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A decrease in phosphorylation of cAMP-response element-binding protein (CREBP) promotes retinal degeneration

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ABSTRACT

Excitotoxicity, induced either by N-Methyl-D-aspartate (NMDA) or kainic acid (KA), promotes irreversible loss of retinal ganglion cells (RGCs). Although the intracellular signaling mechanisms underlying excitotoxic cell death are still unclear, recent studies on the retina indicate that NMDA promotes RGC death by increasing phosphorylation of cyclic AMP (cAMP) response element (CRE)-binding protein (CREBP), while studies on the central nervous system indicate that KA promotes neuronal cell death by decreasing phosphorylation of CREBP, suggesting that CREBP can elicit dual responses depending on the excitotoxic-agent. Interestingly, the role of CREBP in KA-mediated death of RGCs has not been investigated. Therefore, by using an animal model of excitotoxicity, the aim of this study was to investigate whether excitotoxicity induces RGC death by decreasing Ser^{133}-CREBP in the retina. Death of RGCs was induced in CD-1 mice by an intravitreal injection of 20 nmoles of kainic acid (KA). Decrease in CREBP levels was determined by immunohistochemistry, western blot analysis, and electrophoretic mobility gel shift assays (EMSAs). Immunohistochemical analysis indicated that CREBP was constitutively expressed in the nuclei of cells both in the ganglion cell layer (GCL) and in the inner nuclear layer (INL) of CD-1 mice. At 6 h after KA injection, nuclear localization of Ser^{133}-CREBP was decreased in the GCL. At 24 h after KA injection, Ser^{133}-CREBP was decreased further in GCL and the INL, and a decrease in Ser^{133}-CREBP correlated with apoptotic death of RGCs and amacrine cells. Western blot analysis indicated that KA decreased Ser^{133}-CREBP levels in retinal protein extracts. EMSA assays indicated that KA also reduced the binding of Ser^{133}-CREBP to CRE consensus oligonucleotides. In contrast, intravitreal injection of CNQX, a non-NMDA glutamate receptor antagonist, restored the KA-induced decrease in Ser^{133}-CREBP both in the GCL and INL, and inhibited loss of RGCs and amacrine cells. These results, for the first time, suggest that KA promotes retinal degeneration by reducing phosphorylation of Ser^{133}-CREBP in the retina.

1. Introduction

Hyper-stimulation of glutamate receptors (excitotoxicity) in the retina and in the central nervous system leads to neuro-degeneration (Schwarcz and Coyle, 1977; Siliprandi et al., 1992). Although a number of previous studies have suggested that hyper-stimulation of both NMDA and non-NMDA-type glutamate receptors promotes irreversible death of retinal ganglion cells (RGCs) (Chidlow and Osborne, 2003; Siliprandi et al., 1992), the intracellular signals that promote excitotoxicity-induced cell death are unclear. Previous studies have suggested that cyclic AMP (cAMP) regulates a number of transcriptional factors and dictates cell survival (Shaywitz and Greenberg, 1999). One of these transcription factors, cAMP-response element (CRE)-binding protein (CREBP), has been suggested to play a major role in cell survival and synaptic plasticity of neuronal cells (Lonze et al., 2002; Walton et al., 1999; Walton and Dragunow, 2000).

CREBP, a 43 kDa nuclear protein, is constitutively expressed by many neuronal cells, including RGCs (Choi et al., 2003; Harada et al., 1995; Walton et al., 1999; Walton and Dragunow, 2000; Yoshida et al., 1995; Zhang et al., 2005). CREBP consists of three functional domains: a transactivation region containing the site for phosphorylation (also known as the kinase domain), a DNA-binding domain consisting of basic amino acids (basic domain), and a leucine zipper domain (bZip domain). In response to extracellular stimuli, CREBP becomes phosphorylated at $serine^{133}$ (Johannessen et al., 2004; Montminy et al., 1990, 1986; Zhang et al., 2005), binds to CRE site either as a monomer or a homodimer, and activates a number of CRE-target genes. Although CREBP contains many phosphorylation

Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; KA, Kainic acid; TUNEL, TdT-mediated dUTP nick-end labeling; CRE, cAMP-response element; CREBP, cAMP-response element-binding protein; EMSA, electrophoretic mobility shift assay; OCT compound, optimal cutting temperature compound.

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sites, phosphorylation of Ser^{133} has been suggested to play a major role in cell survival. Currently, mechanisms underlying Ser^{133}-CREBP's phosphorylation at the nuclear level are unclear and vary based on the stimuli. Yet, previous studies have suggested that three intermediate-signaling pathways, Calcineurin, mitogen activated protein kinase (MAPK), and Calcium/calmodulin-dependent protein kinase type IV (CaMKIV pathways) may play a role in Ser^{133}-CREBP's phosphorylation at the nuclear level (Bito et al., 1996; Impey et al., 1998; Lee et al., 2005; Sun et al., 1994; Xing et al., 1996).

Although a number of previous reports have indicated that both KA and NMDA promote the death of RGCs (Mali et al., 2005; Manabe and Lipton, 2003; Siliprandi et al., 1992; Zhang et al., 2004a), the role of Ser^{133}-CREBP in RGC death has not been investigated extensively. To date, a few studies reported varying results with regard to Ser^{133}-CREBP expression in the retina. For example, Yoshida et al. (Yoshida et al., 1995) reported that both flashing light and administration of Bay K8644, an L-type Ca2+ channel activator, induced phosphorylation of Ser^{133}-CREBP in the nuclei of amacrine cells and ganglion cells. Another study by Choi et al. (Choi et al., 2003) reported that monosialotetrahexosylganglioside (GM1) protected retinal ganglion cells after optic nerve injury through enhanced Ser^{133}-CREBP's phosphorylation. Finally, by increasing intraocular pressure (IOP), a study by Kim and Park (Kim and Park, 2005) reported that the number of Ser^{133}-CREBP-positive cells decreased with time after injury.

Recent studies on the retina have indicated that NMDA promotes RGC death by increasing phosphorylation of Ser^{133}-CREBP (Isenoumi et al., 2004), while studies on the central nervous system have shown that KA promotes neuronal cell death by decreasing phosphorylation of Ser^{133}-CREBP (Lee et al., 2002). These results suggest that Ser^{133}-CREBP can elicit dual responses on cell survival depending on the excitotoxic-agent used. Interestingly, the role of Ser^{133}-CREBP in KA-mediated survival of RGCs has not been investigated. Therefore, by using an animal model of KA-mediated excitotoxicity, the aim of this study was to investigate whether KA induces RGC death by regulating phosphorylation of Ser^{133}-CREBP in the retina.

2. Materials and methods

2.1. Initiation of excitotoxicity

All experiments on animals were performed under general anesthesia according to institutional protocol guidelines and the guidelines set forth by the association for research in vision and ophthalmology (ARVO). Normal adult CD-1 mice (6–8 weeks old; Charles River Breeding Labs, Wilmington, MA) were anesthetized by an intraperitoneal injection of 1.25% avertin (2,2,2-tribromoethanol in tert-amyl alcohol; 17 μL/g body weight). Throughout this study, unilateral intravitreal injections were performed in a final volume of 2 μL. For control experiments, eyes ($n = 6$; three independent experiments) were injected with 2 μL of 0.1 M phosphate buffered saline (PBS, pH 7.4). Treatment group eyes ($n = 6$; three independent experiments) were injected with 2 μL of 10 mM (corresponding to a final concentration of 20 nmoles) kainic acid (Sigma, St. Louis, MO), prepared in PBS. In a separate set of experiments, eyes ($n = 6$; three independent experiments) were injected with 1 μL of 20 mM KA plus 1 μL of 400 mM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; a non-NMDA-receptor blocker; corresponding to a final concentration of 200 nmoles; Tocris, Ellisville, MO).

2.2. Extraction of cytoplasmic and nuclear proteins

At 6 and 24 h after intravitreal injection of KA, three mice were anesthetized with an overdose of avertin and their eyes were enucleated ($n = 6$ retinas; three independent experiments). After removing the cornea and lens, retinas were carefully peeled off using forceps and washed three times with phosphate buffered saline (pH 7.4). Since Ser^{133}-CREBP is a nuclear protein, proteins were separated into nuclear and cytoplasmic fractions according to our previously published methods (Chintala et al., 1998). Briefly, 6 retinas were placed in Eppendorff tubes, washed in cold PBS, and re-suspended in a buffer containing 400–600 μL of M KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2.0 mg/mL leupeptin, 2.0 mg/mL aprotinin, 0.5 mg/mL benzamidine. The tubes were allowed to stay on ice for 20 min followed by the addition of 12.5 μL of 10% Nonidet P-40. The tubes were vortexed vigorously for 10 s, and the homogenates centrifuged for 30 s. The supernatant containing the cytoplasmic proteins was saved for further experiments. The nuclear pellets were re-suspended in 50 μL of ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2.0 mg/mL leupeptin, 2.0 mg/mL aprotinin, 0.5 mg/mL benzamidine) and incubated on ice for 30 min with intermittent vortexing. Samples were centrifuged for 5 min at 4 °C, and the supernatant containing nuclear proteins was stored at −70 °C until further use. Protein concentration in supernatants was determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

2.3. Western blot analysis

Equal amounts of protein (75 μg) from PBS or KA-treated retinas ($n = 6$ retinas; three independent experiments) were mixed with gel-loading buffer and separated on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to PVDF (Polyvinylidene Fluoride) membranes and incubated for 1 h at room temperature in blocking buffer (10% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 [TBS-T]). Membranes were then probed with antibodies against CREBP (phosphorylated and un-phosphorylated, 1:2000 dilution in TBS-T, Santacurz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. After washing with TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature (1:2500 dilution, Santacruz Biotechnology, Santa Cruz, CA). Proteins on the membranes were detected by using an ECL chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ) and by exposing the membranes to X-ray film. Finally protein bands were scanned with a densitometer, normalized to un-phosphorylated CREBP, and representative results from two independent experiments were shown. Data from 6 different eyes (three independent experiments) were analyzed by ANOVA, followed by a post hoc-Tukey's test by using GB-Stat Software (Dynamic Microsystems, MD) and expressed as mean +/− SEM.

2.3.1. DNA–protein binding assays

DNA–protein interactions were determined by EMSAs according to the procedures described previously (Chintala et al., 1998; Wang et al., 2003). Briefly, 4 μg of nuclear extract was incubated with 16 fmol of ^{32}P-labeled CRE oligonucleotides (5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′) for 15 min at 37 °C. Two-three μg of poly (dI-dC) was included in the binding buffer (25 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1% Nonidet P-40, 5% glycerol, 50 mM NaCl) to inhibit non-specific binding. The DNA–protein complexes were then separated from free oligonucleotides on 7.5% native polyacrylamide gels using a buffer containing 50 mM Tris, 200 mM glycine, pH 8.5, and 1 mM EDTA. The gels were dried, and DNA–protein bands were visualized by capturing the bands on an x-ray film.