Neurochemistry International 53 (2008) 71–78

Contents lists available at ScienceDirect

Neurochemistry International

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

Enhanced expression of RNase L as a novel intracellular signal generated by NMDA receptors in mouse cortical neurons

Chie Sugiyama, Nobuyuki Kuramoto, Reiko Nagashima, Masanori Yoneyama, Kiyokazu Ogita *

Department of Pharmacology, Setsunan University Faculty of Pharmaceutical Sciences, Setsunan University, 45-1 Nagaotoge-cho, Hirakata, Osaka 573-0101, Japan

ARTICLE INFO

Article history:
Received 9 April 2008
Received in revised form 10 May 2008
Accepted 3 June 2008
Available online 8 June 2008

Keywords:
Cortical neurons
Glutamate
Neuronal death
NMDA receptor
RNA
RNase L

ABSTRACT

Recently we showed that the level of mitochondrial mRNA was decreased prior to neuronal death induced by glutamate. As the level of mRNA is regulated by ribonuclease (RNase), we examined RNase activity and its expression in the primary cultures of cortical neurons after glutamate treatment in order to evaluate the involvement of RNase in glutamate-induced neuronal death. A 15-min exposure of the cultures to glutamate at the concentration of 100 μM produced marked neuronal damage (more than 70% of total cells) at 24-h post-exposure. Under the experimental conditions used, RNA degradation was definitely observed at a period of 4–12-h post-exposure, a time when no damage was seen in the neurons. Glutamate-induced RNA degradation was completely prevented by the *N*-methyl-D-aspartic acid (NMDA) receptor channel blocker MK-801 or the NR2B-containing NMDA receptor antagonist ifenprodil. Glutamate exposure produced enhanced expression of RNase L at least 2–12 h later, which was absolutely abolished by MK-801. However, no significant change was seen in the level of RNase H1 mRNA at any time point post-glutamate treatment. Immunocytochemical studies revealed that RNase L expressed in response to glutamate was localized within the nucleus, mitochondria, and cytoplasm in the neurons. Taken together, our data suggest that expression of RNase L is a signal generated by NMDA receptor in cortical neurons. RNase L expression and RNA degradation may be events that cause neuronal damage induced by NMDA receptor activation.

1. Introduction

There is accumulating evidence that activation of glutamate receptors leads to different mitochondrial dysfunctions as a consequence of mitochondrial depolarization elicited by a loss of Ca^{2+} homeostasis (White and Reynolds, 1996; Schinder et al., 1996) and impairment of metabolic homeostasis by a reduction in the activities of different enzymes within mitochondria (Atlante et al., 1999; Delgado-Esteban et al., 2000). In particular, glutamate receptor stimulation is known to cause persistent inhibition of ATP synthesis leading to necrosis in primary cultures of cortical neurons (Delgado-Esteban et al., 2000; Almeida and Bolanos, 2001). This persistent inhibition of ATP synthesis would result from sustained inhibition of mitochondrial cytochrome *c* oxidase and succinate–cytochrome *c* reductase activities by $ONOO^-$, which is generated through activation by glutamate signals of nitric oxide synthase and the formation of reactive oxygen species. In previous reports, we proposed a hypothesis that glutamate-induced long-term attenuation of ATP synthesis is elicited by altered expression of mitochondrial RNA by the transcription factor activator protein-1, which is translocated into mitochondria in response to glutamate signals (Ogita et al., 2002, 2003). This hypothesis has been confirmed by our findings that a decrease in level of mitochondrial mRNA is an event prior to neuronal death induced by glutamate in primary cultures of cortical neurons (Sugiyama et al., 2007). However, the mechanisms underlying the decrease in mitochondrial RNA in glutamate-induced neuronal damage remain to be elucidated.

The regulation of mRNA decay by ribonuclease (RNase) is an important means to control the expression of genes and proteins in eukaryotic cells. RNase constitutes a large superfamily of enzymes having greatly diverse function other than a simple digestive role. Several lines of evidence indicate that many members of the RNase superfamily have important biological actions including neurotoxicity, angiogenic activity, immunosuppressivity, and antitumor activity (Castelli et al., 1998; Silverman, 2007). RNase is divided into endoribonucleases (RNase A, RNase H, RNase III, and RNase L) and exoribonucleases (RNase II, RNase R, RNase D, and RNase T). Of these RNases, RNase L has been shown to be present in mitochondria and importantly implicated in the degradation of

* Corresponding author. Tel.: +81 72 866 3110; fax: +81 72 866 3110.
E-mail address: ogita@pharm.setsunan.ac.jp (K. Ogita).

0197-0186/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2008.06.001

mitochondrial DNA-encoded mRNA in response to interferon treatment (Hassel et al., 1993; Zhou et al., 1993; Le Roy et al., 2001). Of the RNases H in mice, RNase H1 is present in both mitochondria and nucleus and creates RNA primers for replication of mitochondrial DNA (Cerritelli et al., 2003). In the present study, thus, we focused on changes in RNase activity and expression of RNase L and RNase H1 in neurons damaged by excitotoxicity.

The objective of the present study was to investigate whether glutamate triggers enhanced expression and activation of RNases before neuronal death in cortical neurons. The findings of the present study demonstrate that glutamate-induced expression of RNase L is a novel event induced by *N*-methyl-D-aspartic acid (NMDA) receptor activation. Furthermore, our data show that the degradation of RNAs is a preliminary event leading to glutamate-induced death in cortical neurons.

2. Experimental procedures

2.1. Materials

Cytosine-β-D-arabinofuranoside, glucose and Taq DNA polymerase were purchased from Sigma Chemicals (MO, USA). MitoTraker Red CH-H_2XRos, Dulbecco's modified Eagle's medium (DMEM), and 1:1 mixture of DMEM and nutrient mixture F-12 (DMEM/F12) were from Invitrogen Co. (CA, USA). Penicillin–streptomycin solution, poly-L-lysine, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) come from Nacalai Tesque Inc. (Kyoto, Japan). Goat polyclonal antibody against RNase L was purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Fluorescein isothiocyanate conjugated donkey anti-goat IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (PA, USA). All other chemicals used were of the highest purity commercially available.

2.2. Cell culture

Primary neuronal cultures were prepared from the cerebral neocortex of 15-day-old embryonic mice as originally described by di Porzio et al. (1980) with several modifications. In brief, cerebral neocortices were dissected from embryonic Std-ddY mice and incubated with 0.02% EDTA at room temperature for 12 min. The cells were then mechanically dissociated with a fire-narrowed Pasteur pipette in culture medium consisting of DMEM/F-12 supplemented with 10% fetal cow serum, 33 mM glucose, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 mM HEPES and 0.11% sodium bicarbonate. After the culture medium had been removed by centrifugation at 360 × *g* for 4 min and aspiration, the cells pellet was suspended in the same culture medium; and the cells were then plated at a density of 2.0×10^5 cells/cm^2 on poly-L-lysine-coated culture dishes. At 2 days *in vitro* (DIV), the cells were treated with 5 μM cytosine-β-D-arabinofuranoside for 24 h to avoid the growth of proliferative contaminants including glial cells and then maintained for 6 DIV in the culture medium. Before experiments, the culture medium was replaced with glutamate-free assay medium consisting of DMEM supplemented with 33 mM glucose, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 mM HEPES, 0.11% sodium bicarbonate, 50 μg/mL transferrin, 500 ng/mL insulin, 1 pM β-estradiol, 3 nM triiodothyronine, 20 nM progesterone, 8 ng/mL sodium seleniate, and 100 μM putrescine. Cultures were always maintained at 37 °C in 95% air/5% CO_2. Under these culture conditions, microtubule-associated protein 2-positive cells increased in a culture time-dependent manner, representing more than 80% of the cultured cells on 3-12 DIV [positive cells (% of total cells): 3 DIV, 86.4; 6 DIV, 93.9; 9 DIV, 92.1]. Definite expression of NMDA receptor subunit proteins including NR1, NR2A, and NR2B was seen in cortical neurons obtained at least at 8 DIV.

2.3. Drug treatment

At 8 DIV, cortical neurons were exposed to glutamate at the concentration of 100 μM for 15 min. After having been washed with glutamate-free assay medium, the cells were incubated in the same medium for various periods of time. MK-801 and ifenprodil were added at 15 min before glutamate treatment.

2.4. Cell viability

Cell viability was determined by using the MTT assay, as described (Albe et al., 2000). At several time points after a 15-min exposure to glutamate, MTT solution (0.5 mg/mL in PBS) was added into each well of culture dishes; and then the cells were incubated for 2 h at 37 °C. Subsequently, solubilizing solution (0.4 M HCl in isopropanol) equivalent to MTT solution in volume was added, after which the absorbance at 570 nm was measured. Shrunken cells with nuclear condensation were observed as damaged cells by the focal fluorescence of Hoechst 33342 incorporated into the nuclei. At several time points after a 15-min exposure to glutamate, cells were fixed with 4% paraformaldehyde for 20 min and then treated to 10 mg/mL Hoechst 33342 for 20 min at room temperature. The number of damaged cells was counted under a fluorescence microscope.

2.5. Semi-quantitative RT-PCR and real-time PCR

Total RNA was isolated from the cortical neurons with Trizol by following the manufacturer's instructions (Invitrogen Co.). One microgram of total RNA was reverse transcribed to prepare cDNA using Oligo$(dT)_{15}$ primer in accordance with Ready-To-Go You-Prime First-Stranded Beads. An aliquot of cDNA was then amplified with a 0.4 μM concentration of each primer set for the genes of mouse RNase L, RNase H1, and β-actin in 25 μL of reaction mixture containing 0.2 mM concentration of each dNTP, 0.625 units Taq DNA polymerase, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin. The nucleotide sequences of the primers used were the following for RNase L, 5′-ACCATGGAGACCCCGGATTAT-3′ (forward) and 5′-GTCCTTCGTTGTCTGTCGTCG-3′ (reverse), with denaturation at 94 °C for 1 min, annealing at 54.2 °C for 1 min, and elongation at 72 °C for 1 min (Chandrasekaran et al., 2004); RNase H1, 5′-CCATCATGCAAGCCAAGGCT-3′ (forward), and 5′-CTGAGTGAGCTCGTCCAGCT-3′ (reverse), with denaturation at 94 °C for 1 min, annealing at 53.7 °C for 1 min, and elongation at 72 °C for 1 min (Cerritelli et al., 2003); and for β-actin, 5′-CCCAGAGCAAGAGAGGTATC-3′ (forward), 5′-AGAGCATAGCCCTCGTAGAT-3′ (reverse), denaturation at 94 °C for 1 min, annealing at 54.5 °C for 1 min, elongation at 72 °C for 1 min. Reactions were carried out for a total of 25 cycles with the use of a Thermal cycler. After the last cycle, a final extension step was done at 72 °C for 5 min; and then the PCR products were analyzed by 1% agarose gel electrophoresis.

For real-time PCR assay, an aliquot of cDNA was amplified by using a 0.5 μM concentration of each primer set for the genes of mouse RNase L and β-actin in 20 μl of SYBR Premix Ex Taq (Takara Bio Inc.). Reactions were carried out for 40 cycles of 94 °C for 10 s (denaturation), 54.2 °C for 30 s (annealing), and 72 °C for 20 s (elongation).

2.6. Cellular RNA degradation and RNase activity

To determine degraded RNA, we isolated total cellular RNA from the cortical neurons by using Trizol and then heated it at 65 °C for 15 min followed by quick chilling. An aliquot (2 μg) of total RNA was subjected to electrophoresis on a 1.0% agarose gel containing 6.6% formaldehyde.

RNase activity was determined as originally described by Salehzada et al. (1993) with several modifications. Briefly, cells were harvested with phosphate-buffered saline and then centrifuged at 1000 × *g* for 10 min to obtain cell lysates. An aliquot (1.5 μg) of the lysates was incubated at 37 °C for 30 min in Tris–acetate (pH 6.5) containing 1 mM of DTT and EDTA with total RNA (3 μg), which had been isolated from whole brain of naïve mice. RNA degradation was determined by electrophoresis of the incubated samples on a 1.0% agarose gel containing 6.6% formaldehyde. Densitometric analysis for quantification of degraded RNA in both analyses was carried out with the aid of Atto Densitograph software (Atto Co. Tokyo, Japan).

2.7. Immunocytochemical assessments

Cells were washed with 0.03% Tris-buffered saline (pH 7.5) containing 0.03% Tween 20 (TBST) and then fixed with 4% paraformaldehyde for 20 min. After having been subsequently blocked with 1% bovine serum albumin in TBST for 1 h at room temperature, they were reacted overnight at 4 °C with adequately diluted primary antibody against RNase L and MitoTraker Red CH-H_2XRos. After a wash with TBST, the cells were reacted with a fluorescein isothiocyanate conjugated donkey anti-goat IgG for 2 h at room temperature. Finally, the cells were incubated with Hoechst 33342 (5 μg/mL) for 20 min at room temperature and observed under a fluorescence microscope (U-LH100HG, Olympus, Osaka, Japan).

2.8. Data analyses

All data were expressed as the mean ± standard error (S.E.), and the statistical significance was determined by the two-tailed Student *t*-test and the one-way ANOVA with the Bonferroni/Dunnett *post hoc* test.

3. Results

3.1. Glutamate-induced neuronal damage in cortical neurons occurs via the activation of NMDA receptors

Primary cultures of cortical neurons from embryonic mice were exposed to glutamate (10 or 100 μM), NMDA (100 μM), kainic acid (100 μM), or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA, 100 μM) for 15 min. Exposure of the cultures to