereby blocking its binding to the target DNA). However, MG-132 and/or helenalin significantly diminished the increase in IL-6 mRNA levels after the insult. Small interfering RNAs (siRNAs, inhibit target gene expression through the sequence-specific destruction of the target messenger RNA) against NF-κB p65 significantly reduced the increase in NF-κB p65 mRNA levels as well as IL-6 mRNA levels after ischemia. The number of retinal ganglion cells (RGCs) was also significantly decreased using the inhibitors of NF-κB compared with those of the controls after ischemia. These findings support the hypothesis that upregulation of endogenous retinal IL-6 in retinal I/R injury in microglial/phagocytic cells is controlled predominantly by NF-κB p65.

1. Introduction

Interleukin-6 (IL-6) is a multifunctional cytokine that regulates cell differentiation, growth, and survival and may play important roles in a variety of diseases (Gruol and Nelson, 1997). It belongs to the neuropoietic cytokine family that also includes ciliary neurotrophic factor (CNTF), interleukin-11 (IL-11), cardiotrophin-1 (CT-1) (Taga and Kishimoto, 1997), leukemia inhibitory factor (LIF), oncostatin M (OSM) (Gadient and Otten, 1997), and novel neurotrophin-1 (NT-1) (Senaldi et al.,

* *Corresponding author*. Current address: Pharmacology, R&D, Bausch and Lomb, 1400 N. Goodman St., Rochester, NY 14690, USA. Fax: +1 585 338 6223.
E-mail address: lamtt@Bausch.com (T.T. Lam).

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1999), and is expressed in neurons (Dihne and Block, 2001; Dihne et al., 2001), microglia (Block et al., 2000; Suzuki et al., 1999; Suzuki et al., 2000), glial cells (Schiefer et al., 1998), and astrocytes (Van Wagoner and Benveniste, 1999) in the brain. Several in vitro studies showed that IL-6 protects the cerebellar granule neurons against glutamate-induced neurotoxicity (Peng et al., 2005) and increases the survival of retinal ganglion cells (RGCs) (Mendonca Torres and de Araujo, 2001), while some in vivo studies demonstrated that IL-6 is involved in neuronal survival after ischemia (Loddick et al., 1998; Sanchez et al., 2003) or *N*-methyl-D-aspartate (NMDA)-induced excitotoxicity (Inomata et al., 2003).

Nuclear factor kappa-B (NF-κB) is activated by a variety of stimuli such as stress, ischemia/reperfusion (I/R) injury, bacterial endotoxin, viral proteins, tumor necrosis factor-alpha (TNF-α), and IL-1 (Chen et al., 2003; Gabriel et al., 1999; Hu et al., 2005) and is believed to play a central role in regulation of genes encoding inflammatory cytokines, adhesion molecules, chemokines, growth factors, and cyclooxygenase (Baldwin, 1996; Zheng and Yenari, 2004). It consists of two subunits: p65/RelA and p50/p105, and is inactive in the cytoplasm by remaining a complex with its inhibitory unit, IκB. Phosphorylation of IκB results in its ubiquitination and rapid degradation by proteasomes, freeing NF-κB p65 to translocate to the nucleus and bind to DNA, leading to expression of target genes. Because the 5′-flanking sequence upstream of the cytokine IL-6 gene contains several response elements for NF-κB, it has been shown that it is pivotal in the induction of the expression of IL-6 gene in a variety of cell types in response to inflammatory stimuli (Libermann and Baltimore, 1990; Tanabe et al., 1988; Zhang et al., 1990).

In a previous report, we showed that retinal IL-6 is upregulated and that it may be neuroprotective for inner retinal neurons including retinal ganglion cells (RGCs) after retinal ischemia (Sanchez et al., 2003). In the same study, we demonstrated that the major source of retinal IL-6 after the injury might be retinal microglial/phagocytic cells (Sanchez et al., 2003). In this study, we used three different classes of modulators of NF-κB p65: carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132), a proteasome inhibitor that inhibits IκB degradation and hence prevents the activation and translocation of NF-κB into the nucleus; helenalin, an alkylating agent that inhibits the binding of NF-κB p65 to nuclear DNA; and small interfering RNAs (siRNAs) to knock down NF-κB p65 mRNA, to examine the role of NF-κB p65 in retinal IL-6 expression after retinal I/R injury.

2. Results

2.1. *Upregulation of retinal NF-κB after retinal ischemia*

A time-dependent change of retinal NF-κB p65 mRNA levels after retinal ischemia (Fig. 1) was observed showing a significant increase as early as 2 h ($n = 6$, $P < 0.05$, Tukey's test) and a peak at 4 h. There was a trend of gradual decrease from 4 h to 24 h. However, a significant difference was also shown at 8 and 12 h compared with normal ($n = 6$, $P < 0.05$, Tukey's test). There was no significant difference between 24 h and normal ($n = 6$, $P > 0.05$, Tukey's test).

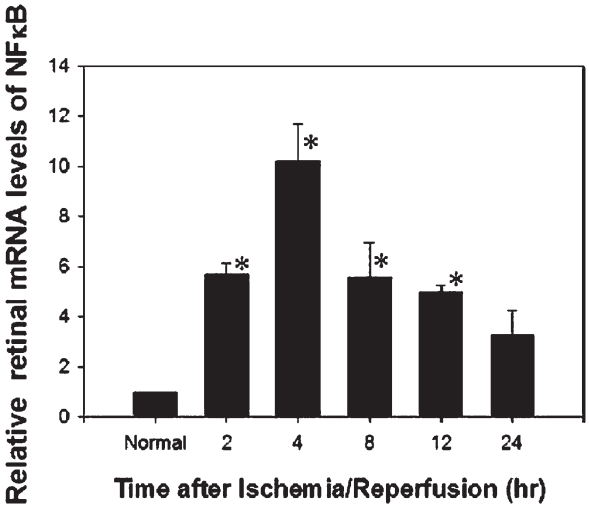

Fig. 1 – Relative mRNA levels of NF-κB p65 in rat retinas after retinal I/R injury. Semi-quantitative real-time RT-PCR showed significant increase ($P < 0.01$, Tukey's test) of NF-κB p65 mRNA at 2 ($n = 6$), 4 ($n = 6$), 8 ($n = 6$), and 12 ($n = 6$) h after I/R injury compared with normal levels ($n = 10$). Note a maximum (10-fold) at 4 h. L-32 was the housekeeping gene.

2.2. *Localization of NF-κB in the retina*

There was a significant increase of NF-κ B p65 positive cells in the retinal ganglion cell layer (RGCL), inner plexiform layer (IPL), and inner nuclear layer (INL) of retinas after ischemia (Figs. 2B–D) compared with that of normal retinas ($P < 0.05$, Tukey's test) (Figs. 2A, D). A peak of NF-κB p65 positive cells was detected in the inner retinas at 12 h after retinal ischemia (Figs. 2B, D). Normal retina showed little activated, nuclear NF-κB p65, ED-1, or IL-6 immunoreactivity (Figs. 3A–C). Activated, nuclear NF-κB p65 labeled by the monoclonal antibody 12H11 was detected in the retinal GCL, IPL and INL at 12 h after I/R injury (Fig. 3F). Triple labeling showed extensive colocalization of activated, nuclear NF-κB p65, IL-6, and ED1 in many cells in the inner retina at 12 h after retinal ischemia (Figs. 3D–F).

2.3. *Inhibition of NF-κB and IL-6 mRNA levels*

To examine the role of NF-κB, we administered MG-132, an inhibitor of NF-κB activation, helenalin, an inhibitor of NF-κB p65 binding to nuclear DNA, or siRNA against NF-κB p65 during or immediately after retinal ischemia. Semi-quantitative real-time RT-PCR assays showed that MG-132 totally abolished I/R injury-induced increases in NF-κB p65 mRNA levels (Fig. 4A) ($P < 0.05$ versus vehicle; Student–Newman–Keuls method), while helenalin had no effect on the elevation of NF-κB p65 mRNA contents after retinal ischemia (Fig. 4A). A combination of MG-132 and helenalin did not suppress NF-κB p65 mRNA levels further. siRNA against NF-κB p65 reduced approximately 50% of the elevation after retinal ischemia (Fig. 5A).

Concomitant with the lowered levels of NF-κB p65 mRNA with MG-132 or siRNA treatment, retinal IL-6 mRNA levels were also significantly lowered in those retinas ($P < 0.05$ versus vehicle; Student–Newman–Keuls method) when compared with their corresponding vehicle-treated ones (Figs. 4B, 5B). MG-132 reduced