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Brain Research 1044 (2005) 227 – 240
BRAIN RESEARCH
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Research report

Contribution of mitogen-activated protein kinases to NMDA-induced neurotoxicity in the rat retina

Yasunari Munemasa[a], Ritsuko Ohtani-kaneko[b,*], Yasushi Kitaoka[a], Kohei Kuribayashi[a], Kazuyuki Isenoumi[a], Jiro Kogo[c], Kayoko Yamashita[b], Toshio Kumai[c], Shinichi Kobayashi[c], Kazuaki Hirata[b], Satoki Ueno[a]

[a]*Department of Ophthalmology, Saint Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki-shi, Kanagawa 216-8511, Japan*
[b]*Department of Anatomy and Cell Biology, Japan*
[c]*Department of Pharmacology, Japan*

Accepted 1 March 2005
Available online 19 April 2005

Abstract

We examined the contributions of the mitogen-activated protein kinases (MAPKs) family [extracellular signal-regulated kinase (ERK), p38 kinase (p38), and c-Jun N-terminal kinase (JNK)] to *N*-methyl-D-aspartate (NMDA)-induced neurotoxicity in the rat retina. Detection of apoptotic cell death in the retinal ganglion cell layer (RGCL) and the inner nuclear layer (INL) by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) staining began 6 h after intravitreal NMDA (100 nmol) injection and continued to increase thereafter. Western blot analysis showed that phosphorylated MAPKs (p-MAPKs) were expressed in the retina following a temporal manner: maximal expression of phosphorylated ERK (p-ERK) at 1 h, maximal expression of phosphorylated p38 (p-p38) at 6 h, and beginning of phosphorylated JNK (p-JNK) significant increase at 6 h after injection. An immunohistochemical/TUNEL co-localization study showed that p-JNK- and p-p38-positive cells in the RGCL were frequently TUNEL-positive, whereas few p-ERK-positive cells were TUNEL-positive. Moreover, co-injection of inhibitors for JNK (0.2 nmol SP600125) and/or p38 (2.0 nmol SB203580) with NMDA was effective in ameliorating NMDA-induced apoptotic cell loss in the RGCL 12 h after injection, as shown by TUNEL-positive cell counts. These inhibitors also protected the inner retina as shown by morphometric studies such as cell counts in the RGCL and measurement of the IPL thickness 7 days after injection. On the other hand, an ERK inhibitor (2.0 nmol U0126) did not suppress NMDA-induced cell death in the RGCL nor thinning of the IPL. These findings suggest that JNK and p38 are proapoptotic in NMDA-induced cell death in the RGCL, but not ERK.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Excitatory amino acids: excitotoxicity

Keywords: Apoptosis; Retina; MAPK; TUNEL; *N*-methyl-D-aspartate

1. Introduction

Glutamate, a major excitatory neurotransmitter, has been shown to cause neuronal cell death in the inner retina [28] and is implicated in certain ocular diseases, including optic neuropathy [47] and proliferative diabetic retinopathy [1,2,6]. Glutamate receptors are classified as metabotropic and ionotropic types, the latter of which are further subcategorized as *N*-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors. In many instances, glutamate neurotoxicity has been predominantly attributed to excessive stimulation of NMDA receptors, which are activated by the co-agonists NMDA (or glutamate) and glycine [24,25,30]. In NMDA receptor-

* Corresponding author. Fax: +81 44 976 7083.
E-mail address: r2kaneko@marianna-u.ac.jp (R. Ohtani-kaneko).

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doi:10.1016/j.brainres.2005.03.014

mediated cell death, the increase in intracellular Ca^{2+} concentration is thought to be the key event [4,23]. However, the detailed molecular mechanisms through which NMDA-induced Ca^{2+} influx leads to retinal cell death remain unclear.

Mitogen-activated protein kinases (MAPKs), which include extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase (p38), are evolutionarily conserved enzymes that link cell-surface receptors to intracellular regulatory targets, and have been shown to be activated by NMDA-induced Ca^{2+} influx [22,34,40]. Members of the mitogen-activated protein kinase (MAPK) family are activated by dual phosphorylation at threonine and tyrosine residues, and are regulated by different MAPK kinase isoforms [7,10]. Upon phosphorylation, the phosphorylated MAPKs (p-MAPKs) in turn phosphorylate several transcription factors to regulate various cellular activities [10,39].

In the central nervous system (CNS), MAPKs are known to respond to various stresses, including ischemia [14,43,44], ultraviolet radiation [36], endotoxins [9], and hyperosmolarity [9], as well as to pro-inflammatory cytokines such as tumor necrosis factor [36] and interleukin-1 [8]. ERK, JNK, and p38 are known to play major roles in these processes and are considered to be intimately involved in cell death induced by these stimuli [8,9,32,33,43].

In the eye, MAPK family members have been shown to be activated and involved in retinal neuronal cell death in retinal ischemia [12,38,48], light damage [27], and axotomy of the optic nerve [18]. Activation of ERK may be related to the expression of some neurotrophic factors such as fibroblast growth factor (FGF) [19] and ciliary neurotrophic factor (CNTF) [27] in photoreceptor. It has recently been reported that NMDA activates p38 in retinal ganglion cells (RGCs) and inhibition of this process protects against NMDA-induced cell death [29]. However, the contribution of other MAPKs to NMDA-induced apoptosis has not been elucidated. In the present study, we examine the time course of activation of MAPKs after NMDA injection and determine their expression in apoptotic cells and the effects of their inhibition on NMDA-induced retinal neuronal cell death.

2. Materials and methods

2.1. Animals

Experiments were performed on 8-week-old male Wistar rats. All studies were conducted according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed in a room in which temperature (23 ± 1 °C), humidity (55 ± 5%), and lighting (light from 6 AM to 6 PM) were controlled.

2.2. Administration of NMDA

Intravitreal injection of NMDA (Sigma, St. Louis, MO) was performed as previously described [20,41]. Briefly, rats were anesthetized by intraperitoneal (ip) injection of sodium pentobarbital (35 mg/kg) and their pupils were dilated with tropicamide. Injection was performed under a microscope with a microsyringe and a 30-gauge needle, which was inserted approximately 1 mm behind the corneal limbus. 5 μl of 20 mM NMDA (a total amount 100 nmol) in 0.01 M phosphate-buffered saline (PBS, pH 7.40) was administered into one eye and PBS was administered into the other eye as a control.

2.3. In situ TUNEL

In situ terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) was performed on sections from 40 rats, 4 of which served as naive controls. The treated rats were deeply anesthetized and perfused pericardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) 1 (n = 4), 3 (n = 4), 6 (n = 8), 12 (n = 12), or 24 (n = 8) h after intravitreal injection, and their eyes were enucleated. The naive control rats were processed similarly. After removal of the anterior segments, the posterior portions of the eyes were post-fixed with 4% PFA in 0.1 M PB overnight at 4 °C and then cryoprotected in 20% sucrose in PBS overnight at 4 °C. 10-μm-thick sections of retina with the optic nerve were then cut with a cryostat.

The TUNEL assay was performed with a fluorescein apoptosis detection system (Promega, Madison, WI), according to the manufacturer's instructions. In brief, after the sections were rinsed in PBS and reacted with 20 μg/ml proteinase K for 10 min at room temperature, they were incubated with the terminal dUTP transferase enzyme together with a mixture of nucleotides in an equilibration buffer for 60 min at 37 °C in a moist chamber. After terminating the reaction by immersing in 2× saline sodium citrate (SSC) for 15 min, the sections were washed three times with PBS for 5 min each. For nuclear staining, they were counterstained with 4′,6-diamidino-2-phenylindole dihydrochlorid (DAPI, Molecular Probes, Eugene, OR). We counted TUNEL-positive cells in the retinal ganglion cell layer (RGCL) and the inner nuclear layer (INL) manually at 1.0–1.5 mm (both sides) from the center of the optic nerve using a fluorescence microscope. The average number of TUNEL-positive cells/eye was obtained from three sections of each retina.

2.4. Western blot analysis

Fifty-four rats were used for the Western blot analysis. Nine rats were used as naive controls and 45 rats were used for injection. Eyes were enucleated at various time