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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.

2.7. IP and WB

The whole cell lysate (~0.25–5 mg of protein) was used for IP with anti-Nrf2 antibodies at 4 °C for overnight at continuous agitation. Afterwards mix of antibody-protein extracts was incubated with 70 μL of G-protein coupled to agarose at 4 °C for 2 h at continuous agitation. Samples were centrifuged in a Nanofuge and pellets were washed three times with cold TNTE 0.1% (50 mM Tris–HCl buffer pH 7.4, 150 mM NaCl, 5 mM EDTA and 0.1% Triton X-100). Pellets were resuspended in Laemmli buffer (100 mM Tris–HCl pH 6.8, 20% glycerol, 2% SDS, 0.05% bromophenol blue and 100 mM DTT) and boiled for 3 min. Samples were stored at −20 °C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and assayed by WB using specific antibodies. Data shown are representative from 3 or more independent experiments. For protein analysis, nuclear and cytosolic fractions were used for IP with specific antibodies. Proteins were separated by SDS-PAGE and assayed by WB using specific antibodies as previously described [13]. Densitometry was carried out with image processing and analysis program ImageJ 1.48 and the data were expressed as relative units.

2.8. Statistics

Data were expressed as mean ± SD. They were analyzed with the software Prism 5 (GraphPad, San Diego, CA, USA) by one-way analysis of variance (ANOVA) followed by multiple comparisons according to Dunnett or Bonferroni, as appropriate. $p < 0.05$ was considered as significant.

3. Results

3.1. Cell damage induced by H_2O_2 on CGNs

CGNs at 9 DIV were treated with different concentrations of H_2O_2 (control group, 5, 10, 12.5, 15, 20, 25, 50 μM) for 1 h. Fig. 1 shows a typical field of CGNs culture at 9 DIV and also the morphological changes induced to the cell bodies and neurite outgrowth after H_2O_2 treatment with a contracted appearance when compared to untreated controls. It was identified cell body degeneration starting from 15 μM H_2O_2 that becomes more evident at 50 μM H_2O_2. This observation correlates the data obtained with the MTT assay (Fig. 2a). It was observed that cell viability of CGNs exposed to H_2O_2 for 1 h was reduced significantly in a concentration dependent manner (Fig. 2a) starting from 12.5 μM H_2O_2. The MTT reduction rate decreased to 95.70%, 82.88%, 80.08%, 77.66%, 66.11%, 44.62% and 25.30% with 5 μM, 10 μM, 12.5 μM, 15 μM, 20 μM, 25 μM and 50 μM of H_2O_2, respectively.

3.2. MLN4924 does not affect CGNs viability but it prevents H_2O_2-induced cell viability reduction

MTT assays were performed in order to determine the effect of MLN4924 on cell viability. It was observed that CGNs viability was unchanged when the neurons were incubated by 24 h with different concentrations of MLN4924 (Fig. 2b). Then, it was examined whether the inhibitor could prevent the H_2O_2-induced decrease on cell viability. To study the cytoprotective effects of MLN4924, we analyzed cell viability by MTT and FDA methods in CGNs pretreated with MLN4924 and stimulated with H_2O_2. Consistent with our last experiments, pretreatment for 1 h with 330 nM MLN4924 attenuated significantly cell damage induced by H_2O_2 above 15 μM concentration (Fig. 2c and d). Altogether, these results evidence that MLN4924 exerts a cytoprotective effect against oxidative damage in CGNs.

3.3. MLN4924 ameliorates H_2O_2-induced ROS production increase in CGNs

To additionally characterize the cytoprotective effect of MLN4924, the ability of the compound to modulate ROS production induced by H_2O_2 exposition in CGNs was examined. First, it was evaluated the effects on ROS production of H_2O_2 and

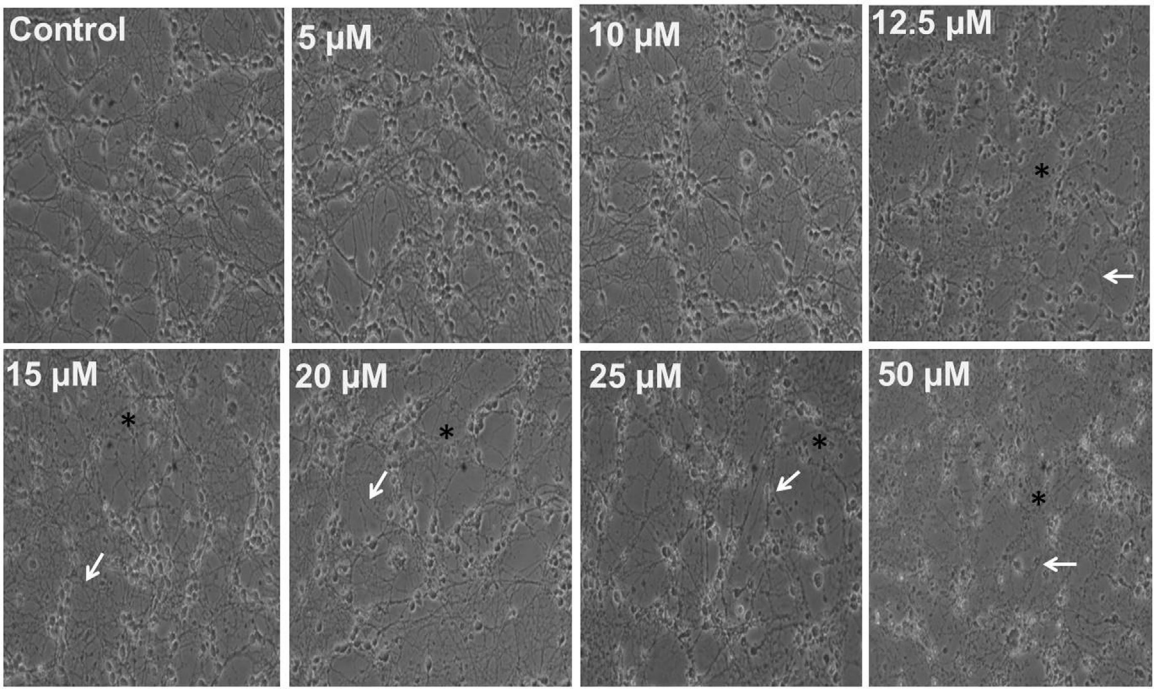

Fig. 1. Cell damage induced by hydrogen peroxide (H_2O_2) on cerebellar granule neurons (CGNs). Representative images of the phase contrast micrographs showing the effect of different concentrations of H_2O_2 (5–50 μM) for 1 h on CGNs morphology. Images were obtained at 24 h of recovery (40X). Soma reduction (*) and decrease in neurite length (↑) are indicated.

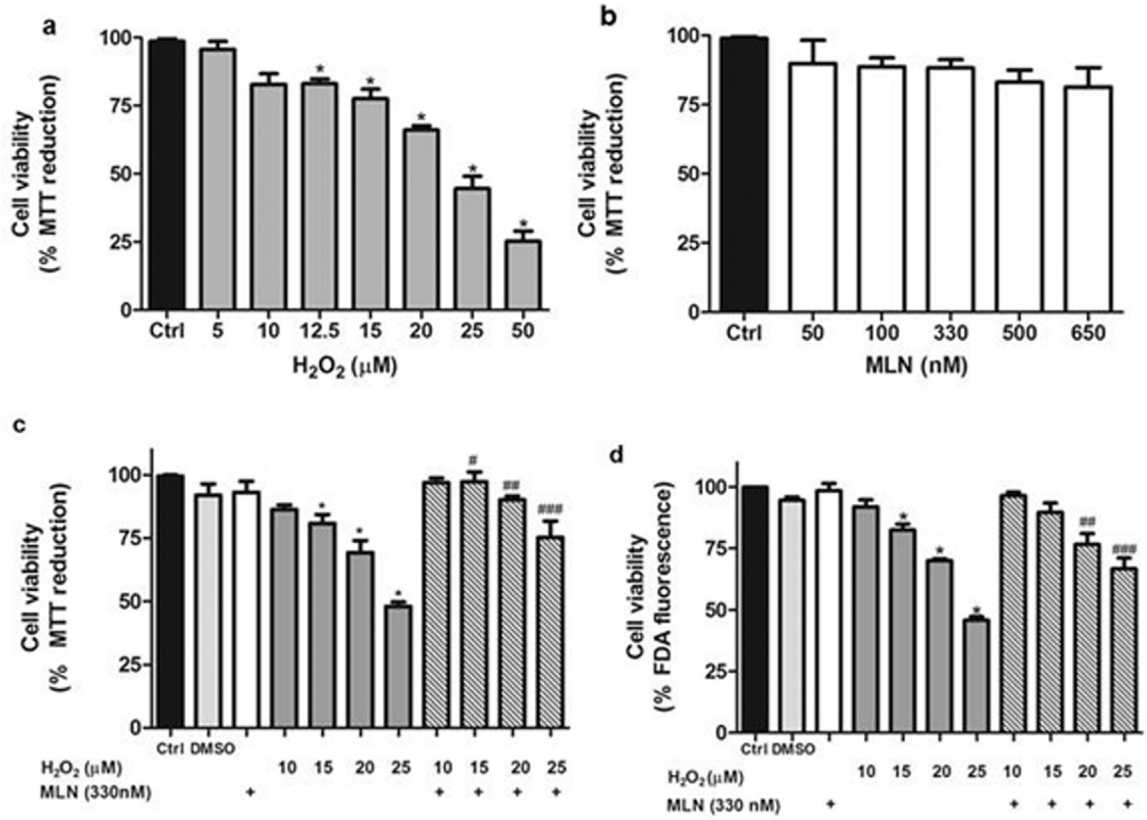

Fig. 2. MLN4924 does not affect cerebellar granule neurons (CGNs) viability but it prevents hydrogen peroxide (H_2O_2)-induced cell viability reduction. (a) Effect of H_2O_2 (1 h exposure) and (b) MLN4924 (MLN, 24 h) and both stimuli (c and d) on CGNs viability. Cell viability was determined 24 h after H_2O_2 addition. CGNs were incubated with different concentrations of H_2O_2 for 1 h after the incubation with MLN (for 1 h); cell viability was determined 24 h after by MTT (c) and FDA (d) assays. DMSO = dimethylsulfoxide. Data are mean ± SD. $^{*}p < 0.05$ vs control (Ctrl), $^{\#}p < 0.05$ vs. H_2O_2 (15 μM), $^{\#\#}p < 0.05$ vs. H_2O_2 (20 μM), $^{\#\#\#}p < 0.05$ vs. H_2O_2 (25 μM), n = 3.

MLN4924 in independent experiments. The exposure by 1 h to 15, 20 and 25 μM H_2O_2 led to a significant increase on ROS production (Fig. 3a). The highest ROS production was reached with 25 μM H_2O_2. In contrast, when CGNs were incubated by 1, 2, 4 and 6 h with 330 nM MLN4924, it was found that the inhibitor modulated ROS production in a biphasic way. First, the ROS levels were increased between 2 and 4 h and they returned to the basal levels at 6 h of stimulation. These results indicate that MLN4924 may modulate ROS production in CGNs (Fig. 3b). Finally, it was observed that MLN4924 pretreatment attenuated significantly H_2O_2-induced ROS production in CGNs (Fig. 3c). Taken together, these results suggest that MLN4924 induces protective effects against oxidative stress, by modulating ROS production against cytotoxic H_2O_2 effects.

3.4. MLN4924 induces Nrf2 protein accumulation in CGNs and prevents downregulation induced by H_2O_2

To study the antioxidant potential of MLN4924, it was examined its ability to modulate Nrf2 protein stability, which plays a critical role in the response against oxidative stress. Cellular fractionation and protein immunoprecipitation assays were assessed in CGNs stimulated with MLN4924 330 nM for 2 h to analyze Nrf2 protein dynamic. Interestingly, it was observed basal levels of Nrf2 protein in the nucleus, but not in cytosolic fraction in absence of MLN4924 (Fig. 4a). However, in presence of MLN4924, Nrf2 protein was accumulated in both cytoplasm and nucleus (Fig. 4a).

To assess the role of Nrf2 in the cytoprotective effect of MLN4924, it was investigated if the inhibitor would modulate Nrf2 protein against H_2O_2 induced damage. H_2O_2 treatment substantially reduced the basal levels of Nrf2 protein in cytoplasm and nucleus of CGNs. It was observed that cells stimulated with H_2O_2, show a downregulation in the levels of Nrf2 protein in nucleus (Fig. 4b). However, when cells are pre-treated with the inhibitor MLN4924 and stimulated with H_2O_2, downregulation of Nrf2 protein was abolished (Fig. 4b). Collectively, these results suggest that MLN4924 suppresses H_2O_2-induced oxidative damage by blocking downregulation of Nrf2 protein.

4. Discussion

Oxidative stress is one of the main factors involved in neuronal loss and damage but also in neurodegenerative disorders. The toxicological consequences of H_2O_2 treatment in several reports include ROS production, neurite degeneration and cell death [8,9]. For that reason, H_2O_2 represents an important model for studying the neuropathology of oxidative stress and to investigate neuroprotective mechanisms. The present study shows that H_2O_2 stimulates ROS generation and cell damage associated with neuronal cell death.

Beyond their antiproliferative and proapoptotic properties, proteasome inhibitors could be also able to provide neuroprotection. Several groups have reported that proteasome inhibition induces an elevation of Nrf2 activity on different models [15,24,27,31]. However, toxicity events have been associated with the generalized inhibition of protein degradation, so that more specific inhibitors have been searched. MLN4924 is a selective inhibitor of NAE and has been widely used for its ability to regulate activity of cullins by inhibiting their neddylation. In this way, MLN4924 is capable of stabilizing a variety of proteasome target proteins but with much more specificity than classical proteasome inhibitors [28].

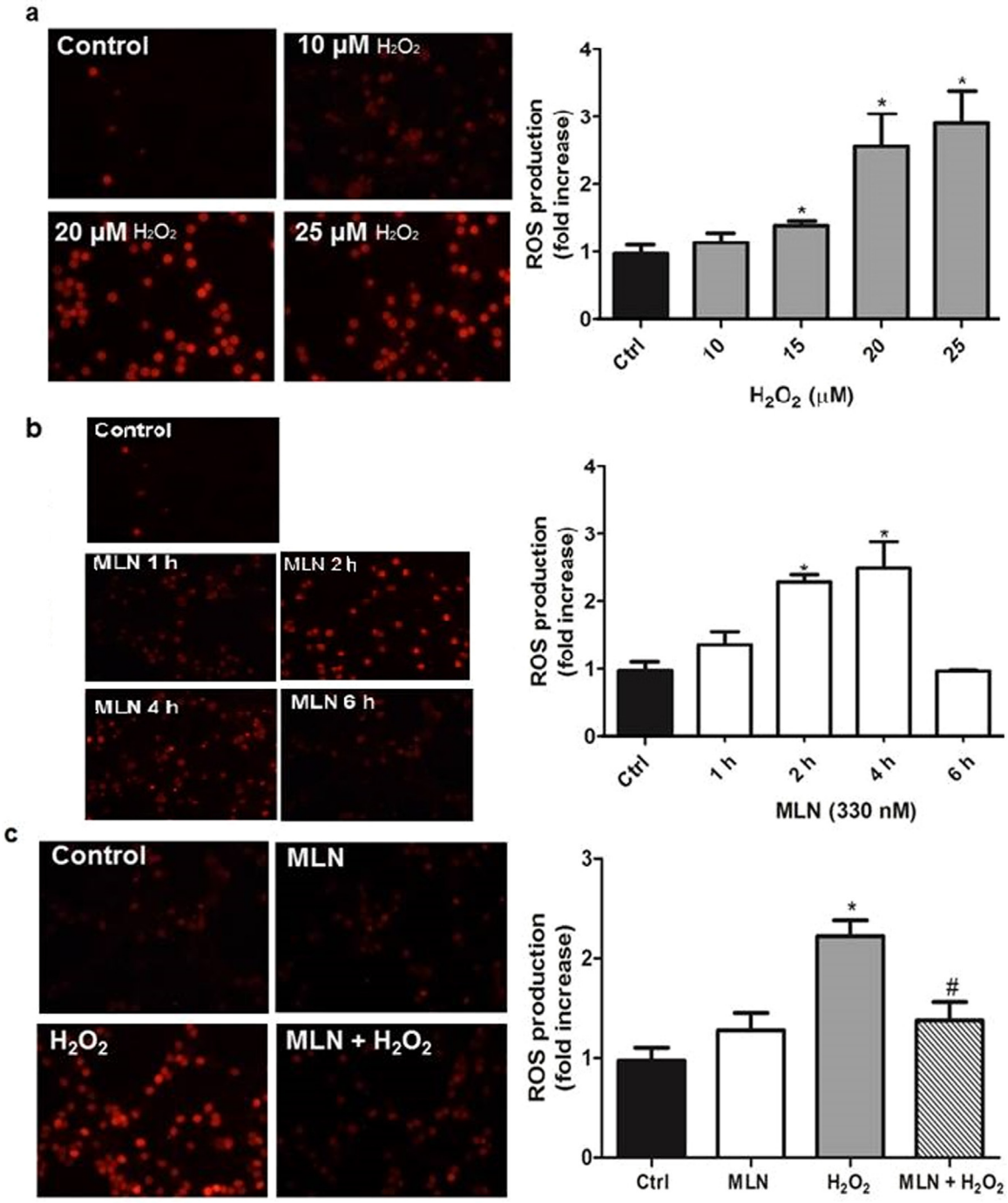

Fig. 3. MLN4924 ameliorates hydrogen peroxide (H_2O_2)-induced reactive oxygen species (ROS) production increase in CGNs. (a) Representative images and quantitative data of ROS induced by different concentrations of H_2O_2 (0–25 μM) for 1 h. (b) Representative images and quantitative data of ROS production induced by MLN4924 (MLN) 330 nM for 1 h, 4 h and 6 h. Data are mean ± SD, $^*p < 0.05$ vs control (Ctrl). (c) Representative images of ROS in cells treated with H_2O_2 (20 μM, 1 h), MLN (330 nM, 1 h) and MLN pretreatment + H_2O_2. H_2O_2 was added for 1 h after the incubation with MLN (330 nM for 1 h). Quantitative data were obtained at the end of each treatment. The intensity of ethidium fluorescence was measured using an image analyzer in 5 different fields per well per condition in three independent experiments. Data are mean ± SD. $^*p < 0.05$ vs control (Ctrl), $^\#p < 0.05$ vs H_2O_2.

Studies focused on neuroprotection have attempted to recognize drugs with antioxidant properties and therapeutic potential by its ability to induce the Kelch-like ECH*-associated protein 1 (Keap1)/Nrf2/antioxidant response element (ARE) pathway; nonetheless the effect of regulating the neddylation process in oxidative stress and its relationship with neurodegenerative diseases has not been yet explored. Here we show that MLN4924 increased Nrf2 protein stability and attenuated oxidative stress and cellular damage on CGNs. Therefore, our findings suggest that prevention of Nrf2 protein degradation by inhibiting neddylation has a therapeutic potential for the prevention of neuronal damage.

Clinical trials conducted with human cancer patients have concluded that MLN4924 is safe and highly effective as an anticancer therapy, but there are no many reports of MLN4924 effects on normal cellular context [7,26]. It was observed that MLN4924 has no effect on cell viability. Moreover, it was observed that pretreatment with nanomolar concentrations of MLN4924 prevented H_2O_2 induced cell death. The effect of MLN4924 in

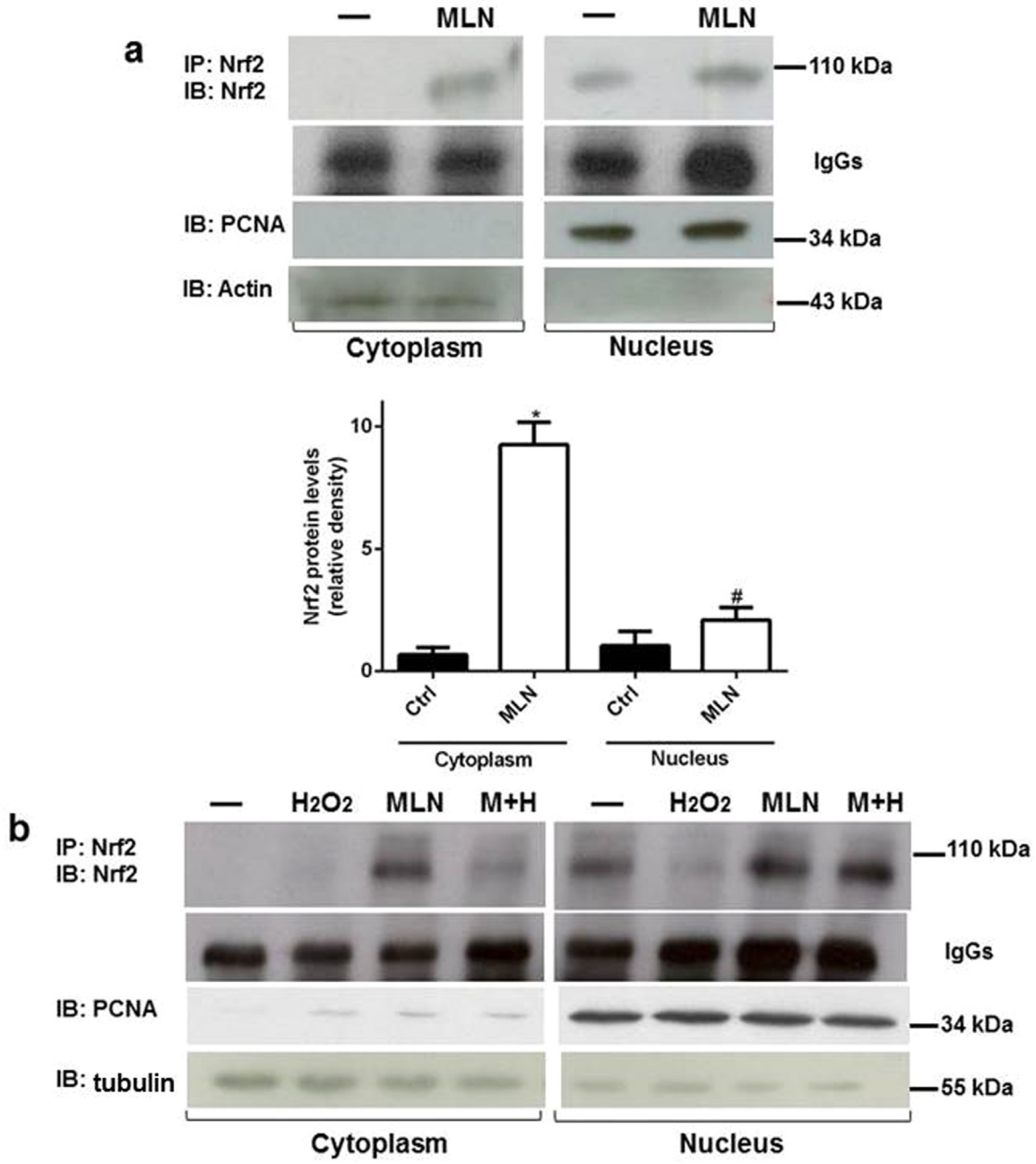

Fig. 4. MLN4924 induces Nrf2 protein accumulation in cerebellar granule cells (CGNs) and prevents downregulation induced by hydrogen peroxide (H_2O_2). Effect of MLN4924 treatment on nuclear Nrf2 protein by immunoblot (IB) after immunoprecipitation (IP) in CGNs. (a) Representative images of Nrf2 IB. Cells were treated with or without MLN4924 (MLN, 330 nM, 2 h). Densitometry was carried out with image processing and analysis program ImageJ 1.48 and data were expressed as relative units, i.e. the Nrf2 protein levels relative to the basal levels (-). Data are mean ± SD. $^{*}p < 0.05$ vs. cytoplasm control (Ctrl), $^{\#}p < 0.05$ vs nucleus Ctrl, *t*-student test, n = 5. (b) Representative image of effect of hydrogen peroxide (20 μM H_2O_2 for 1 h), MLN4924 (330 nM MLN for 2 h) and for the MLN (M) + H_2O_2 (H) group on Nrf2 levels. H_2O_2 was added for 1 h after the incubation with MLN (330 nM, for 1 h). PCNA = Proliferating cell nuclear antigen.

hippocampal neurons has been described, showing synaptic strength, surface glutamate receptor levels and dendritic spine width reductions, suggesting that Nedd8 is involved in the maintenance of excitatory synapses compromising neuron viability [25]. In contrast, during neurodegenerative disorders, Nedd8 accumulation has been observed in Lewy bodies in Parkinson's disease and neuronal and glial inclusions of Alzheimer's disease [5,16]. Therefore, it is plausible that Nedd8 mediates important mechanisms in order to maintain homeostasis against neuronal damage, but an upregulation in its expression promotes neurodegenerative disorders. This study also provides evidence for the beneficial effect of pharmacological inhibition of Nedd8 by promoting Nrf2 accumulation and by preventing oxidative cell damage.

One of the main mechanisms of action of MLN4924 in cancer cells is the induction of ROS-dependent apoptosis, however it remains unknown the effect of MLN4924 over the ROS production in a normal cell context [18,30]. It was found that MLN4924 induces transiently ROS production in a time-dependent manner suggesting that MLN4924 could generate changes in the cellular redox homeostasis without affecting cell viability.

Moreover, concentrations and exposition times used in this work differ from the utilized in antitumoral therapy. The effect of the MLN4924 would be similar as that for several proteasome

inhibitors, where concentrations in the micromolar range become toxic for cells, but not at lower concentrations [15,24]. This effect may reside mainly in the modulation of protein degradation and accumulation. Since neddylation could also be involved in accumulation of proteasome target proteins, it was assessed the effects of MLN4924 on Nrf2 protein and it was found that this inhibitor induces Nrf2 accumulation in both cytoplasm and nucleus. According to Nguyen et al. [20], it was found that Nrf2 protein is located mainly in the nucleus of cells incubated with H_2O_2. Nrf2 protein degradation mechanisms may be mediated by Keap1 dependent and independent process. In the latter mechanism is involved the cullin 1/beta-transducin repeats-containing proteins (βTRCP). In this context, it has also been demonstrated that H_2O_2 can induce the activity of glycogen synthase kinase-3 beta (GSK-3β) and thereby promotes the degradation of Nrf2 pathway cullin 1/βTRCP [3,23]. Therefore Nrf2 protein downregulation may result from this pathway, suggesting that this effect is associated with increased ROS levels and cell death. However it was observed an accumulation of Nrf2 protein in nucleus and cytoplasm with MLN4924 even in presence of H_2O_2. These findings suggest that Nrf2 accumulation induced by MLN4924 could be involved on its ability to diminish the H_2O_2 induced ROS production cell death. In fact, Nrf2 accumulation may be followed by the enhanced expression of cytoprotective proteins against H_2O_2. It is possible that Nrf2 accumulation may be consequence of the inhibition of cullin neddylation that decreases the cullin activity involved in the Nrf2 degradation. Because neddylation is an important process for cullin activity, further studies to evaluate the neddylation status of cullins in neuronal cells treated with MLN4924 must be conducted to verify that the cytoprotective effect of MLN4924 observed in CGNs is associated with neddylation inhibition.

5. Conclusion

MLN4924 inhibitor prevents oxidative stress and neuronal damage by accumulation of Nrf2 protein. Further research will be crucial in determining how this inhibitor mediates neuronal protection and its use with a clinical approach in neurodegenerative diseases.

Compliance with ethical standards

Conflicts of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in this study were in accordance with the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) at the Faculty of Chemistry, UNAM (FQ/CICUAL/095/15).

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