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Comparison between 5-aminosalicylic acid (5-ASA) and para-aminosalicylic acid (4-PAS) as potential protectors against Mn-induced neurotoxicity

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Abstract

Manganese (Mn) is an essential metal for biological systems, however occupational or clinical exposure to high levels of Mn can produce a neurological disorder called manganism. Oxidative stress and neuroinflammation play major roles in the Mn-induced neurodegeneration leading to dysfunction of the basal ganglia. We investigated the toxic effects of $MnCl_2$in an immortalized rat brain endothelial cell line (RBE4) and the protective effects of the radical scavenging aminosalicylic acids, 5-aminosalicylic acid (5-ASA) and 4-aminosalicylic acid (4-PAS). Mn cytotoxicity was determined with 3-[4,5-dimethylth-iazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) activity. A significant decrease in MTT reduction concomitant with increased LDH release was noted in RBE4 cells exposed for 24 h to $MnCl_2$ (600 and 800 μM)($p<0.0001$). Our results establish that compared to 4-PAS, 5-ASA has greater efficacy in protecting RBE4 cells from Mn-induced neurotoxicity after pre-exposure to $MnCl_2$ 800 μM ($p<0.0001$).

Index Entries

manganese; neurotoxicity; neuroprotection; RBE4 cells; blood; brain barrier; endothelium

Introduction

Mn is an essential nutrient and it plays a fundamental role in cellular homeostasis. However, excessive Mn exposure causes neurotoxicity (Cotzias et al. 1968). Clinical signs, such as Parkinsonism, characterized by an extrapyramidal movement disorder, commonly appear after a primarily psychiatric presentation (*locura manganica*) (Mergler et al. 1999, Roels et al. 1985). Symptoms associated with manganism have been treated with limited efficacy with psychiatric and anti-parkinsonian drugs (Herrero Hernandez et al. 2006, Koller, Lyons and Truly 2004, Blanc 1990). In contrast to Parkinson's disease, Mn predominantly targets the globus pallidus (Herrero Hernandez et al. 2006). Chelation serves to alleviate the acute symptoms, but there is little evidence to support that such therapy has chronic consequences (e.g. Parkinsonism) (Rosenstock and Cullen 1994). Treatment of workers with occupational Mn-induced parkinsonism with ethylene diamine tetra-acetic acid (EDTA) and anti-parkinsonian drugs has led to improvement in clinical outcome (Herrero Hernandez et al. 2006).

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Herein, we utilized an established blood–brain barrier (BBB) model of immortalized rat brain endothelium 4 (RBE4) cells to evaluate Mn-induced cytotoxicity. The RBE4 cell line preserves features of the *in vivo* brain endothelium(Demeuse et al. 2002), expressing a number of BBB transporters (P-glycoprotein) and endothelial markers (VIII-related antigen) (Durieu-Trautmann et al. 1993). RBE4cells were derived from rat brain microvascular endothelial cells immortalized with the plasmid pE1A-neo, containing the E1A region of adenovirus 2 and a neomycin-resistance gene (Bressler et al. 2004, Roux et al. 1994).

Given the limited success in treating chronic states of Mn-induced disease with anti-parkinsonian drugs(Sadek, Rauch and Schulz 2003, Herrero Hernandez et al. 2006, Koller et al. 2004), an increasing and immediate need exists for efficacious therapy against Mn-induced neurological impairment. We evaluated the efficacy of two carboxylic acids**,** 4-aminosalicylic acid (4-PAS) and 5-aminosalicylic acid (5-ASA), analyzing two biochemical indicators of cell toxicity, lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), following RBE4 cells treatment with $MnCl_2$ for 24 hours (h).

Previous studies in rats demonstrated that treatment with 4-PAS markedly reduced brain Mn levels in animals sub-chronically and sub-acutely exposed to this metal (Zheng et al. 2009, Santos et al. 2012a). However, 4-PAS is used as a second line anti-tuberculosis (TB) agent. Given (1) the indiscriminate use of antibiotics (Fitzwater et al. 2012) that threatens their clinical efficacy,(2) the documented efficacy of 5-ASA as an antioxidant and anti-inflammatory agent (Pearson, Jourd'heuil and Meddings 1996, Goncalves, Almeida and Dinis 1998), and (3) the role of reactive oxygen species (ROS) and neuroinflammation in the etiology of Mn-induced neurotoxicity (Milatovic and Aschner 2009, Zhang et al. 2001), the present study was designed to assess the efficacy of 5-ASA (*vs.* 4-PAS) in mitigating the cytotoxicity of this metal in RBE4 cells.

Materials and Methods

Chemicals

Manganese chloride tetrahydrate ($MnCl_2.4H_2O$), 4-PAS, 5-ASA and penicillin/streptomycin solution were obtained from Sigma Aldrich. Heat- inactivated fetal bovine serum (FBS), trypsin, nutrient mixture Ham F10, Geneticin G-418 and minimal essential medium (MEM) were purchased from Gibco. Basic fibroblastic growth factor (bFGF) were purchased from Life Technologies - Invitrogen.

Cell Culture

RBE4 cultures were cultured in 44.5% minimum essential medium (MEM), 44.5 %Ham F10 with glycine, 10% fetal bovine serum (FBS), and 1% of a penicillin/streptomycin solution and kept at 37 °C with 5% CO_2. For subculturing, the cells were dissociated with 0.25% trypsin, split 1:3, and subcultured in flasks coated with collagen with 75 cm^2 of growth area. The cells reached confluence at a density of $1{,}48 \times 10^5$cells/ml.

Cells Treatment

The RBE4 cells in 96-well culture plates were treated with $MnCl_2.4H_2O$ at 400, 600 and 800 μM and combinations of $MnCl_2.4H_2O$ and the anti- oxidants 4-PAS (Mn + 4-PAS 1 mM; Mn + 4-PAS 2 mM) and 5-ASA (Mn + 4- PAS 1 mM; Mn + 4-PAS 2 mM). Cells were treated with manganese chloride for 24 hours followed by the anti-oxidants 4-PAS or 5-ASA for 45 minutes. Mn (Marreilha dos Santos et al. 2008), 4-PAS and 5-ASA concentrations were based on previous experiments in the same cell line.

LDH Toxicity Assay

RBE4 cells were grown for 24 h, in 96-well culture plates, at 37°C, until confluency at a density of 1.48×10^5cells/ml. Forty-five minutes after 4-PAS or 5-ASA or medium (control) treatment, cultures were processed in accordance with the LDH-based colorimetric assay (Legrand et al. 1992). The LDH released into the supernatant medium was analyzed according to the manufacture's protocol (Promega) and quantified with an ELISA plate reader (Zenyth 3100) at 490 nm.

MTT Toxicity Assay

The MTT assay was performed according to the manufacturer's protocol (Sigma). After treatments (see above) and at the end of the respective incubation periods, 50 μl of MTT solution was added to each well, followed by 2 h incubation of the plates at 37°C in a CO_2 incubator. The reaction was terminated by the addition of 50 μl of MTT solubilization solution, and the results were quantified by measuring the absorbance at 570 nm. Treated samples are reported as percentage of viability in comparison to controls.

Statistical Analysis

All results are expressed as means +standard error of the mean (SEM) of 3 or more individual assays. Multiple comparisons were performed by utilizing one-way analysis of variance (ANOVA), followed by Bonferroni test for comparison of treatments with the controls. Results were analyzed by GraphPad Prism 4 (GraphPad software, San Diego, California); P values less than 0.05 were considered to be significant.

Results

4-PAS (2 mM) was effective in protecting RBE4 cells treated for 24 h with 600 μM of $MnCl_2$, as evidenced by significant ($p<0.0001$) increase in MTT levels (figure 1B) and decrease in LDH release (figure 1A).

Figure 2A shows that 1 and 2 mM 5-ASA significantly attenuated LDH release in RBE4 cells exposed for 24 h to400 ($p<0.0001$ and $p<0.001$), 600 ($p<0.0001$ and $p<0.0001$) or 800 μM ($p<0.001$ and $p<0.001$) $MnCl_2$. 5-ASA (1 and 2 mM) also significantly increased the conversion of MTT to formazan in RBE4 cells treated for 24h with $MnCl_2$within the tested concentration range ($p<0.0001$) (figure 2B).

Discussion

4-PAS and 5-ASA were assessed for their ability to attenuate Mn-induced cytotoxicity in RBE4 cells. The present study confirms that in cells exposed to $MnCl_2$ alone, the increase in LDH release is concordant with the decrease in MTT levels, corroborating previous reports (Marreilha dos Santos et al. 2008, dos Santos et al. 2010). 5-ASA significantly decreased Mn cytotoxicity in RBE4 cells exposed to $MnCl_2$ for 24 h.

Several recent reports have suggested that inflammation and oxidative stress contribute to Mn-induced neurodegeneration (Zhao et al. 2009, Milatovic et al. 2009, Santos et al. 2012b). The aminosalicylic acids used 4-PAS and 5-ASA provide an anti-oxidative protection through the deactivation of excited oxygen species (Yppolito et al. 2002).

For the first time, our results show that 5-ASA has greater efficacy in protecting RBE4 cells from Mn-induced cytotoxicity in comparison to 4-PAS after pre-exposure to $MnCl_2$ 800 μM ($p<0.0001$).

The efficacy of 4-PAS has been ascribed to its role in reducing Mn concentrations in body fluids and brain, acting as a Mn-chelating agent (Zheng et al. 2009). A recent report in rats co-exposed to $MnCl_2$and 4-PAS showed that 4-PAS was also effective in attenuating $MnCl_2$-induced neurotoxicity (Santos et al. 2012a).

5-ASA is clinically used as an anti-inflammatory agent against intestinal mucosa and joint synovial inflammation (Svartz 1988), where oxidative stress has been implicated (Miles and Grisham 1994). Brain cells are especially susceptible to oxidative stress. 5-ASA showed protection against the oxidation in gerbil cortical synaptosomes system caused by azo-bis(isobutyronitrile) (AIBN) and 2,2′-azobis (amidino propane) dihydrochloride (AAPH) (Kanski, Lauderback and Butterfield 2001).

Recent studies also showed that 5-ASA inhibits nuclear factor-kB (NF-kB) activation preventing the expression of genes encoding proinflammatory cytokines, chemokines, adhesion molecules and inflammatory mediators (Barnes and Karin 1997). In PC12 cells, Mn induced NFκ-B (Ramesh, Ghosh and Gunasekar 2002) expression, lending support to the role of oxidative stress in its neurotoxicity. Pretreatment with either vitamin E or an NFκ-B inhibitor protected against Mn toxicity in mesencephalic cells (Prabhakaran et al. 2008). Notably, NFκ-B is not a target of 4-PAS. Induction of Mn-superoxide dismutase (Mn-SOD) activity by 5-ASA may also contribute to the therapeutic mechanism of 5-ASA (Valentine 2001). Notably, MnSOD is not a target of 4-PAS.

Although 5-ASA protects RBE4 cells from the deleterious effects of Mn,, it doesn't cross the BBB(UKPAR 2009). These findings suggest that new strategies to deliver it to the brain should be developed in order to prevent Mn-induced degeneration or other neurotoxicities associated with oxidative stress. Furthermore, we suggest the use of *in vivo* and *in vitro* models to study the effectiveness of other NFκ-B inhibitors and/or enhancers of Mn-SOD activity that readily cross the BBB. These studies should profitable advance strategies to control and mitigate the adverse effects of Mn in the brain.

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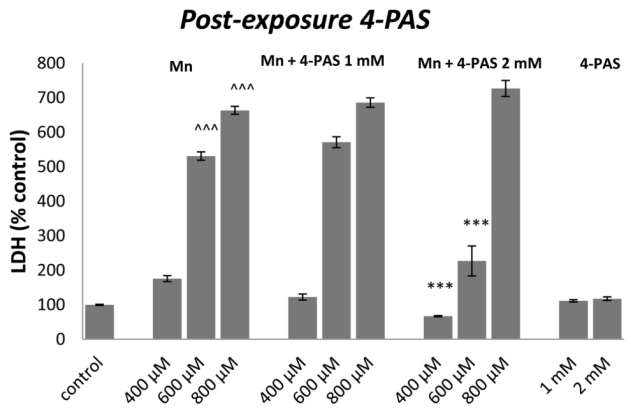

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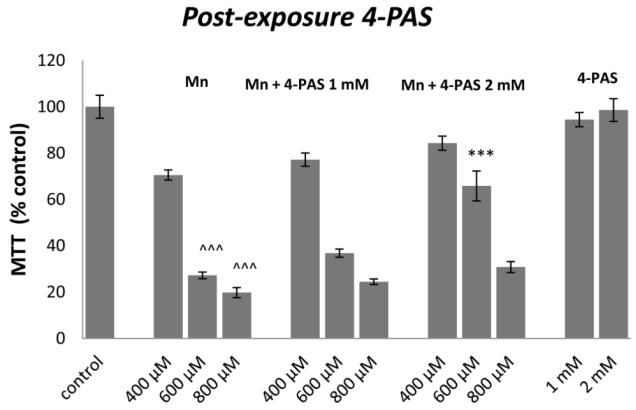
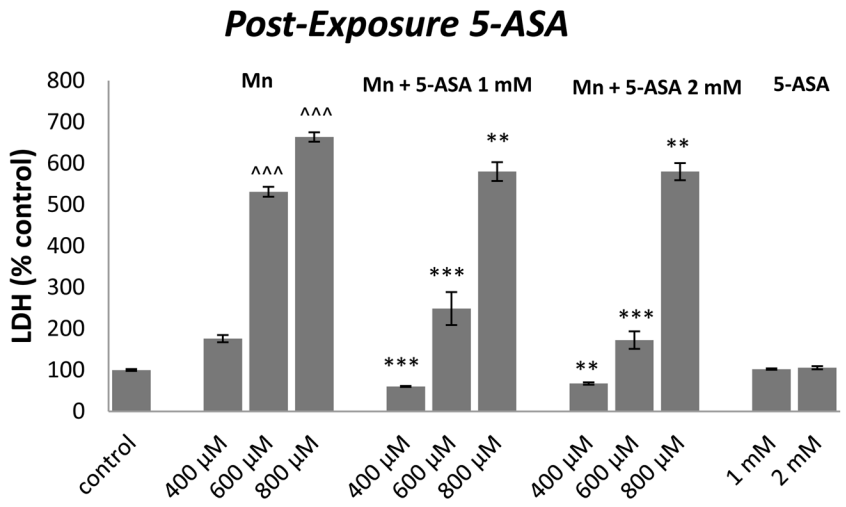


Figure 1.

The effect of 4-PAS on MTT reduction (A) and membrane integrity (B) in RBE4 cells cultured in 96-well culture plates treated for 24 h with 400, 600 or 800 μM $MnCl_2$ or medium alone. After 24 h RBE4 cells were treated for 45 minutes with medium or 1 or 2 mM 4-PAS in DMSO. Values of MTT (% control) (A) and LDH (% control) (B) represent mean ± SEM (n=12). ^^^ $p<0.0001$ significantly different from control group, *** $p<0.0001$,** $p<0.001$ significantly different from RBE4 cells exposed to the respective Mn 400, 600 and 800 μM group by one-way ANOVA followed by Bonferroni's multiple comparison tests.

A



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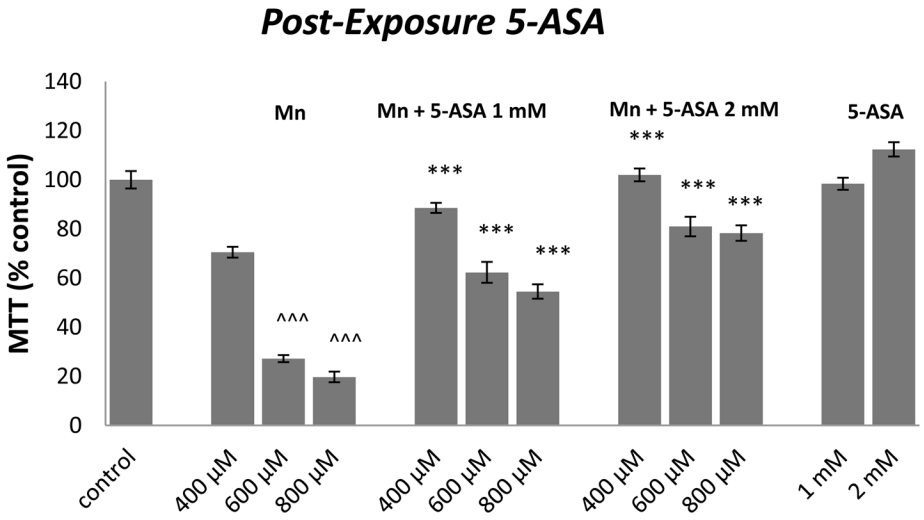

Figure 2.

The effect of 5-ASA on MTT reduction (A) and membrane integrity (B) in RBE4 cells cultured in 96-well culture plates treated for 24 h with 400, 600 or 800 μM $MnCl_2$ or medium alone. After 24 h RBE4 cells were treated for 45 minutes with medium, or 1 or 2 mM 5-ASA in HCl. Values of MTT (% control) (A) and LDH (% control) (B) represent mean ± SEM (n=12). ^^^ $p<0.0001$ significantly different from control group, *** $p<0.0001$, ** $p<0.001$ significantly different from RBE4 cells exposed to the respective Mn 400, 600 and 800 μM group by one-way ANOVA followed by Bonferroni's multiple comparison tests.