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Research Paper

The MLN4924 inhibitor exerts a neuroprotective effect against oxidative stress injury via Nrf2 protein accumulation

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ABSTRACT

It was explored the cytoprotective and antioxidant effect of MLN4924, a specific inhibitor of Nedd8-activating enzyme (NAE), against hydrogen peroxide (H_2O_2)-induced damage in cerebellar granule neurons (CGNs). Primary cultures of CGNs were exposed to H_2O_2 after preincubation with MLN4924. The compounds were removed, and CGNs were incubated in culture medium for 24 h in order to determine cell viability by 3-[4,5-dimethylthiazol-2-yl)]-2,5-diphenyl-tetrazolium bromide (MTT) and fluorescein diacetate (FDA) assays. It was demonstrated that MLN4924 remarkably attenuated H_2O_2-induced cell damage. Meanwhile reactive oxygen species (ROS) production was evaluated with the fluorescent probe dihydroethidium (DHE). Interestingly H_2O_2-induced ROS production was inhibited by pretreatment with MLN4924. MLN4924 treatment in CGNs resulted in nuclear factor E2-related factor 2 (Nrf2) protein accumulation. Intriguingly this effect was observed in the cytosolic and nuclear compartments of the CGNs. The cytoprotective effect of MLN4924 was associated with its ability to diminish ROS production induced by H_2O_2 and the accumulation of Nrf2 protein levels in the cytoplasm and nucleus of the CGNs.

1. Introduction

The central nervous system is very vulnerable to oxidative stress, the imbalance between generation of reactive oxygen species (ROS) and antioxidant defenses results in functional and structural damage to neuronal cells; this fact has been associated to neurodegenerative diseases [12]. A key for the defense is the nuclear factor E2-related factor 2 (Nrf2) that enhances the expression of multiple cytoprotective proteins [19]. It has been shown that nuclear accumulation of Nrf2 is an essential event in the cytoprotection against oxidative stress, thereby in past decades, several natural and synthetic compounds and antioxidants (as curcumin, sulforaphane and nordihydroguayaretic acid) have been assessed as beneficial agents for modulating the Nrf2 activity as a therapeutic strategy versus oxidative stress and neurodegeneration [10,11,14,17].

Nrf2 stability is regulated by the ubiquitin-proteasome system, specifically by cullin 3 (Cul3) that has an important role in Nrf2 ubiquitination [4]. Recently, cullin neddylation process has been emerged as a promising target to modulate the activity or degradation of specific proteins. Neddylation process is a posttranslational modification by Nedd8 protein and regulates the ubiquitin ligase activity of cullins [1,6]. MLN4924, whose chemical name is ((1S,2S,4R)-4-(4-(((S)-2,3-dihydro-1H-inden-1-yl)amino)-7H-pyrrolo [2,3-d]pyrimidin-7-yl)-2-hydroxycyclopentyl)methyl sulfamate, is a first-in-class selective inhibitor of Nedd8-activating enzyme (NAE), the first specific enzyme component of the Nedd8 conjugation pathway that prevents the subsequent neddylation of cullins and promotes the cullin direct substrates accumulation, including the transcription factor Nrf2 [29].

Taking into account the recent evidences, it was decided to study if the use of the MLN4924 neddylation pathway inhibitor could induce Nrf2 accumulation and prevent the hydrogen peroxide (H_2O_2) induced neurotoxicity in primary cultures of cerebellar granule neurons (CGNs).

Abbreviations: CGNs, Cerebellar granule neurons; Cul3, Cullin 3; DHE, Dihydroethidium; DIV, Days *in vitro*; FBS, Fetal bovine serum; FDA, Fluorescein diacetate; H_2O_2, Hydrogen deroxide; MLN4924, Selective inhibitor of NAE; MTT, 3-[4,5-dimethylthiazol-2-yl)]-2,5-diphenyl-tetrazolium bromide; NAE, Nedd8-activating enzyme; Nedd8, Neural precursor cell expressed developmentally down-regulated protein 8; Nrf2, Nuclear factor E2-related factor 2; PCNA, Proliferating cell nuclear antigen; ROS, Reactive oxygen species

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2. Materials and methods

2.1. Chemicals

The following reagents were obtained from Sigma Aldrich Co. (St. Louis, MO, USA): trypsin, deoxyribonuclease I, cytosine arabinose, L-glutamine, Basal Medium Eagle, poly-L-lysine, fluorescein diacetate (FDA), Tris–HCl, Triton X-100, Nonidet P-40, sodium dodecyl sulfate (SDS), dimethylsulfoxide (DMSO), 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES), bromophenol blue, glycerol, dithiothreitol (DTT), sodium deoxycholate, imidazole, 3-(4,5-dimetiltiazol-2)-2,5-diphenyl tetrazolium bromide (MTT), protease inhibitor cocktail containing 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) at 104 mM, aprotinin at 80 μM, bestatin at 4 mM, L-trans-epoxysuccinyl-leucyl-amido(4-guanidino)butane (E-64) at 1.4 mM, leupeptin at 2 mM and pepstatin A at 1.5 mM (catalog P8340) and anti-tubulin antibodies. Sodium chloride, H_2O_2 and ethylenediaminetetraacetic acid (EDTA) were obtained from JT Baker (Xalostoc, Edo. Méx., México). Dulbecco's Modified Eagle Medium (DMEM), dihydroethidium (DHE), 0.4% trypan blue, trypsin inhibitor, penicillin-streptomycin and fetal bovine serum (FBS) were obtained from ThermoFisher Scientific (Walthman, MA, USA). Protein G agarose, Fast Flow was from Merck Millipore (Billerica, MA, USA). MLN4924 was obtained Active Biochem (Maplewood, NJ, USA). The anti-Nrf2 and anti-proliferating cell nuclear antigen (PCNA) antibodies (rabbit polyclonal) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). The anti-actin antibodies were from Chemicon International (Billerica, MA, USA). All other reagents were of analytical grade and commercially available.

2.2. Cell culture

CGNs have been widely used as an in vitro model to investigate and characterize mechanisms of neuronal death or survival, as well as mechanisms of neurodegeneration and neuroprotection [2]. For these reasons, primary cell cultures greatly enriched (> 90%) in CGNs were used in this work; they were obtained from 7 to 8-day-old Wistar rat cerebellum as previously described [10]. Cytosine arabinose (10 mM) was added 24 h after plating and glucose (5 mM) was added to the cultures at 4 days in vitro (DIV). With the use of this protocol, more than 95% of the cultured cells were CGNs [21].

2.3. Culture treatments

Neurons were used after 9 DIV. First, the effect of the addition of H_2O_2 and MLN4924 on neuron viability was studied. Cell cultures were exposed to several concentrations of H_2O_2 (0–50 μM) in Ringer buffer (134 mM NaCl, 2.2 mM $CaCl_2$, 56.4 mM KCl, 108 mM $NaHCO_3$, 0.43 mM KH_2PO_4, 10 mM glucose and 109 mM HEPES, pH 7.4) for 60 min. After this time, H_2O_2 was removed and CGNs were incubated in culture medium for 24 h to determine cell viability. Similarly, cell cultures were exposed to several concentrations of MLN4924 (0–650 nM) in Ringer buffer for 24 h; after this time, MLN4924 was removed and cell viability was determined. FBS was absent during the exposure to H_2O_2 and MLN4924.

In further experiments, the effect of the MLN4924 on H_2O_2-induced cell death was studied. MLN4924 (330 nM) was added for 1 h to the CGNs, then it was removed and H_2O_2 was added at indicated concentrations for 1 h (pretreatment groups). After this time, H_2O_2 was removed and CGNs were incubated in culture medium for 24 h to determine cell viability. In order to evaluate the effect of MLN4924 present during H_2O_2 treatment, the cell cultures were exposed to 330 nM MLN4924 for 1 h before and during the addition of increasing concentrations of H_2O_2 for 1 h. After this time, H_2O_2 and MLN4924 were removed and CGNs were incubated in culture medium for 24 h to determine cell viability.

The effect of the H_2O_2 and MLN4924 on ROS production and Nrf2 levels was also studied. To evaluate the effect of MLN4924 on H_2O_2-induced ROS production and Nrf2 levels, cell cultures were exposed to 330 nM MLN4924 for 1 h before and during the incubation of H_2O_2 (20 μM, 1 h). After this time, MLN4924 and H_2O_2 were removed and both ROS production and Nrf2 levels were determined.

2.4. Determination of cell viability

The number of viable cells (% of control) was estimated using the colorimetric MTT assay and FDA assay as previously described [10]. MTT is reduced to formazan by the activity of mitochondrial dehydrogenases; absorbance is directly proportional to viable cells. Cell viability was expressed as a percentage of MTT reduction or fluorescence emission. On the other hand, FDA is a cell permeable probe that is converted it to the fluorescent compound fluorescein by the esterases of viable cells. Cells were treated with 12 μM FDA for 5 min at 37 °C and the fluorescence was quantified in a Synergy HT MultiMode Microplate Reader (Biotek, Winooski, VA, USA) using the following wavelengths filters: excitation 485/20 nm and emission 528/20 nm. Viability of control cells (without treatment) was considered as 100% in both assays. The value of cells incubated with different treatments was compared with that obtained for control cells.

2.5. ROS assay

The determination of ROS was performed by using the fluorescent probe DHE as previously described [22]. DHE is oxidized to ethidium in the cytosol mainly by superoxide anion ($O_2^{\bullet -}$) and it is then retained within the nucleus which is stained with bright red fluorescence. After treatment, 20 μM DHE was loaded in DMEM without phenol red during 30 min at 37 °C. Cells were visualized under epifluorescence microscope using the fluorescent cube G-2A (excitation 510–560 nm, emission of 590 nm) from Nikon Corporation (Tokyo, Japan) for the ethidium detection. The intensity of ethidium was measured in five different fields per well per condition in three independent experiments using the NIS Elements software V2.3 for Image acquisition and V3.0 for Image Analysis (Nikon, Tokyo, Japan).

2.6. Cell fractionation

Cellular fractionation was modified from a previously described method [13]. Briefly, CGNs were washed with cold phosphate buffered saline and then resuspended in homogenization buffer (3 mM imidazole pH 7.4, 250 mM sucrose, plus a cocktail of protease inhibitors) by 20 passages through a 22-gauge needle. The homogenate was centrifuged at 840*g* for 15 min at 4 °C; the supernatant contained the cytosolic fraction, and the pellet contained the nuclei. Nuclei were lysed in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet and 0.1% SDS) for 2 h min at 4 °C and later centrifuged at 12,662*g* for 5 min. Protein extracts for each fraction were used for immunoprecipitation (IP) assays with Nrf2 specific antibodies and then analyzed by Western blot (WB). PCNA was used as control for the nuclear fraction and tubulin and actin were used as controls for the cytoplasmic fraction.