Arch Toxicol (1994) 68: 566–570

Archives of
Toxicology
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Dopaminergic system activity and cellular defense mechanisms in the striatum and striatal synaptosomes of the rat subchronically exposed to manganese

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Received: 21 February 1994/Accepted: 18 April 1994

Abstract. In 6-month-old male Wistar rats, levels of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), ascorbic acid (AA), dehydroascorbic acid (DHAA), uric acid and glutathione (GSH) were determined by HPLC in the striatum and striatal synaptosomes after subchronic oral exposure to $MnCl_2$ 50–100–150 mg/kg. Mn significantly decreased levels of DA and GSH and increased levels of DHAA and uric acid both in the striatum and synaptosomes. In synaptosomes, individual total Mn doses/rat were directly correlated with individual DOPAC/DA ratio values ($r = +0.647$), uric acid ($r = +0.532$) and DHAA levels ($r = +0.889$) and inversely correlated with DA ($r = -0.757$) and GSH levels ($r = -0.608$). In turn, GSH levels were inversely correlated with uric acid ($r = -0.451$) and DHAA levels ($r = -0.460$). In conclusion, the response of striatal cellular defense mechanisms (increase in AA oxidation, decrease in GSH levels) correlated well with changes in markers of dopaminergic system activity and increase in uric acid levels. The latter provides evidence of an Mn-induced oxidative stress mediated by xanthine oxidase.

Key words: Manganese neurotoxicity – Oxidative stress – GSH – Ascorbic acid – Uric acid – Striatum

Introduction

The mechanism of manganese (Mn) neurotoxicity, which is associated with a deficiency of striatal dopaminergic system activity (Seth and Chandra 1984), is to date still controversial. The metal's ability to enhance the formation of reactive oxygen species and oxidation byproducts of catecholamines (quinones) (Graham 1984; Parenti et al. 1988) has been suggested as the underlying mechanism for toxicity; indeed, Mn may be involved in neuronal degeneration owing to its ability to participate in the toxic free radical reaction (Donaldson 1987).

Mn accumulates in striatal mitochondria after subchronic exposure, its efflux from mitochondria being extremely slow (Gavin et al. 1990, 1992). Moreover, Mn can accumulate in dopaminergic neurons binding to neuromelanim pigment (Lyden et al. 1984).

An impairment of the cellular antioxidant defense mechanism has been claimed by Liccione and Maines (1988), since subchronic exposure to Mn induced a marked decrease in glutathione (GSH) levels and GSH peroxidase activity in the rat striatum. In addition, Vescovi et al. (1988) showed that Mn inhibited brain GSH-*S*-transferase (GST), a family of enzymes which can catalyze the conjugation of GSH with toxic semi-orthoquinones arising from catechol autoxidation (Graham 1978).

It is well known that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) damages the nigrostriatal dopaminergic system by means of its four-electron oxidation product, 1-methyl-4-pyridinium ion (MPP^+) (Niklas et al. 1985). The detailed mechanism of the MPP^+ neurotoxic effects seems to be related to oxidative stress (Adams and Odunze 1991). Cleeter et al. (1992) demonstrated that irreversible MPP^+-induced inhibition of mitochondrial complex I is prevented by free radical scavengers GSH and ascorbic acid (AA), which suggests a possible involvement of free radicals. MPTP also depletes GSH in brainstem (Yong et al. 1986) and striatum (Ferraro et al. 1986) and its metabolites inhibit GST in rat brain (Awasthi et al. 1987). In previous studies (Desole et al. 1993 a, b), we showed that the response of the neuronal antioxidant system (increase in AA oxidation, decrease in GSH levels) well correlated with the MPTP-induced changes in markers of striatal dopaminergic system activity; moreover, we provided evidence for a mechanism of MPTP neurotoxicity involving oxidative stress produced by xanthine oxidase. Taken together, all these findings indicate that the neuronal antioxidant system (AA, GSH) may play an important role in preventing neuronal damage by MPTP.

The present study was thus undertaken to assess the role of the endogenous antioxidant system (AA oxidation, GSH

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levels) during the Mn-induced oxidative stress in the striatum and striatal synaptosomes (taken as a model of neuronal terminals) of the rat.

Materials and methods

All experiments were conducted on 6-month-old male Wistar rats (Morini), 400–590 g body weight (bodywt), maintained, under standard animal care conditions, on a 12-h day/night cycle and given food and water ad libitum. Groups of eight rats were treated, by gavage, with $MnCl_2$ 25, 50 and 75 mg/5 ml per kg twice per day for 6 consecutive days, respectively, according to Kontur and Fetcher (1988). The last half daily dose was given on day 7, 1 h before death. Controls were given tap water by gavage (5 ml/kg). The calculated total $MnCl_2$ mg/rat on the basis of the bodywt, at the end of the treatment period, turned out to be [mean ± standard deviation (SD)] 168.6±19.4, 310.0±24.4 and 503.3±42.7, respectively.

All studies were carried out in accordance with the Decreto n° 116/1992 of the Italian Ministry of the Public Health (Directive 86/609/EEC).

DA, dihydroxyphenylacetic acid (DOPAC), uric acid, AA and dehydroascorbic acid (DHAA) determinations were performed by HPLC-ECD according to the method previously described (Desole et al. 1993 a, b). GSH analysis was performed according to the enzymatic recycling method of Anderson (1985).

Rats were killed by decapitation 1 h after the last dose. Heads were cooled in liquid nitrogen and thereafter striata of both sides were rapidly removed; the striata of the left side were immediately processed for synaptosome preparations, those on the right side frozen at –40° C; thereafter they were weighed and homogenised in EDTA 1 mM containing meta-H_3PO_4 1%. After centrifugation (17500 *g* for 10 min at 4° C), the supernatant was divided into two aliquots. The first was filtered and immediately injected into the HPLC system for monoamines, metabolites, uric acid and ascorbic acid determinations. The second aliquot was adjusted to pH 7.0 with 45% K_2PO_4 and 1% DL-homocysteine was added to reduce DHAA to AA. The sample was incubated for 30 min at 25° C, then adjusted to pH 3.0 with 30% metaH_3PO_4, filtered and injected (20 µl) for total AA determination. DHAA concentration was calculated from the difference in AA content between the first and second aliquots.

Crude synaptosomes of left striata were prepared according to a modification of the Gray and Whittaker (1962) method. Striata were rapidly removed and homogenized in 30 vol ice cold 0.32 M sucrose buffered at pH 7.4 with phosphate, using a Teflon-glass system. The homogenate was centrifuged at 4° C for 10 min at 1500 *g* to remove nuclei and debris, and crude synaptosomes were isolated from the supernatant by centrifugation at 4° C at 22000 *g* for 20 min. In order to lyse synaptosomes, the pellet was resuspended by sonication in 0.9 ml ice cold metaphosphoric acid and an aliquot of 50 µl was taken for protein analysis. After centrifugation (17200 rpm for 7 min), the supernatant was divided into two aliquots for DA, DOPAC, uric acid, ascorbic acid, DHAA and GSH determinations.

All values were expressed in nmol or pmol/mg protein and given as mean ± SD. Biochemical data were analysed with ANOVA, and then with Student's two-tailed *t*-test.

Results

DA and DOPAC levels, DOPAC/DA ratio

In the whole striatum, higher Mn doses (100 and 150 mg/kg) led to a significant decrease (–13% and –23%, respectively) in DA levels, compared to the control group. In the three groups of rats given Mn, individual DA levels were inversely correlated with individual Mn dose/rat [$r = -0.536$, $p < 0.01$, degree of freedom (df) = 22].

DOPAC contents underwent a biphasic change. The lower dose (50 mg/kg) significantly increased DOPAC levels (+87.5%), the higher doses led to a decrease (–31% and –53%, respectively). Individual DOPAC levels were inversely correlated with individual Mn dose/rat ($r = -0.670$, $p < 0.0005$, $df = 22$).

Changes in the DOPAC/DA ratio were consistent with DOPAC levels changes: significant increase with the lower Mn dose (+69%) and decrease with higher doses (–19% and –50%, respectively); again, individual Mn doses/rat were inversely correlated with DOPAC/DA ratio values ($r = -0.848$, $p < 0.00001$, $df = 22$).

In striatal synaptosomes, Mn treatment led to a dose-dependent decrease in DA levels; individual DA levels were inversely correlated with individual Mn dose/rat ($r = -0.757$, $p < 0.00002$, $df = 22$).

DOPAC changes were of minor importance and did not reach statistical significance.

Changes in the DOPAC/DA ratio were consistent with DA levels changes: significant and individual dose-dependent increase ($r = +0.647$, $p < 0.001$, $df = 22$).

Uric acid levels

Mn treatment led to an increase in uric acid levels both in striatum and striatal synaptosomes; only in the latter did the individual Mn doses/rat correlate with uric acid levels ($r = +0.532$, $p < 0.01$). In turn, individual uric acid levels were inversely correlated with synaptosomal GSH levels ($r = -0.451$, $p < 0.02$).

GSH levels

The higher Mn doses led to a decrease in GSH levels both in the striatum and striatal synaptosomes; individual GSH levels were inversely correlated with individual Mn dose/rat both in striatum ($r = -0.727$, $p < 0.0001$, $df = 22$) and synaptosomes ($r = -0.608$, $p < 0.002$, $df = 22$) (Fig. 1). In turn, individual GSH levels were significantly and inversely correlated with DHAA levels both in the striatum and striatal synaptosomes ($r = -0.530$ and -0.460, respectively).

AA and DHAA levels, DHAA/AA ratio

The lower Mn dose decreased DHAA levels both in striatum and striatal synaptosomes, with a consequent decrease in the DHAA/AA ratio. AA levels were slightly increased but not to the point of statistical significance, compared to control group. Conversely, the higher Mn doses decreased AA levels and increased DHAA levels both in the striatum and striatal synaptosomes, with a consequent increase in the DHAA/AA ratio. Both in the striatum and striatal synaptosomes individual Mn doses/rat were significantly correlated with individual AA levels ($r = -0.689$ and -0.756, respectively), DHAA levels ($r = +0.632$ and $+0.889$, respectively) and DHAA/AA ratios values ($r = +0.686$ and $+0.757$, respectively (Tables 1 and 2).

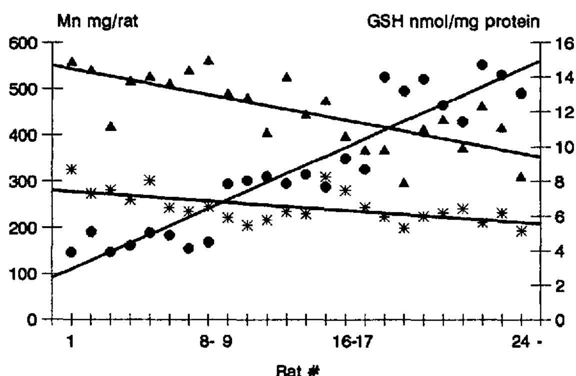

Fig. 1. Correlations between individual total Mn dose/rat and GSH levels in the striatum and striatal synaptosomes of rats subchronically exposed to oral $MnCl_2$. Rats 1–8, $MnCl_2$ 50 mg/kg per day; rats 9–16, $MnCl_2$ 100 mg/kg per day; rats 17–24, $MnCl_2$ 150 mg/kg per day. Pearson's correlation coefficient ($df = 22$): individual total Mn dose/rat ($df = 22$) vs: striatal GSH, $r = -0.727$, $p < 0.0001$; synaptosomal GSH, $r = -0.608$, $p < 0.02$. -●- Mn mg/rat; -▲- striatal GSH; ✳ synaptosomal GSH

Discussion

Metabolism of dopamine may be associated with an increased production of H_2O_2 and increased oxidative damage of the dopaminergic system (Spina and Cohen 1989). One of the proposed mechanism of Mn neurotoxicity (Graham 1984; Parenti et al. 1988) is the metal's ability to enhance the formation of reactive oxygen species and oxidation byproducts of catecholamines (quinones). Although it is well established that Mn decreases striatal DA in experimental animals, the effect varies with experimental conditions, form of Mn, route of administration and length of exposure (Seth and Chandra 1984). In the present study, Mn induced a dose-dependent decrease in striatal and synaptosomal DA levels and a selective increase in the synaptosomal DOPAC/DA ratio, a reliable marker of DA turnover (Westerink 1975). In addition, Mn treatment increased uric acid levels both in the striatum and synaptosomes. It is well known that the catabolism of ATP leads to xanthine and hypoxanthine, both of which are metabolised by xanthine

Table 1. Effect of subchronic orally administered $MnCl_2$ on levels of DA, DOPAC, uric acid (pmol/mg protein), GSH, AA and DHAA (nmol/mg protein), and on DOPAC/DA and DHAA/AA ratios in the rat striatum

	Control (n = 10)	$MnCl_2$ mg/kg per day			ANOVA	
		50 (n = 8)	100 (n = 8)	150 (n = 8)	F	P
Total $MnCl_2$ mg/rat	–	168.6 ± 19.4	310.0 ±24.4	503.3 ±42.7		
DA	614.8 ±53.8	657.0 ±196	532.3 ±45.1[a]	473.2 ±58.3[b]	5.0	<0.01
DOPAC	152.2 ±21.8	285.4 ±142[c]	105.7 ±16.1[d]	71.5 ±16.9[a]	14.1	<0.0001
DOPAC/DA ratio	0.247± 0.03	0.418± 0.09[f]	0.199± 0.02[g]	0.154± 0.05[b]	37.5	<0.00001
Uric acid	27.8 ± 4.6	36.9 ± 6.5[a]	37.8 ± 9.5[c]	41.0 ± 9.2[h]	5.2	<0.01
GSH	14.4 ± 1.4	13.9 ± 1.2	11.9 ± 1.4[a]	10.2 ± 1.6[i]	15.9	<0.00001
AA	14.6 ± 1.2	15.3 ± 1.9	13.4 ± 0.6[l]	12.8 ± 1.1[c]	6.5	<0.002
DHAA	1.35 ± 0.2	1.00 ± 0.3[m]	1.36 ± 0.4	1.61 ± 0.2[l]	6.0	<0.005
DHAA/AA ratio	0.093± 0.02	0.067± 0.02[m]	0.103± 0.03	0.123± 0.03[c]	8.0	<0.0005

p vs the Control group: [a]<0.005; [b]<0.0001; [c]<0.01; [d]<0.0002; [e]<0.000001; [f]<0.00005; [g]<0.001; [h]<0.002; [i]<0.00002; [l]<0.03; [m]<0.02
Pearson's correlation coefficient
Individual total $MnCl_2$ mg/rat ($df = 22$) vs individual:
a) DA levels, $r = -0.536$, $p < 0.01$; b) DOPAC levels, $r = -0.670$, $p < 0.0005$; c) DOPAC/DA ratio values, $r = -0.792$, $p < 0.00001$; d) uric acid levels, $r = +0.271$, $p > 0.2$; e) GSH levels, $r = -0.727$, $p < 0.0001$; f) AA levels, $r = +0.689$, $p < 0.0002$; g) DHAA levels, $r = +0.632$, $p < 0.001$; h) DHAA/AA ratio values, $r = +0.686$, $p < 0.0005$

Table 2. Effect of subchronic orally administered $MnCl_2$ on levels of DA, DOPAC, uric acid (pmol/mg protein), GSH, AA and DHAA (nmol/mg protein), and on DOPAC/DA and DHAA/AA ratios in the striatal synaptosomes of the rat

	Control (n = 10)	$MnCl_2$ mg/kg per day			ANOVA	
		50 (n = 8)	100 (n = 8)	150 (n = 8)	F	P
Total $MnCl_2$ mg/rat	–	168.6 ±19.4	310.0 ±24.4	503.3 ±42.7		
DA	107.7 ±12.2	102.8 ±16.3	66.9 ±15.5[a]	54.4 ± 6.03[b]	31.8	<0.00001
DOPAC	86.5 ± 4.7	94.2 ±24	87.1 ± 5.6	87.4 ± 5.3	0.7	>0.5
DOPAC/DA ratio	0.844± 0.10	0.948± 0.27	1.362± 0.38[c]	1.621± 0.18[b]	18.4	<0.00001
Uric acid	28.4 ± 5.1	34.2 ± 5.2[d]	42.5 ±12[e]	45.8 ±11[f]	7.4	<0.001
GSH	7.83 ± 1.0	7.22 ± 0.8	6.47 ± 0.9[e]	5.86 ± 0.5[g]	9.5	<0.0002
AA	7.30 ± 0.9	8.09 ± 0.8	7.17 ± 0.9	6.26 ± 0.4[g]	7.2	<0.001
DHAA	0.76 ± 0.2	0.65 ± 0.3	0.97 ± 0.2	1.25 ± 0.2[e]	10.4	<0.0001
DHAA/AA ratio	0.104± 0.02	0.084± 0.04	0.146± 0.05[h]	0.199± 0.04[b]	16.5	<0.00001

p vs the Control group: [a]<0.00005; [b]<0.00001; [c]<0.001; [d]<0.005; [e]<0.01; [f]<0.002; [g]<0.0001; [h]<0.02
Pearson's correlation coefficient
Individual total $MnCl_2$ mg/rat ($df = 22$) vs individual:
a) DA levels, $r = -0.757$, $p < 0.00002$; b) DOPAC levels, $r = -0.186$, $p > 0.2$; c) DOPAC/DA ratio values, $r = +0.647$, $p < 0.001$; d) uric acid levels, $r = +0.532$, $p < 0.01$; e) GSH levels, $r = -0.608$, $p < 0.002$; f) AA levels, $r = -0.756$, $p < 0.00002$; g) DHAA levels, $r = +0.889$, $p < 0.0002$; h) DHAA/AA ratio values, $r = +0.757$, $p < 0.00002$

oxidase. The products of xanthine oxidase include uric acid and superoxide radical anion (Adams and Odunze 1991). Therefore, the present study provides evidence of Mn-induced oxidative stress mediated by xanthine-oxidase, which may contribute to the neuronal damage.

The hypothesis that Mn neurotoxicity might result from an impairment of cellular antioxidant system has been advanced by Graham (1984), Donaldson (1987), Archibald and Tyree (1987) and Liccione and Maines (1988), on the basis of decreased GSH levels and GSH peroxidase activity. According to Martenson and Meister (1991), an important in vivo function of GSH is to maintain tissue AA, which may have reducting functions that are not efficiently performed by GSH. Oxygen free radicals may be involved in the pathogenesis of the Parkinson's disease (PD) (Adam and Odunze 1991). Riederer et al. (1989) showed that in PD the regional depletion of GSH correlates well with the disease severity; in addition, a consistent decrease in regional AA levels was found (–30% in putamen, –33% in globus pallidus, –75% in the amygdaloid nucleus). Indeed, the present study provides evidence that Mn total dose per rat correlated well with the response of the neuronal antioxidant system (increase in AA oxidation, decrease in GSH levels), thus suggesting an involvement of the system in limiting neuronal damage by Mn-induced oxidative stress. In this regard, the uric acid's own role as a free radical scavenger (Becker 1993) remains to be elucidated.

The question arises as to whether an impaired neuronal antioxidant system may play an enabling role in Mn neurotoxicity. Ageing is a factor known to increase nigrostriatal MPTP toxicity (Jarvis and Wagner 1990). In a previous study (Desole et al. 1993a) we showed that aged rats have a neuronal antioxidant system (levels of AA and GSH in the striatum and brainstem) considerably lower than in young rats. Consistent with the hypothesis that an impaired neuronal antioxidant system may play an enabling role in Mn neurotoxicity are the findings (Desole et al. 1994) that, in 20-month-old rats, noradrenergic and serotonergic systems in the brainstem were impaired after subchronic exposure to Mn doses which did not affect the same monoaminergic systems in 3-month-old rats.

In conclusion, in 6-month-old rats subchronically exposed to Mn, the response of striatal cellular defense mechanism (increase in AA oxidation, decrease in GSH levels) correlated well with changes in markers of dopaminergic system activity and increase in uric acid levels. The latter provides evidence of a Mn-induced oxidative stress mediated by xanthine oxidase.

Acknowledgements. The research was supported by Italian Ministry of the University and Scientific and Technological Research quota 40% 1992/1993 (Project: New assessment approaches in toxicology) and quota 60% 1994.

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