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Short Communication

Protective effects of sodium para-amino salicylate on manganese-induced neuronal death: the involvement of reactive oxygen species

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Abstract

Objectives This study tested whether sodium para-amino salicylic dihydrate, an antibacterial drug for tuberculosis, could block manganese-induced apoptosis in SK-N-MC neurons.

Methods Cell viability, Hoechst staining, dichlorofluorescin diacetate analysis for reactive oxygen species measurement, and immunoblotting were performed.

Key findings *In vitro*, manganese chloride significantly decreased the viability of SK-N-MC cells, accompanied by apoptotic features such as changes in nuclear morphology. Sodium para-amino salicylic dihydrate inhibited these apoptotic characteristics through reducing intracellular reactive oxygen species generation, protecting mitochondrial membrane potential and caspase-3 activation.

Conclusions Sodium para-amino salicylic dihydrate inhibits manganese-induced apoptosis in neurons and may reduce manganese-mediated neurodegeneration.

Keywords apoptosis; manganese; manganism; PAS-Na; ROS

Introduction

The neurometabolism of heavy metals has received very little attention from neuroscientists until the last decade, but there is a direct relationship between heavy metal neurometabolism and neurodegenerative diseases. Environmental heavy metals, including manganese (Mn), may cause neuronal degeneration.[1–3] Mn is an abundant, naturally occurring element in the Earth's crust, most frequently found in the form of oxides, carbonates and silicates.[4] Mn exposure represents a significant public health concern because of its use as a catalyst in countless industrial processes and its presence in gasoline additives and in fungicides.[5,6] Excessive exposure to Mn is an important occupational hazard, and inhalation of particulate Mn compounds is associated with the deposition of Mn within the striatum and cerebellum.[7] Occupational exposure to Mn for periods from 6 months to 2 years can cause an extrapyramidal syndrome, referred to as manganism, closely resembling Parkinson's disease (PD) at both the molecular and clinical levels.[8–9] PD risk is also increased with exposure to industrial emissions of Mn and Mn-containing compounds such as fuel additives.[10] Therefore, exposure to ambient Mn may accelerate the incidence of PD.[10] Mn in the brain is normally deposited in degenerative lesions of the globus pallidus, subthalamic nucleus, caudate nucleus and putamen,[11] but is sometimes deposited in lesions of the substantia nigra, the primary degenerative structure in PD.[12]

Non-steroidal anti-inflammatory drugs, including analogues of salicylates, may have neuroprotective benefits, because inflammatory processes play a role in the pathogenesis of neurodegenerative diseases.[13,14] Interestingly, sodium para-amino salicylic dihydrate (PAS-Na) improved Mn-induced PD in several cases.[15,16] However, the mechanism

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underlying this effect is unclear. We therefore examined the effects of PAS-Na on Mn-damaged neurons to elucidate its mode of action.

Materials and Methods

Materials

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum, trypsin and other tissue culture reagents were purchased from Life Technologies, Inc (Gaithersburg, MD, USA). $MnCl_2$ and PAS-Na were purchased from Sigma (St Louis, MO, USA). 3,3′-Dihexyloxacarbo-cyanine iodide ($DiOC_6(3)$) and 2,7-dichlorofluorescein diacetate were from Molecular Probes (Eugene, OR, USA). All other chemicals were purchased from either Sigma or Aldrich (St Louis, MO, USA) and stored according to the manufacturer's instructions. Plasticware was obtained from Falcon Inc. (Franklin, NJ, USA).

Cell culture and viability

SK-N-MC human neuroblastoma cells were obtained from the American Type Culture Collections (Manassas, VA, USA). The SK-N-MC neuroblastoma cell line was maintained in DMEM containing 10% (v/v) heat-inactivated fetal bovine serum, penicillin G (10 000 U/ml), streptomycin (10 mg/ml), and L-glutamine (200 mM). The cultures were maintained in a humidified incubator (5% CO_2, 95% air at 37°C) and half of the medium was changed three times a week. Cells were used in experiments at 80–90% confluency. Cells were assessed by the trypan blue exclusion process for dead cells. Cell viability was calculated by dividing the non-stained (viable) cell count by the total cell count.

Fluorescent staining of nuclei

Cells were treated with $MnCl_2$ in the presence or absence of PAS-Na. Briefly, cells were fixed with 3.7% paraformaldehyde for 10 min at room temperature, rinsed twice for 5 min with phosphate-buffered saline (PBS) and incubated with 10 μM Hoechst 33258 in PBS at room temperature for 30 min. After three washes in PBS, the cells were observed under a fluorescence microscope (MPS 60; Leica, Wetzlar, Germany).

Determination of caspase-3 activity

SK-N-MC cells (3×10^6) were washed with PBS and incubated for 30 min on ice with 100 ml of a lysis buffer (10 mM Tris-HCl, 10 mM $NaH_2PO_4/NaHPO_4$, pH 7.5, 130 mM NaCl, 1% Triton1 X-100 and 10 mM sodium pyrophosphate). Cell lysates were spun, supernatants were collected, and the protein concentrations were determined using the bicinchoninic acid method. For each reaction, 30 μg of the protein was added to 1 ml of a freshly prepared protease assay buffer (20 mM HEPES pH 7.5, 10% glycerol, 2 mM dithiothreitol) containing 20 mM of AC-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) (BD Biosciences Pharmingen, San Diego, USA). The reaction mixtures in the absence of cellular extracts were used as the negative controls (fluorescence background). The reaction mixtures were incubated for 1 h at 37°C, and the level of aminomethyl-coumarin liberated from AC-DEVD-AMC was recorded using a spectrofluorometer (Hitachi F-2500, Tokyo, Japan) at an excitation wavelength of 380 nm and an emission wavelength range of 400–550 nm. The data was recorded as the integral of the relative fluorescence intensity minus the background fluorescence.

Cytofluorometric assessment of mitochondrial membrane potential

A stock solution of $DiOC_6$ (4 mmol/l) was prepared in ethanol and stored in small lots at 20°C; working solution (dilution 1 : 2000 for $DiOC_6(3)$) was produced in medium (DMEM) immediately before use. A total of 5×10^5 cells were incubated in DMEM that contained 100 nM $DiOC_6(3)$ at 37°C, and analysed using a PAS cytofluorometer (Partec) equipped with Partec software (Becton Dickinson, Trenton, USA). Forward and side scatters were gated for the major population of normal-sized cells and a minimum of 10 000 cells was analysed. The fluorescent probe $DiOC_6$ was excited using a 488 nm argon laser and emissions were collected through a FL1 detector fitted with a 525 ± 5 nm band pass filter.

Dichlorofluorescein diacetate assay

Intracellular reactive oxygen species (ROS) levels were measured as described elsewhere.[11] The cells were incubated with 1 mM $MnCl_2$ for 2.5 h and then treated with PAS-Na. Separate samples were incubated with $MnCl_2$ for 24 h and then treated with 100 μM 2,7-dichlorofluorescein diacetate (Molecular Probes) at 37°C for an additional 30 min. After chilling with ice, cells were washed with cold PBS, removed by scraping, and then resuspended at 1×10^6 cells/ml in PBS containing 10 mM EDTA. The fluorescence intensity of dichlorofluorescein diacetate formed by the reaction between dichlorofluorescein diacetate and the intracellular ROS of >10 000 viable cells in each sample was analysed by PAS Vantage flow cytometry (Partec), at 488 nm excitation and 525 nm emission wavelengths, respectively. Data were collected and analysed using Partec software. The experiments were repeated at least three times, with similar results. Histogram data are representative of the three independent experiments.

Statistical analysis

The data were analysed by analysis of variance in the dose–response experiments as well as by two-tailed Student's *t*-tests. $P < 0.05$ was considered significant.

Results

PAS-Na suppresses Mn-induced cell death

To determine if PAS-Na modifies Mn-induced responses in SK-N-MC cells, cells were exposed to 1000 μM $MnCl_2$ followed by PAS-Na for 24 h. $MnCl_2$ (1000 μM) alone reduced cell viability to about 54% of controls (93% of cell viability) (Table 1), but higher levels (e.g. 10 mM) induced almost complete cell death, which is not appropriate for examining the protective effects of PAS-Na. We therefore used 1 mM $MnCl_2$ for all experiments. PAS-Na (0.1 to 10 μM) significantly inhibited $MnCl_2$-induced cell death by

Table 1 Inhibitory effect of sodium para-amino salicylic dihydrate on SK-N-MC cell death caused by manganese chloride

Group	Cell viability (%)
Control	93.1 ± 1.5
$MnCl_2$ 1 mM	54.2 ± 4.2*
$MnCl_2$ 1 mM + PAS-Na 0.1 μM	67.2 ± 2.5**
$MnCl_2$ 1 mM + PAS-Na 1 μM	71.3 ± 1.9**
$MnCl_2$ 1 mM + PAS-Na 10 μM	67.9 ± 3.2**
$MnCl_2$ 1 mM + PAS-Na 100 μM	57.8 ± 3.8
$MnCl_2$ 1 mM + PAS-Na 1000 μM	42.5 ± 5.2

Cells were pretreated with 1 mM manganese chloride ($MnCl_2$) for 2.5 h and then with sodium para-amino salicylic dihydrate (PAS-Na) for 24 h. Cell viability was assessed by the trypan blue assay. *$P < 0.05$, significantly different compared with control; **$P < 0.05$, significantly different compared with $MnCl_2$.

about 12–16% compared with Mn alone, but 1000 μM PAS-Na was toxic (Table 1). PAS-Na (1 μM) also inhibited Mn-induced toxicity as shown by apoptotic parameters such as nuclear shrinkage (Figure 1). We added PAS-Na after $MnCl_2$ to mimic a therapeutic paradigm (e.g. restoring lost function) rather than a preventive paradigm. At 30 min, 1, 1.5, 2, 2.5 or 3 h after treatment with $MnCl_2$, PAS-Na was added to the cells to determine the appropriate PAS-Na treatment interval. Cell viability was similar among the various treatment intervals, and so in this study we treated all SK-N-MC cells with PAS-Na at 2.5 h after $MnCl_2$.

PAS-Na regulates Mn-induced intracellular ROS production

We tested whether PAS-Na could block ROS induction by 1000 μM $MnCl_2$. Pretreatment with $MnCl_2$ increased intracellular ROS levels to 168% (100% is baseline for controls) as measured by flow cytometry (Figure 2), and PAS-Na treatment (1 and 10 μM for 24 h) reduced this effect to 136 and 132%, respectively (Figure 2). PAS-Na treatment (1 and 10 μM) alone did not change ROS generation.

PAS-Na regulates Mn-induced mitochondrial membrane potential collapse and caspase-3 activation

To determine whether Mn-induced neurotoxicity contributes to mitochondrial dysfunction, we treated SK-N-MC cells with 1000 μM $MnCl_2$ and measured mitochondrial membrane potential ($\Delta\Psi_m$). $MnCl_2$ induced the collapse of $\Delta\Psi_m$, as illustrated by decreased mitochondrial $DiOC_6$ fluorescence (Figure 3), but PAS-Na blocked this effect. $MnCl_2$ also increased the activity of caspase 3, an executor caspase, and PAS-Na blocked this effect (Figure 4). Consistent with the cell viability data (Table 1), higher concentrations of PAS-Na (e.g. 100 μM) did not regulate caspase-3 activation (data not shown).

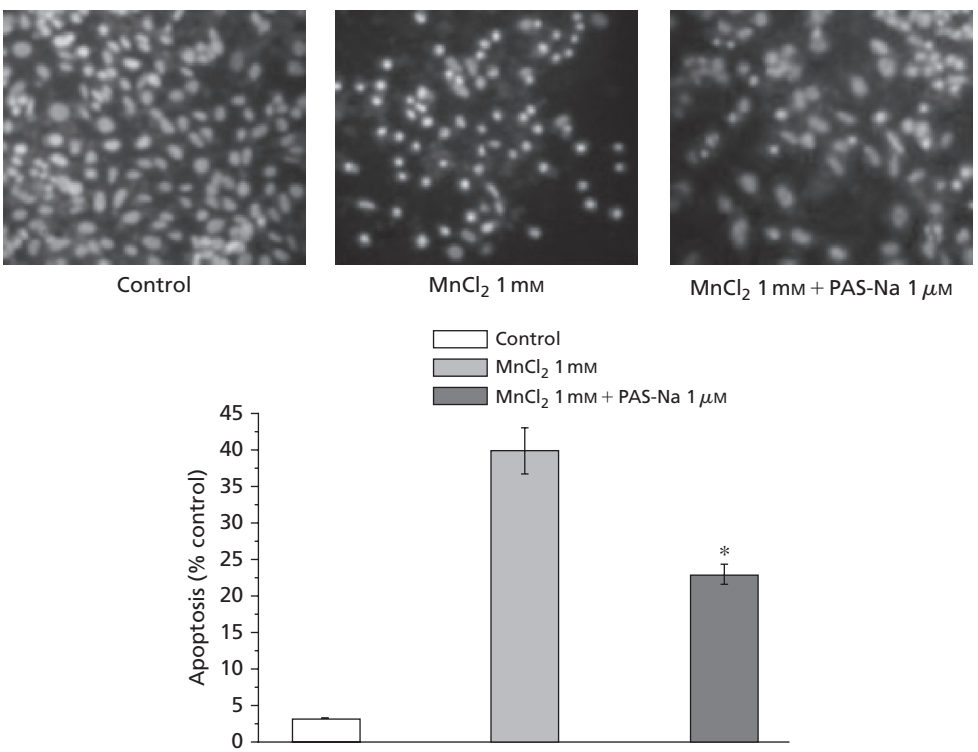

Figure 1 Sodium para-amino salicylic dihydrate inhibits manganese-induced cell death. Cells were pretreated with 1000 μM manganese chloride ($MnCl_2$). After 2.5 h, cells were treated with 1 μM sodium para-amino salicylic dihydrate (PAS-Na) and then incubated for 24 h. Cells were stained with Hoechst 33258 and observed under a fluorescence microscope. The shrunken nuclei were quantified. Data represent means ± SE ($n = 3$). *$P < 0.05$, significantly different compared with $MnCl_2$-treated cells.

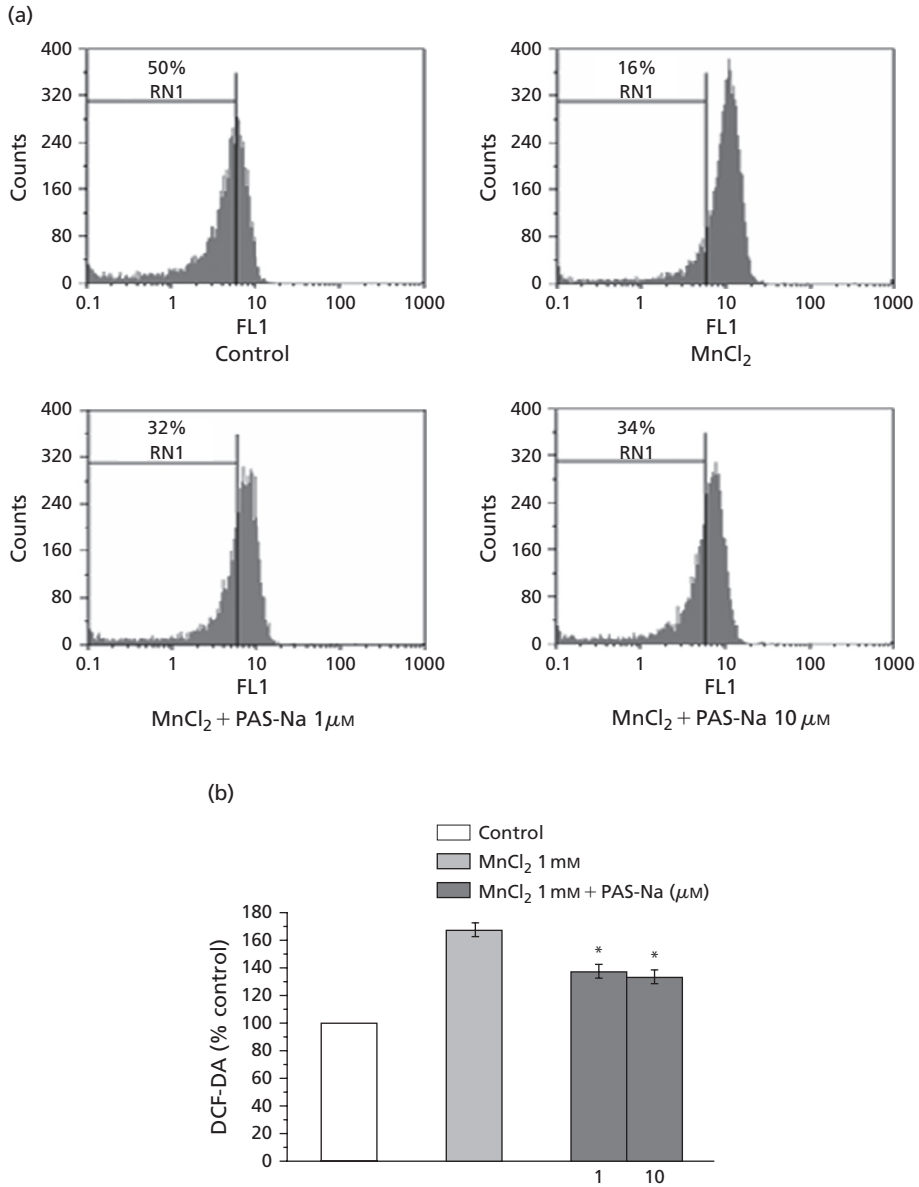

Figure 2 Sodium para-amino salicylic dihydrate inhibits manganese-induced intracellular reactive oxygen species production. (a) Cells were pretreated with 1000 μM manganese chloride ($MnCl_2$). After 2.5 h, cells were treated with 1 μM sodium para-amino salicylic dihydrate (PAS-Na) and then incubated for 24 h. The cells were then incubated with 100 μM 2′,7-dichlorofluorescein diacetate (DCF-DA) and the fluorescence intensity of lots of 10 000 cells were analysed by flow cytometry. (b) DCF-DA fluorescence was quantified. Data represent means ± SE (n = 3). *$P < 0.01$, significantly different compared with $MnCl_2$-induced reactive oxygen species production.

Discussion

We showed that PAS-Na can block the apoptotic effects of Mn in SK-N-MC neurons. Apoptotic pathways involve the mitochondria, death receptors and sub-organelle associated pathways.[17] Mitochondria play an important role via ROS, mitochondrial membrane potential collapse and caspase-3 activation.[18] Excess exposure to Mn, an essential micro-element, causes a Parkinson-like syndrome[19] and motor neuron disease.[20] Hundreds of cases of Mn exposure occur all over the world, mostly from airborne sources of occupational origin (e.g. groups of exposed miners, railway, construction, steel-smelting and other workers).[21,22] Mn-induced neurological manifestations indicate a significant relationship with the regulation of neurotransmitters, including glutamate and GABA.[23] The sensitivity of different behaviour and the integration of different (motor, sensory, motivational) functions make behavioural tests an important tool in the functional toxicology of Mn. *In vitro*, one of the earliest cellular responses after Mn exposure is ROS generation.[24] Oxidative stress mediates Mn-induced apoptosis in several in-vitro models, such as PC-12 cells,[24,25] and *in vivo* in Mn-induced neurotoxicity in the rat.[19] We showed that Mn induces apoptotic signals, including ROS, in SK-N-MC

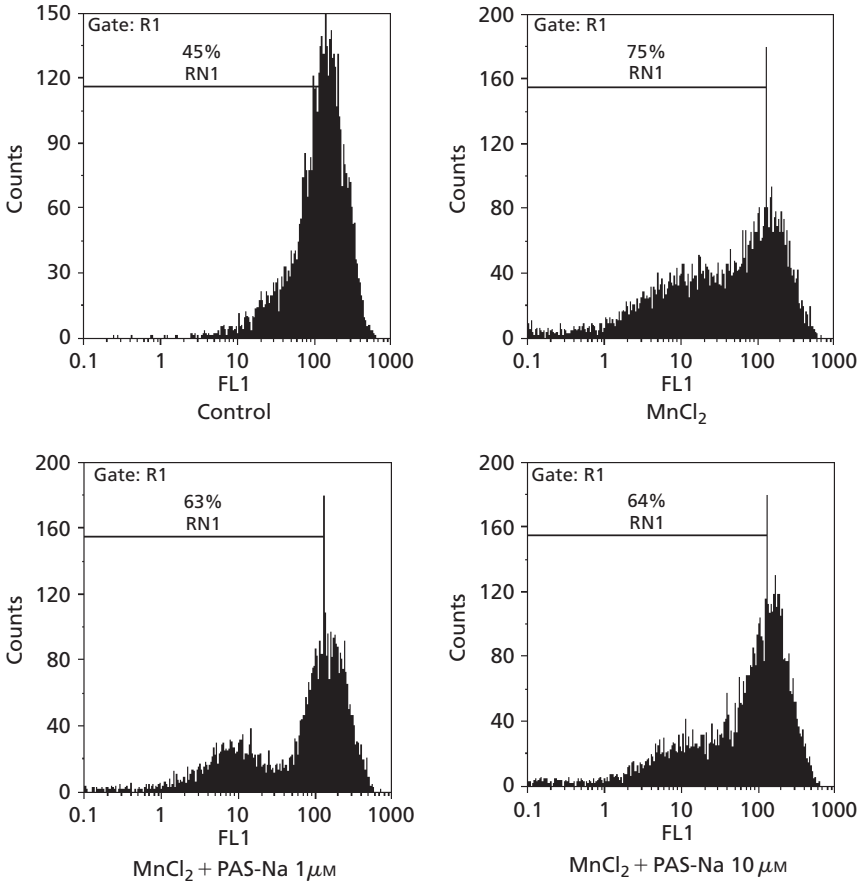

Figure 3 Sodium para-amino salicylic dihydrate regulates the manganese-induced collapse of mitochondrial membrane potential. Cells were pretreated with 1000 μM manganese chloride ($MnCl_2$). After 2.5 h, cells were treated with 1 or 10 μM sodium para-amino salicylic dihydrate (PAS-Na) and then incubated for 24 h. Cells were then incubated with 100 nM $DiOC_6$. Fluorescence intensity of 10 000 cells was determined by flow cytometry.

neurons. ROS can be both beneficial and deleterious.[26] At low/moderate concentrations, ROS have important roles in cell signalling, such as signal transduction.[27] However, excessive amounts of ROS can cause harmful effects.[28] Accumulating oxidative damage can then affect the efficiency of mitochondria and further increase the rate of ROS production.[29] ROS can influence learning and memory and may lead to cognitive dysfunction.[30]

Excessive Mn can induce mitochondrial dysfunction[31] via ROS, which also influences ageing,[10,32–34] PD and Alzheimer's disease. We showed that PAS-Na can inhibit Mn-induced apoptosis. PAS-Na is a moiety of salicylic acid, a non-steroidal anti-inflammatory drug. Non-steroidal anti-inflammatory drugs may be neuroprotective through inhibiting cyclooxygenase enzymes and scavenging ROS and reactive nitrogen species activity. Anti-inflammatory compounds may suppress microglial proliferation, modulating cell cycle progression and apoptosis.[35,36] Drugs that inhibit inflammation and microglial activation could have therapeutic potential in PD and dementia.[31] Here, PAS-Na attenuated the release of ROS induced by Mn (Figure 2). PAS-Na alone has antioxidant properties through quenching of singlet molecular oxygen,[37,38] but here did not influence ROS generation alone.

Mn toxicity may target mitochondria. Mn can dissipate mitochondrial membrane potential ($\Delta\Psi_m$) and induce mitochondrial dysfunction.[31] Accumulated oxidative stress and mitochondrial energy failure cause the induction of the mitochondrial permeability transition Ca^{2+}-dependent process characterized by the opening of the permeability transition pore of the inner mitochondrial membrane. This process increases permeability to ions, protons and other solutes, which consequently dissipate the mitochondrial inner membrane potential ($\Delta\Psi_m$).[33,35] The collapse of the $\Delta\Psi_m$ results in the colloid osmotic swelling of the mitochondrial matrix, movement of metabolites across the inner membrane, defective oxidative phosphorylation, cessation of ATP synthesis and further generation of ROS.[39] Here, PAS-Na inhibited the collapse of the mitochondrial membrane potential caused by Mn (Figure 3). Activation of pro-apoptotic proteins leads to the formation of pores in the mitochondria and the collapse of the electrochemical gradient across the mitochondrial membrane, an important step in the apoptosis cascade that includes caspase-3.[15,35]

In summary, PAS-Na treatment of SK-N-MC neurons suppresses apoptosis and inhibits mitochondrial dysfunction caused by Mn through mitochondrial alteration. Our experiments also indicate that PAS-Na may have therapeutic

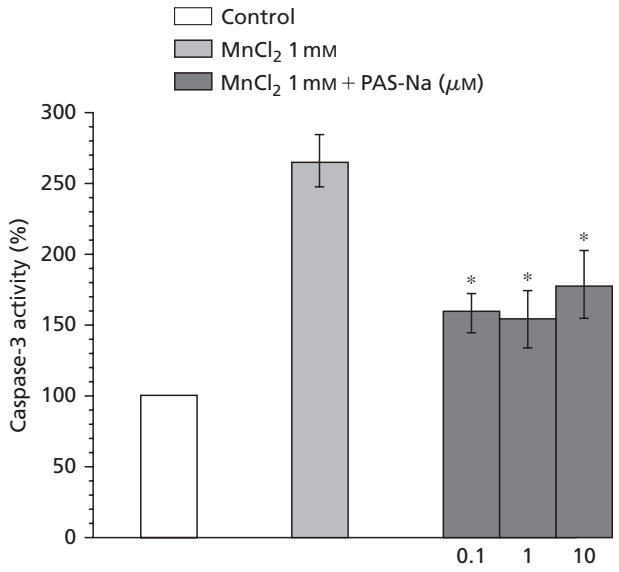

Figure 4 Sodium para-amino salicylic dihydrate inhibits manganese-induced caspase-3 activation. Cells were pretreated with 1000 μM manganese chloride ($MnCl_2$). After 2.5 h, cells were treated with 0.1, 1 or 10 μM sodium para-amino salicylic dihydrate (PAS-Na) and then incubated for 24 h. Caspase-3 activity was then measured. $^*P < 0.01$, significantly different compared with $MnCl_2$-stimulated caspase-3 activity.

potential in manganism. However, our results need to be confirmed in more sophisticated model systems *in vivo*.

Conclusions

PAS-Na inhibits Mn-induced apoptosis in neurons *in vitro* by regulating ROS production, the collapse of mitochondrial membrane potential and caspase-3 activation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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