Journal of Pharmacological Sciences 135 (2017) 72–80

Contents lists available at ScienceDirect

Journal of Pharmacological Sciences

journal homepage: www.elsevier.com/locate/jphs

Full paper

Activation inhibitors of nuclear factor kappa B protect neurons against the NMDA-induced damage in the rat retina

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ARTICLE INFO

Article history:
Received 5 April 2017
Accepted 13 September 2017
Available online 29 September 2017

Keywords:
N-methyl-D-aspartic acid
Nuclear factor kappa B
High-mobility group Box-1
Retina

ABSTRACT

We reported that high-mobility group Box-1 (HMGB1) was involved in excitoneurotoxicity in the retina. HMGB1 is known to activate nuclear factor kappa B (NF-κB). However, the role of NF-κB in excitotoxicity is still controversial. Here, we demonstrated that NF-κB activation induced by NMDA led to the retinal neurotoxicity. Male Sprague–Dawley rats were used, and NMDA (200 nmol/eye) and bovine HMGB1 (15 μg/eye) were intravitreally injected. Triptolide (500 pmol/eye), BAY 11-7082 (500 pmol/eye), and IMD-0354 (7.5 nmol/eye), NF-κB inhibitors, were co-injected with NMDA or HMGB1. Retinal sections were obtained seven days after intravitreal injection. Cell loss in the ganglion cell layer was observed in the HMGB1- and the NMDA-treated retina. All of the NF-κB inhibitors used in this study reduced the damage. BAY 11-7082 reduced the expression of phosphorylated NF-κB 12 h after NMDA injection, upregulation of GFAP immunoreactivity induced by NMDA 12 and 48 h after NMDA injection, and the number of TUNEL-positive cells 48 h after NMDA injection. The results suggest that NF-κB activation is one of the mechanisms of the retinal neuronal death that occurs 48 h after NMDA injection or later. Prevention of NF-kB activation is a candidate for the treatment of retinal neurodegeneration associated with excitotoxicity.

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1. Introduction

It has been well-known that retinal ganglion cell death occurs in the patients of glaucoma, but the mechanism of the neuronal death is not completely understood. Ionotropic glutamate receptor channels are known to be activated[1] by hypoxia[1] and ischemia-reperfusion,[2] which causes neuronal cell death. Stimulation of the N-methyl-D-aspartic acid (NMDA) receptor, one of ionotropic glutamate receptor subtypes,[3,4] leads to a large increase of intracellular Ca^{2+}, which causes excitotoxicity in neuron. It has been thought that excitotoxicity at least partially causes glaucomatous degeneration.[5]

Intravitreal NMDA injection evokes apoptosis and necrosis in the retinal neurons.[6] Damage-associated molecular patterns (DAMPs), including High-mobility group box-1 (HMGB1), released by damaged cells induce the activation of immune system.[7–9] We recently reported that co-injection of neutralizing antibody against HMGB1 reduced the NMDA-induced excitoneurotoxicity.[10]

HMGB1 binds to receptor for advanced glycation end products (RAGE) and Toll-like receptor (TLR) 2/4, and evokes inflammatory response via direct stimulation of these receptors.[11–15] Stimulation of TLR 2/4 and RAGE is known to cause NF-κB activation in neuron and other tissues.[16–21] In fact, glutamate is reported to activate NF-κB in neurons,[22–24] and that calpain is involved in the underlying mechanisms.[25] We recently reported that calpain was activated by intravitreal NMDA in the retina.[26] It is still controversial whether activation of NF-κB is pro-apoptotic or anti-apoptotic,[27,28] and it has not been clarified whether activation of NF-κB causes the intravitreal-NMDA-induced retinal neuronal death. The aim of the present study is to elucidate the role of NF-κB in the retinal neuronal death induced by NMDA using NF-κB activation inhibitors in the rats, *in vivo*.

2. Materials and methods

2.1. Animals

The experimental procedures in the present study conformed to the "Guiding Principles for the Care and Use of Laboratory Animals"

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Peer review under responsibility of Japanese Pharmacological Society.

https://doi.org/10.1016/j.jphs.2017.09.031
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approved by The Japanese Pharmacological Society, and were approved by the Institutional Animal Care and Use Committee of Kitasato University. Male Sprague–Dawley rats (7–8 weeks old, 230–300 g body weight; Japan SLC, Hamamatsu, Japan) were used in the present study. Ketamine (90 mg/kg, i.p., Daiichi-Sankyo, Tokyo, Japan) and xylazine (10 mg/kg, i.p., Tokyo Kasei, Tokyo, Japan) were used for anesthesia. A heating pad and a heating lamp were used to maintain rectal temperature of the rats at 37 °C during experiments.

2.2. Intravitreal injection

Activation inhibitors of NF-κB were injected intravitreally. A 33-gauge needle connected to a 25-μL microsyringe (MS-N25, Ito Seisakujo, Fuji, Japan) was used for intravitreal injection as previously described.[10,29–33] The volume of the drug solution is 5 μL and the solution with HMGB1 or NMDA was administered into one eye, and the drug solution without HMGB1 or NMDA was injected into the contralateral eye.

2.3. Preparations of the drugs

Bovine HMGB1 protein solution (1 μg/μL, 15 μg/eye) was obtained from Chondrex (Redmond, WA). NMDA was obtained from Nacalai Tesque (Kyoto, Japan) and dissolved in saline (200 nmol/eye). Triptolide (500 pmol/eye), BAY 11-7082 (500 pmol/eye), and IMD-0354 (7.5 nmol/eye), NF-κB activation inhibitors, were purchased from Abcam (Cambridge, UK). These NF-κB activation inhibitors were dissolved in DMSO and diluted with saline. DMSO concentration of the injected drug solution was 2% (triptolide and BAY 11-7082) or 30% (IMD-0354).

2.4. Histological evaluation

The methods for histological evaluation have been described previously.[10,29–33] Briefly, rats were euthanized seven days after injection of HMGB1 or NMDA. Both eyes were enucleated and fixed with a Davidson solution, comprised of 37.5% ethanol, 9.3% paraformaldehyde and 12.5% acetic acid, for 24 h at room temperature. A microtome (HM325, Microm International, Walldorf, Germany) and a microtome blade (PATH BLADE + PRO by Kai, Matsunami Glass) were used to cut 5-μm-thick horizontal sections through the optic nerve head. The sections were stained with hematoxylin and eosin (HE). The number of cells in 0.5 mm of the retinal ganglion cell layer (GCL), and width of the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL) and the outer nuclear layer (ONL) were evaluated on the photographs of the HE-stained sections. These measurements from the HMGB1 or NMDA-injected eye were expressed as percentages of those from the contralateral eye.

2.5. TUNEL staining and immunohistochemistry

ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Chemicon, Temecula, CA) was used according to the manufacturer's instruction. Immunohistochemistry was performed as previously described.[10] Rabbit polyclonal anti-phospho-NF-κB p65 antibody (Ser 276) (1:50; Santa Cruz Biotechnology, Dallas, TX), mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:500, Proteintech, Rosemont, IL), Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (1:200, Abcam), and Alexa Fluor® Plus 488-conjugated goat anti-mouse IgG (1:200, Life Technologies, Carlsbad, CA) were used. Nuclear counterstaining was performed with DAPI-Fluoromount-G Mounting Medium (Southern Biotech, Birmingham, AL). LSM 710 confocal laser microscope (Carl Zeiss, Jena, Germany) was used to collect images. Fluorescent intensity of the phosphorylated NF-κB p65 signal in GCL was measured with Image J software (National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2012).

2.6. Statistical analyses

All data are presented as mean ± standard error of the mean (SEM). To compare the two groups, Student's *t*-test was used. For multiple comparisons, Tukey–Kramer test was used. If $P < 0.05$, we considered the differences to be statistically significant.

3. Results

3.1. Involvement of NF-κB in neurotoxicity induced by HMGB1 in the retina

To clarify whether NF-κB activation causes in the HMGB1-induced neurotoxicity, the effects of triptolide (500 pmol/eye), BAY 11-7082 (500 pmol/eye), and IMD-0354 (7.5 nmol/eye), NF-κB activation inhibitors, on the neurotoxicity were tested. HMGB1 solution (1 μg/μL, 15 μg/eye) was mixed with the solution of the inhibitor, and intravitreally injected. Fig. 1A shows typical pictures of the HE-stained retinal sections taken seven days after HMGB1 injection. The cell density in GCL, and the thickness of IPL, INL, OPL and ONL from five independent experiments are shown in Fig. 1B. The NF-κB activation inhibitors protected the cells in GCL against the HMGB1-induced injury.

3.2. Involvement of NF-κB in excitoneurotoxicity in the retina

To clarify whether NF-κB activation causes the retinal neurotoxicity induced by NMDA, we tested the effects of triptolide (500 pmol/eye), BAY 11-7082 (500 pmol/eye), and IMD-0354 (7.5 nmol/eye), NF-κB activation inhibitors, on the neurotoxicity. Fig. 2A shows typical pictures of the HE-stained retinal sections taken seven days after NMDA injection. The cell density in GCL, and the thickness of IPL, INL, OPL and ONL from five independent experiments are shown in Fig. 2B. The NF-κB activation inhibitors significantly reduced cell loss in GCL induced by intravitreal NMDA, although these compounds did not reverse the NMDA-induced decrease of the thickness of IPL.

3.3. Reduction of apoptosis by an NF-κB activation inhibitor in the NMDA-induced neurotoxicity

To determine whether an NF-κB activation inhibitor has an anti-apoptotic effect, we tested the effects of BAY 11-7082 (500 pmol/eye) on apoptosis induced by NMDA using TUNEL staining. Typical photomicrographs of TUNEL staining taken 12–48 h after NMDA injection (200 nmol/eye) are shown in Fig. 3A. The numbers of the TUNEL-positive cells from three independent experiments are shown in Fig. 3B. BAY 11-7082 significantly decreased the number of the TUNEL-positive cells in GCL and INL 48 h after intravitreal injection of NMDA.

3.4. Effect of the intravitreal NMDA on distribution of phosphorylated NF-κB

To clarify whether intravitreal NMDA affected the distribution of phosphorylated NF-κB p65 (Ser 276), an active form of NF-κB, immunohistochemical analyses were performed. Representative photomicrographs 12 h after intravitreal NMDA are demonstrated in Fig. 4A. Fluorescent intensities of the phosphorylated NF-κB p65 signal in GCL from three independent experiments are shown in Fig. 4B. In the saline-treated group, positive signals were found in

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