Protection by Edaravone, a Radical Scavenger, against Manganese-Induced Neurotoxicity in Rats

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ABSTRACT: Manganese (Mn) is a required element for biological systems; however, its excessive exposure may lead to a neurological syndrome known as manganism. The aim of the present study was to assess the toxic effects of subacute exposure of Mn by measuring weight gain, motor performance, and biochemical parameters (complex I activity, lipid peroxides, and protein carbonyls) in brain mitochondria in rats. We also examined whether edaravone (EDA), a radical scavenger, exerts protective effects against Mn-induced neurotoxicity. In addition, we evaluated the accumulation of Mn in brain regions using magnetic resonance imaging. Mn-exposed rats revealed significantly impaired motor performance, weight loss, and Mn accumulation in particular brain area. Lipid peroxides and protein carbonyls were significantly increased in Mn-exposed rats, whereas complex I activity was found to be decreased. EDA treatment significantly prevented mitochondrial oxidative damage and improved motor performance. These findings suggested that EDA might serve as a clinically effective agent against Mn-induced neurotoxicity. © 2016 Wiley Periodicals, Inc. J. Biochem. Mol. Toxicol. 30:217–223, 2016; View this article online at wileyonlinelibrary.com. DOI 10.1002/jbt.21780

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INTRODUCTION

Manganese (Mn) is an essential trace element for normal growth and functioning of many physiological processes. Mn acts as a cofactor in the functioning of several enzymes such as arginase, superoxide dismutase, and pyruvate carboxylase [1-3]. However, despite its essentiality in cell biology, exposure to excessive Mn due to occupational and environmental sources results in a neurological syndrome with cognitive, psychiatric, and motor disturbances known as manganism. Manganism displays itself via extrapyramidal motor symptoms such as postural instability, generalized bradykinesia, rigidity, and mimics Parkinson’s disease [1-3].

Mn is mainly used for the production of steel, batteries, and electronics all over the world. Other examples of important applications of Mn are methylene-bis-dithiocarbamate (MANEB), which is an agricultural fungicide, and gasoline adjuvant methyl cyclopentadienyl manganese tricarbonyl [1–3].

Clinical diagnosis of manganism is based on combination of clinical and radiological examination. Most of these patients typically show bilateral T1-weighted magnetic resonance imaging (MRI) hyperintensive signals in the basal ganglia region indicating Mn accumulation. Mn is also a paramagnetic contrast agent for MRI due to its efficient positive contrast enhancement in T1-weighted images. The use of Mn as a contrast agent, known as manganese-enhanced MRI (MEMRI), offers benefits for clinical and preclinical studies. Recent reports suggest that MEMRI can be used effectively to investigate axonal transport, neuronal connectivity, and brain lesions in several animal models [1, 4, 5].

Mechanisms of Mn-induced neurotoxicity have been suggested to include dysfunction of mitochondrial respiration, generation of reactive oxygen species (ROS), and impairment of dopaminergic, glutamatergic, and GABAergic systems. Mn enters the mitochondria via the calcium uniporter, and mainly binds to the inner mitochondrial membrane or matrix proteins [3, 6, 7]. Mn also leads to an increase in cellular uptake of other metals such as iron. The excess accumulation of Fe in brain may consequently trigger cellular oxidative stress that leads to neuronal damage. Mn can
participate in Fenton reactions and causes subsequent oxidative damage within cells [3, 6, 7].

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one; EDA), a free-radical scavenger, has been proposed as a novel neuroprotective compound. It has been demonstrated to ameliorate neuronal damage by reducing hydroxyl radical and superoxide anion levels in various models of cerebral infarction [8], Parkinson’s disease [9], and Alzheimer’s disease [10]. Given that EDA seems to offer protection from a number of neurological injuries in which oxidative damage plays a significant role, we hypothesized whether EDA may display beneficial effects against Mn-induced neurotoxicity. To test our hypothesis, we assessed the toxic effects of subacute Mn exposure and the possible protective effects of EDA against Mn toxicity by measuring weight gain, motor performance, mitochondrial electron transport chain (ETC) enzyme complex I (NADH: CoQ oxidoreductase) activity, lipid peroxidation, and protein carbonylation in rat brain mitochondria. We also examined the Mn accumulation in brain regions by using MRI.

MATERIALS AND METHODS

Animals

Thirty-two adult male Sprague–Dawley rats were included in the study. The rats were housed in cages and maintained under standard conditions with 12-hour light/dark cycles at room temperature (22 ± 2°C). They were fed by standard pellet diet and tap water ad libitum. All animal care and experimental procedures were approved by the Institutional Animal Care and Ethical Committee (2011-079).

Chemicals

All reagents used in experiments were of analytical grade and purchased from Sigma-Aldrich (Stemheim, Germany). EDA was obtained from Tocris Bioscience (Ellisville, MO).

Experimental Design

The rats were randomly separated into four groups as follows; Group I (n = 8): 1 mL/kg saline, Group II (n = 8): 10 mg/kg MnCl₂+1 mL/kg saline, Group III (n = 8): 10 mg/kg MnCl₂+1 mg/kg EDA, Group IV (n = 8): 10 mg/kg MnCl₂+5 mg/kg EDA. Mn solution was prepared freshly by dissolving MnCl₂ in saline. To induce Mn toxicity, rats were received 10 mg/kg MnCl₂ in a volume of 1 mL/kg once daily and intraperitoneally (i.p.) through 28 days. EDA was freshly prepared in saline. It was given once daily and intramuscularly 30 min following MnCl₂ injection. The dose of EDA was selected based on previous studies [11, 12]. For the vehicle group, an identical volume of saline was given i.p.

The rats were monitored daily for behavior and health conditions throughout the study. Body weights of the animals were recorded on days 0, 7, 14, 21, and 28 of treatments and percent change of body weight gain was calculated as

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\text{Percent change of body weight} = \frac{100 \times (\text{Final weight} - \text{Initial weight})}{\text{Initial weight}}
\]

At the end of the 28-day period, MRI and motor test were performed and then rats were euthanized by decapitation. Brains were quickly removed, and mitochondria isolation and biochemical analysis including complex I activity, lipid peroxide, and protein carbonyl levels were achieved.

Demonstration of Brain Mn Accumulation by MRI

For examining the Mn accumulation in the brain, we performed MR scanning on the study groups. First, the rats were anesthetized using a mixture of ketamine hydrochloride (40 mg/kg; Alfamine®, Alfasan International, Woerden, the Netherlands) and xylazine hydrochloride (4 mg/kg; Alfazyme®, Alfasan International), i.p. The body temperature was controlled by a rectal temperature probe during the scan and maintained at approximately 36–37 °C by a heating pad. All images were acquired using a 3 Tesla (T) MR scanner (Magnetom Verio, Siemens Healthcare, Erlangen, Germany) and an eight-channel wrist coil resonator (Siemens Healthcare). The optimum flip angle and time for repetition (TR) were chosen for adequate T1 enhancement due to Mn as well as a short time to echo (TE) for compensation of T2-weighted signal loss. Data acquisition: coronal fluid-attenuated inversion-recovery (FLAIR) (TR/TE/time for inversion [TI], 2040/11/873.8 ms; field of view, 3 cm); number of excitations (NEX), 8; number of slices, 8; slice thickness, 1 mm; gap, 0.1 mm; flip angle, 150°; resolution, 128 x 128; voxel, 0.4 x 0.4 x 1 mm; SNR, 1; bandwidth, 260 HZ/Px [13].

Assessment of Gross Motor Function

The motor performances of the rats were evaluated by inclined-plate test according to the method described by Rivlin and Tator [14]. The device was an 18-x-18-cm² platform, which could be adjusted to provide a slope of varying degrees. Briefly, the rats were
placed with their body axis perpendicular to the inclined plane. The initial angle of the inclined plate was 50 deg. The incline angle slowly enhanced, and the maximum angle of the plate on which the rat preserved its position for 5 s without falling was recorded as motor score. This procedure was repeated five times per rat, and the average value of the climbing angles was recorded.

**Isolation of Mitochondria**

Mitochondria isolation was achieved at 4°C using differential centrifugation according to Ikegaya et al. [15]. Briefly, each tissue was homogenized in buffer (10 mM Tris, 0.2 mM EDTA, 0.25 M sucrose, pH 7.6) and centrifuged at 1500×g for 10 min to remove cell debris and nuclei. The mitochondria were separated from the supernatant by centrifugation at 8000×g for 20 min. The pellet was resuspended in buffer (10 mM Tris, 0.2 mM EDTA, pH 7.6). Mitochondria samples from each brain region were divided into aliquots and stored at −80°C.

**Determination of Mitochondrial Complex I Activity**

Complex I activity in mitochondrial samples were determined according to the method of Janssen et al. [16]. The rate of oxidation of NADH to NAD⁺ was monitored spectrophotometrically at 340 nm. The enzyme activity was expressed as nmol/mg protein.

**Determination of Lipid Peroxidation**

Lipid peroxidation was determined in brain tissues by measuring thiobarbituric acid reactive substances (TBARS) as described by Demougeot et al. [17]. Tetramethoxypropane was used as standard for measuring the amount of TBARS at 535 nm, and the levels of TBARS were expressed as nmol/mg protein.

**Determination of Protein Oxidation**

Protein oxidation was determined in brain tissues by measuring protein carbonyls according to Fagan et al. [18]. The carbonyl content was analyzed using 2,4 dinitrophenylhydrazine and calculated from the maximal absorbance at 360 nm employing a molar absorbance coefficient of 22 000 M/cm. Levels of protein carbonyls were expressed as nmol/mg protein.

**Determination of Tissue Protein Levels**

The total protein concentration in the brain homogenates was determined spectrophotometrically according to Bradford’s method using bovine serum albumin as standard [19].

**Statistical Analysis**

Statistical analyses were performed using SPSS for Windows, version 15.0. Results were presented as mean ± standard error of mean (SE). All data were analyzed by one-way analysis of variance (ANOVA). Post hoc testing of pairwise comparisons was performed using the Bonferroni test. The differences were considered statistically significant when \( p < 0.05 \).

**RESULTS**

**Evaluation of Body Weight**

Body weights of each group were recorded weekly to assess general health of the animals. As presented in Fig. 1, there was no significant difference in initial body weights between the groups \( (p > 0.05) \). However, at the end of the 28-day period, while control rats gained considerable weight (18.91% of initial body weight), Mn + saline rats showed significantly lower percentage gain in body weight compared to the control group (3.78% of initial body weight, \( p < 0.005 \)). Treatment of the rats with EDA (1 and 5 mg/kg) significantly preserved weight loss in Mn-exposed rats (10.93% and 17.67%, respectively; \( p < 0.05 \)).

**Evaluation of Brain Mn Distribution**

Figure 2 represents MRI scans obtained from saline, Mn + saline, and Mn + 5 mg/kg/day EDA groups at the end of the study. T1-weighted images displayed a considerable hyperintensity in hippocampus, pituitary gland, olfactory bulb, and globus pallidi of the animals. However, Mn accumulation did not show any difference with EDA treatment (Fig. 2).

**Effects of Mn Toxicity and EDA Treatment on Gross Motor Function**

We assessed the effects of Mn toxicity and EDA treatment on motor performance of the rats using inclined plate method. ANOVA results revealed significant difference among the groups \( F_{3,25} = 11.16, \ (p < 0.0005) \). The Mn-received rats showed significantly lesser degrees than those of controls (61.68...
FIGURE 1. Weekly body weight gain. All rats were weighed weekly throughout the study. The Mn + saline group showed significantly lower weight gain compared to controls (p < 0.005). EDA treatments significantly preserved weight loss compared to the Mn + saline group (p < 0.05).

FIGURE 2. MR images representing Mn accumulation in rat brain. Animals were imaged at week 4 to validate regional Mn deposition in T1-weighted images. Arrows indicate significantly positive contrast enhancement in Mn-exposed rats. (A) Saline, (B) Mn + saline, and (C) Mn + 5 mg/kg EDA.

± 0.72 vs. 68.43 ± 0.72, p < 0.0005). However, both 1 and 5 mg/kg EDA effectively improved motor performance in Mn-exposed rats (p < 0.05 and < 0.005, respectively), (Fig. 3).

Effects of Mn Toxicity and EDA Treatment on Complex I Activity

Statistical analysis of the data showed significant difference among the study groups for complex I activity [F₃,25 = 14.97, (p < 0.0005)]. Complex I activity was significantly decreased in the Mn + saline group compared to the vehicle group (p < 0.0005). Treatment of Mn-exposed rats with 5 mg/kg EDA significantly prevented the reduction in complex I activity compared to the Mn + saline group (p < 0.05). However, no significant difference was found between the 1 mg/kg EDA and Mn + saline groups (Fig. 4).

FIGURE 3. Alterations in gross motor function in Mn-exposed rats. Motor performance of the rats was assessed by measuring maximum climbing angle (degree) on an inclined plate. One-way ANOVA and post hoc Bonferroni test revealed significant differences between the groups. Results were presented as mean ± SE. #p < 0.0005 different from saline. *p < 0.05 different from Mn + saline. **p < 0.005 different from Mn + saline.
FIGURE 4. Effects of Mn exposure and EDA treatments on brain mitochondrial ETC complex I activity, lipid peroxide and protein carbonyl levels. One-way ANOVA and post hoc Bonferonni test showed significant differences between the groups. Results were presented as mean ± SEM. # $p < 0.0005$ different from saline. * $p < 0.05$ different from Mn $+$ saline. ** $p < 0.0005$ different from Mn $+$ saline.

Effects of Mn Exposure and EDA Treatment on Brain Protein Carbonyls

The protein carbonyl levels were used to detect the toxic effects of Mn-induced oxidative damage on proteins. One-way ANOVA revealed a significant main effect of treatment [$F_{3,25} = 43.03$, ($p < 0.0005$)]. The Mn $+$ saline group exhibited higher levels of protein carbonyls compared to the vehicle group ($p < 0.0005$). Under these conditions, both low and high doses of EDA significantly counteracted the generation of protein carbonyls in the Mn-exposed groups ($p < 0.0005$) (Fig. 4).

DISCUSSION

Cumulative evidence suggests that excess brain Mn generates neurotoxicity and may lead to a Parkinsonian syndrome. Radiological evaluations of the patients have confirmed the relationship between Mn exposure and high signal intensities on T1-weighted brain MRIs. In the present study, we examined the specific brain regions in which Mn mostly accumulated following Mn exposure by using 3T-MRI. We observed a significant positive contrast enhancement particularly in the basal ganglia of Mn-exposed rats. We also observed a decline in weight gain and gross motor performance in the Mn $+$ saline group. In accordance with our results, Avila et al. have suggested that adult rats exposed to either 10 or 25 mg/mL MnCl$_2$ in their drinking water revealed reduced locomotor activity and increased striatal lipid peroxidation [20]. Similarly, O'Neal et al. demonstrated significant loss in body weights and exploratory behavior of animals exposed to 6 and 15 mg/kg Mn for consecutive 4 weeks [21]. Although locomotor activity and food consumption were not monitored in our study, we believe that weight loss in Mn-exposed rats is probably associated with hypoactivity, reduced appetite, and food intake.

It was evident that Mn neurotoxicity occurs as a result of oxidative stress and mitochondrial dysfunction [5, 22–25]. Oxidative stress causes the impairment of mitochondrial function by destroying the inner membrane of mitochondria and induces apoptotic pathways. Zhang et al. have demonstrated a substantial Mn accumulation in the rat striatal mitochondria and accompanying ROS overproduction upon administration of 15 mg/kg/day Mn for 6 weeks [22]. Also, it has been shown that treatment of primary rat astrocytes with Mn at a concentration of 100 µM stimulated a significant increase of F2-isoprostanes, a marker of lipid peroxidation [24]. In the present study, treatment of rats with Mn through 28 days resulted in a significant suppression in complex I activity and elevation in lipid.
peroxidation and protein carbonylation. These findings seem to be parallel with previous studies indicating the role of oxidative stress in Mn-induced neurotoxicity.

Until now numerous studies have indicated the antioxidant, antiapoptotic, and anti-inflammatory properties of EDA against toxic agents such as paraquat [26], 6-hydroxydopamine [27], and rotenone [9]. It has been reported that EDA could successfully preserve whole nigrostriatal dopaminergic systems against a 6-hydroxydopamine-induced rat model [27]. Another study demonstrated that a 5-week treatment with EDA eliminated the toxic effects of rotenone on dopaminergic neurons by preventing ROS generation and mitochondrial damage [9]. Recently, we reported the beneficial effects of EDA against Mn-induced toxicity and apoptotic cell death in astrocytes [28]. In the current study, we further examined whether EDA have in vivo protective effects against Mn toxicity in rats. Similar to in vitro findings, we found that EDA markedly counteracted the oxidative stress by reducing the generation of lipid peroxides and protein carbonyls following Mn exposure. Moreover, EDA at a dose of 5 mg/kg/day significantly prevented the reduction in mitochondrial ETC complex I activity in Mn-exposed rats.

In conclusion, although this is the first report to evaluate the effects of EDA on Mn neurotoxicity, the absence of behavioral and histological assessment, and the examination of the chemical interaction between EDA and Mn are the limitations of our study. From the evidences of this study, we can propose that EDA may have beneficial effects against Mn-induced toxicity. Overall, these findings can contribute to better understanding of mechanism of clinical manganese.

REFERENCES


