OVEREXPRESSION OF HUMAN COPPER/ZINC SUPEROXIDE DISMUTASE IN TRANSGENIC MICE ATTENUATES OXIDATIVE STRESS CAUSED BY METHYLENEDIOXYMETHAMPHETAMINE (ECSTASY)

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Abstract—Administration of 3,4-methylenedioxymethamphetamine (4×20 mg/kg) to non-transgenic CD-1 mice caused marked depletion in dopamine, 3,4-dihydroxyphenylacetic acid and 5-hydroxytryptamine in the caudate–putamen. There were no significant changes in serotonergic markers in the hippocampus and frontal cortex. Homozygous and heterozygous copper/zinc superoxide dismutase transgenic mice show partial protection against the toxic effects of 3,4-methylenedioxymethamphetamine on striatal dopaminergic markers. In addition, 3,4-methylenedioxymethamphetamine injections caused marked decreases in copper/zinc superoxide dismutase activity in the frontal cortex, caudate–putamen and hippocampus of wild-type mice. Moreover, there were concomitant 3,4-methylenedioxymethamphetamine-induced decreases in catalase activity in the caudate–putamen and hippocampus, decreases in glutathione peroxidase activity in the frontal cortex as well as increases in lipid peroxidation in the frontal cortex, caudate–putamen, and hippocampus of wild-type mice. In contrast, administration of 3,4-methylenedioxymethamphetamine to homozygous superoxide dismutase transgenic mice caused no significant changes in antioxidant enzyme activities nor in lipid peroxidation.

These results provide further substantiation of a role for oxygen-based radicals in 3,4-methylenedioxymethamphetamine-induced neurotoxicity. The present data also suggest that free radicals generated during 3,4-methylenedioxymethamphetamine administration may perturb antioxidant enzymes. Consequently, there might be further overproduction of free radicals with associated peroxidative damage to cell membranes and associated terminal degeneration.

Key words: MDMA, superoxide dismutase, transgenic mice, antioxidant enzymes, biogenic amines.

3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy") is a ring-substituted phenyl-isopropylamine that is related to both amphetamines and hallucinogens, such as mescaline28 (Fig. 1). Its entry into the brain may be through P-glycoprotein located in the blood–brain barrier.26 MDMA has been reported to enhance empathy and to cause euphoria, anxiety, depression and paranoid disorders in human subjects.2,12,34 Neurologic effects of MDMA include seizures with secondary anoxic events and associated oxidative damage to the brain.30 Moreover, MDMA can cause decrements in cerebrospinal fluid 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA),27 and at high doses, this drug of abuse might also damage dopaminergic as well as serotonergic neurons in humans.14

Administration of MDMA to animals elicits variable neurotoxic effects that depend on the species under study.28 In rats5,15,16,24,39,42 and non-human primates36,37 MDMA can produce long-lasting toxic effects on serotonergic neurons. In contrast, injections of MDMA to mice caused striatal dopamine (DA) depletion without significantly affecting serotonergic systems, when the animals were killed three to eight days24 or two weeks later.7,31 Moreover, large differences in sensitivity to the DA-depleting properties of MDMA have been reported among different strains of mice.45

Although the cause of MDMA-induced neurotoxicity has not been fully determined, a number of possible scenarios have been put forward. For example, because direct infusion of MDMA into cerebroventricular space did not cause any toxicity, it was suggested that the neurodegenerative effects of the drug might be related to its metabolites.29,33,40 This argument is supported, in part, by the demonstration that MDMA is metabolized into catechol...
and quinone metabolites. Further metabolism of these compounds could result in the formation of free radicals which might induce oxidative stress, and subsequent cellular damage. Other possible mechanisms also include: (i) increased metabolism of DA released by MDMA; and (ii) formation of DA metabolites such as 6-hydroxydopamine which is known to cause oxidative stress. The possible role of reactive oxygen species in MDMA-induced toxicity is further supported by our previous observations that transgenic mice that have high levels of the antioxidant enzyme copper/zinc superoxide dismutase (Cu/ZnSOD) in the brain were protected against the acute lethal effects of MDMA and against the subacute and long-term toxic effects of the drug on brain DA systems.

Thus, we reasoned that, if MDMA was indeed causing oxidative stress in the brain, it might perturb the activities of various antioxidant enzymes (Cu/ZnSOD, catalase, glutathione peroxidase) which participate in a concerted collaboration with each other in order to protect the brain against oxidative damage. We also tested the possibility that MDMA injections might be associated with increased lipid peroxidation in the mouse brain. Finally, in order to try to relate the changes in enzymatic activities to the neurotoxic effects of MDMA, we also measured the effects of MDMA on monoamine levels in mice killed at similar time-points following administration of the drug. The data on the perturbations of antioxidant enzymes presented herein support the view that MDMA-induced toxicity may indeed occur via the production of reactive oxygen species.

**EXPERIMENTAL PROCEDURES**

**Chemicals**

Glutathione reductase and NADPH were obtained from Sigma Chemical (St Louis, MO). Reduced glutathione (GSH) and t-butyl hydroperoxide was from Aldrich Chemical (Milwaukee, WI). Hydrogen peroxide was procured from Fischer Scientific Co. (Fairlawn, NJ). SOD assay kit (Cat. No. 574600) and lipid peroxidation assay kit (Cat. No. 473634) were obtained from Calbiochem-Novabiochem (San Diego, CA). All the other chemicals were of analytical grade quality.

**Animals**

Male heterozygous and homozygous SOD-transgenic (Tg) mice of strain 218/3 on a CD-1 background were used in these experiments. These animals carry the complete human Cu/ZnSOD gene and were produced as described previously. They were obtained from a colony maintained at our Institute. Male non-transgenic CD-1 mice (non-Tg) (Charles River, Raleigh, NC), weighing 30–45 g, were used as the wild control. These mice have been used in several experiments in which we have tested the role of oxidative stress in the deleterious effects of various toxins. All animal use procedures were according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee.

**Drug administration and preparation of samples**

Mice received doses of 20.0 mg/kg of MDMA or saline via the intraperitoneal route. MDMA was given at 2-h intervals for a total of four injections. Control mice received saline. Mice were killed 16 h later and their brains were rapidly removed. The frontal cortex (FC), caudate–putamen (CPu) and hippocampus were dissected out on ice and frozen immediately at −70°C.

**Analysis of monoamines and their metabolites**

Brain regions were analysed for monoamines and their metabolites using high-performance liquid chromatography utilizing electrochemical detection at +0.75 V as previously described. In brief, individual samples were sonicated in 400–500 µl 0.1 M perchloric acid and centrifuged at 7200 g (12,000 r.p.m.) for 10 min. The supernatant (50 µl) was injected on to a 10 cm X 4.6 mm Spherisorb 3 µM ODS reverse-phase chromatography column (Thomson Instruments, Springfield, VA) in a mobile phase containing 0.1 M citric acid, 8% acetonitrile, 0.5 g/l octanesulfonic acid, 0.3% triethylamine, and 10 µM EDTA at a flow rate of 0.7 ml/min. DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and HVA, serotonin (5-HT), and...
5-HIAA were separated and detected in a single chromatogram and were quantified as relative peak areas versus the internal standard, 5-hydroxy Nα-methyltryptamine. The detection limit was 100 pg. Protein content was determined by the method of Lowry et al.25

Antioxidant enzyme activities

To quantify the antioxidant enzymes, Cu/ZnSOD, catalase and glutathione peroxidase (GSH-Px) activities, the samples were weighed and homogenized using a Teflon glass homogenizer in 10 volumes of appropriate buffer required for the specific enzyme assay.

Cu/Zn-SOD was measured using the Calbiochem’s SOD-525 kit. The assay is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydroxybenzo[c]-fluroene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. One enzyme unit is defined as the activity that doubles the autoxidation background. Catalase activity was quantitated from the decrease in the absorbance of H2O2 at 240 nm in the presence of the enzyme. One unit of catalase was defined as 1 µmol of H2O2 consumed per minute per milligram protein. GSH-Px activity was quantitated from the decrease in NADPH absorbance at 340 nm using the method of Gunzler and Flohe.19 The amount of GSH-Px inducing a net fall in GSH to 10% of the initial concentration in 1 min at 37°C and pH 7.0 is defined as one unit. Protein concentrations for calculation of specific activity were determined using Pierce BCA Protein assay kit (Rockford, Illinois) with bovine serum albumin as the standard and absorbance measured at 526 nm.

Lipid peroxidation

Lipid peroxidation was measured by the release of malondialdehyde (MDA), the end product derived from the breakdown of polyunsaturated fatty acids and related esters.21 The LPO-586 assay kit from Calbiochem uses 10.3 mM N-methyl-2-phenylindole in acetonitrile, a chromogenic reagent which reacts with MDA to yield a stable chromophore with maximal absorbance at 586 nm.

Data analysis

Data were analysed using statistical software (StatView 4.02) on a Macintosh computer (Quadra 700). Statistical analyses were done by a one-way ANOVA followed by Fischer’s protected least significant difference (PLSD) test.

RESULTS

Effects of methylenedioxymethamphetamine on levels on striatal dopamine, 3,4-dihydroxyphenylacetic acid and homovanillic acid

Figure 2 shows that administration of MDMA caused a significant reduction of DA (−78%) in the CPu of non-Tg mice. These changes were somewhat attenuated in the CPu of heterozygous (−51%) and homozygous (−45%) SOD-Tg mice (Fig. 2). The CPu content of the major DA metabolite, DOPAC, was also reduced in the non-Tg control (−63%) and heterozygous SOD-Tg mice (−47%) but not in homozygous SOD-Tg mice (Fig. 2).

Effects of methylenedioxymethamphetamine on antioxidant enzymes

Copper/zinc-superoxide dismutase activity. In comparison to non-Tg mice, heterozygous SOD-Tg mice showed a 2.1-fold increase while homozygous SOD-Tg mice showed a 4.3-fold increase in SOD activity in the FC (Fig. 4). In the CPu and hippocampus, heterozygous SOD-Tg mice showed respective increases of threefold and 2.8-fold while homozygous SOD-Tg mice demonstrated changes corresponding to 3.6-fold and threefold increases compared to control Cu/ZnSOD values (Fig. 4). Administration of MDMA caused significant
Fig. 3. Effects of repeated MDMA administration (4 × 20 mg/kg) on 5-HT (A) and 5-HIAA (B). Values represent means ± S.E.M. (percentage of respective controls) (n = 6–9 mice/group). There were no significant differences in 5-HT or 5-HIAA levels between the saline-injected groups. The absolute values (ng/mg protein) for the saline groups are as follows: 5-HT: CPu, 6.12 ± 0.31 (non-Tg); 4.77 ± 0.32 (Hetero); 5.37 ± 0.26 (Homo). FC, 8.71 ± 0.29 (non-Tg); 7.53 ± 0.32 (Hetero); 8.48 ± 0.39 (Homo). Hippocampus, 8.94 ± 0.64 (non-Tg); 8.15 ± 0.41 (Hetero); 8.45 ± 0.33 (Homo). 5-HIAA: CPu, 4.27 ± 0.16 (non-Tg); 4.09 ± 0.41 (Hetero); 4.38 ± 0.23 (Homo). FC, 1.94 ± 0.07 (non-Tg); 2.42 ± 0.16 (Hetero); 2.52 ± 0.14 (Homo). Hippocampus, 4.18 ± 0.17 (non-Tg); 5.10 ± 0.46 (Hetero); 5.61 ± 0.19 (Homo). Key for statistics: **P < 0.001 vs respective controls. †P < 0.01 vs non-Tg mice treated with MDMA.

Fig. 4. Effects of MDMA on Cu/ZnSOD activity in the frontal cortex, caudate–putamen and hippocampus of non-Tg and SOD-Tg mice (n = 10 mice/group). Values represent means ± S.E.M. (percentage of non-Tg control). The absolute values (units/mg protein) for saline and MDMA-treated are as follows: FC: 0.42 ± 0.01 and 0.06 ± 0.001 (non-Tg); 0.94 ± 0.03 and 0.61 ± 0.05 (Hetero); 2.30 ± 0.16 and 2.14 ± 0.17 (Homo). CPu: 0.81 ± 0.06 and 0.38 ± 0.05 (non-Tg); 2.46 ± 0.07 and 2.00 ± 0.04 (Hetero); 2.83 ± 0.14 and 2.70 ± 0.11 (Homo). Hippocampus: 0.73 ± 0.03 and 0.27 ± 0.03 (non-Tg); 2.25 ± 0.11 and 1.78 ± 0.18 (Hetero); 2.42 ± 0.14 and 2.17 ± 0.08 (Homo). Key for statistics: **P < 0.001, ***P < 0.0001 in comparison to saline-injected wild-type mice. $P < 0.05, $$$P < 0.001 in comparison to saline-injected animals of the same strain (PLSD after ANOVA).
decreases in enzyme activity in all three brain regions examined in the non-Tg mice (Fig. 4). These corresponded to 85%, 55% and 65% in the FC, CPu and hippocampus, respectively. MDMA-induced decreases in SOD activity were attenuated in the FC (40%), the CPu (25%) and hippocampus (30%) of heterozygous SOD-Tg mice (Fig. 4) whereas MDMA caused no significant changes in SOD activity in the homozygous SOD-Tg mice (Fig. 4).

Catalase activity. Catalase activity was significantly higher in the FC (1.8-fold), CPu (1.25-fold) and hippocampus (1.5-fold) of homozygous SOD-Tg mice in comparison to the activity measured in non-Tg mice (Fig. 5). Catalase activity was also slightly but significantly increased in the CPu (1.2-fold) and hippocampus (1.3-fold) of heterozygous SOD-Tg mice. MDMA caused significant decreases in catalase activity in both the CPu and hippocampus but not in the cortices of non-Tg and heterozygous SOD-Tg mice (Fig. 5) whereas there were no significant changes in catalase activity in the brains of homozygous SOD-Tg mice.

Glutathione peroxidase activity. GSH-Px activity was slightly but significantly increased in the CPu (1.2-fold) of homozygous SOD-Tg mice (Fig. 6). MDMA treatment caused significant decreases only in the FC of the non-Tg mice (Fig. 6). Unexpectedly, MDMA treatment led to increases in GSH-Px activity in all three brain regions of heterozygous and homozygous SOD-Tg mice.

Effects of methylenedioxymethamphetamine on lipid peroxidation

There were no differences in MDA released in saline-treated mice of the three strains. MDMA injections caused increased MDA release in the three brain regions of both non-Tg and heterozygous SOD-Tg mice (Fig. 7). The percentage increases were more prominent in the non-Tg and consisted of respective increases of +40% (FC), +35% (CPu) and +50% (hippocampus) in the non-Tg mice but of +25% (FC), +32% (CPu) and +30% (hippocampus) in the heterozygous SOD-Tg mice. MDMA did not affect MDA release in the homozygous SOD-Tg mice (Fig. 7).

DISCUSSION

The major MDMA effects reported here include: (i) neurotoxic effects on striatal dopaminergic terminals; (ii) decreases in Cu/ZnSOD activity in the FC, CPu and hippocampus regions of non-Tg mice; (iii) decreases in catalase activity in the CPu and hippocampus in non-Tg mice; (iv) decreases in GSH-Px activity.
Fig. 6. GSH-Px activity (percentage of non-Tg control) in the frontal cortex, caudate–putamen and hippocampus of non-Tg and of SOD-Tg mice. Data are means ± S.E.M. values measured in 10 animals/group. The absolute values (×10^−2 units/mg protein) for saline and MDMA-treated are as follows: FC: 0.56 ± 0.04 and 0.24 ± 0.01 (non-Tg); 0.63 ± 0.04 and 1.10 ± 0.04 (Hetero); 0.67 ± 0.02 and 1.00 ± 0.07 (Homo). CPu: 0.97 ± 0.05 and 0.89 ± 0.10 (non-Tg); 1.00 ± 0.08 and 1.30 ± 0.05 (Hetero); 1.10 ± 0.08 and 1.40 ± 0.01 (Homo). Hippocampus: 0.37 ± 0.01 and 0.33 ± 0.01 (non-Tg); 0.36 ± 0.01 and 0.48 ± 0.03 (Hetero); 0.40 ± 0.01 and 0.45 ± 0.03 (Homo). Statistical significance was analysed as above. Key for statistics: *P < 0.05 in comparison to saline-injected wild-type mice. $$$P < 0.0001 in comparison to saline-injected animals of the same strain (PLSD after ANOVA).

Fig. 7. MDA released in the frontal cortex, caudate–putamen and hippocampus of non-Tg and of SOD-Tg mice. Data are means ± S.E.M. values (percentage of non-Tg control) measured in 10 animals per group. The absolute values (µM MDA released/mg protein) for saline and MDMA-treated are as follows: FC: 1.27 ± 0.02 and 1.80 ± 0.11 (non-Tg); 1.33 ± 0.01 and 1.57 ± 0.07 (Hetero); 1.10 ± 0.05 and 1.12 ± 0.04 (Homo). CPu: 2.24 ± 0.17 and 2.99 ± 0.26 (non-Tg); 2.24 ± 0.17 and 2.99 ± 0.28 (Hetero); 2.27 ± 0.07 and 2.34 ± 0.19 (Homo). Hippocampus: 1.55 ± 0.11 and 2.96 ± 0.23 (non-Tg); 1.52 ± 0.07 and 2.08 ± 0.22 (Hetero); 1.58 ± 0.07 and 1.61 ± 0.19 (Homo). Statistical significance was analysed as above. Key for statistics: *P < 0.05, **P < 0.001, ***P < 0.0001 in comparison to saline-injected wild-type mice (PLSD after ANOVA).
activity in the FC of non-Tg mice; (v) increase in lipid peroxidation in the non-Tg mice; and (vi) significant attenuation of these MDMA effects in SOD-Tg mice.

In what follows, we discuss possible scenarios for these observations. It is to be noted that both catalase (Fig. 5) and GSH-Px (Fig. 6) activities are increased in the untreated SOD-Tg mice, with the greatest increases being observed in the FC of the animals. The fact that Cu/ZnSOD activity (Fig. 4) also showed greater percentage increases in the FC suggests that there might be a relationship between these observations. Specifically, because the high Cu/ZnSOD activity in the FC would generate more H$_2$O$_2$ from the enzymatic breakdown of superoxide radicals, it is likely that the enzymes, catalase and GSH-Px responsible for the catabolism of H$_2$O$_2$, might have shown a much greater compensatory response in the FC compared to the CPu or the hippocampus.

The present data on the effects of MDMA on monoaminergic systems are in accord with previous observations reported in mice. For example, a single large dose of MDMA caused decreases in DA but not in 5-HT levels while multiple large doses of the drug caused only small and transient decreases in striatal 5-HT levels. The present attenuation of the effects of MDMA in SOD-Tg mice is also consistent with data obtained previously. In those experiments, a different schedule of MDMA injections was used. Specifically, one large dose (50 mg/kg) or three large doses given one day apart were used. In both cases, however, SOD-Tg mice showed a substantial degree of protection against the toxic effects of the drug. Using a dose schedule similar to the one in the present study (20 mg/kg × 4), we also observed marked decreases in $[^{125}\text{I}]$-RTI-labeled DA uptake sites in non-Tg mice killed two weeks after MDMA administration; these were attenuated in SOD-Tg mice (unpublished observations). These observations indicate that the neurotoxicity of the MDMA may indeed be mediated, in part, by the increased production of superoxide radicals. As suggested in the introduction, superoxide anions (O$_2^-$) might be produced as by-products of reactions that involve redox-cycling or through the autoxidation of excess DA released by MDMA. Interaction of H$_2$O$_2$ with O$_2^-$ could lead to the production of the more toxic hydroxyl radicals with subsequent degeneration of the nigrostriatal dopaminergic pathway. Although formation of superoxide radicals and hydroxyl radicals have been reported after administration of methamphetamine, this remains to be demonstrated for MDMA. If MDMA does indeed increase O$_2^-$ production, O$_2^-$ could be responsible for the marked decreases in Cu/ZnSOD and catalase activities observed in the brain regions of the non-Tg mice since O$_2^-$ have been reported to inactivate antioxidant enzymes. The protection afforded in the SOD-Tg mice support that view.

It is also of interest that injections of MDMA to heterozygous and homozygous SOD-Tg mice resulted in significant increases in GSH-Px in all the three brain regions examined (see Fig. 6). These observations suggest that these mice were able to increase their production of GSH-Px in order to get rid of excess H$_2$O$_2$ generated from the dismutation of O$_2^-$ formed subsequent to the administration of MDMA. The SOD-Tg mice might have been able to respond in that way because the GSH-Px system in SOD-Tg mice was already primed to react because of the increased basal levels of H$_2$O$_2$ being generated in SOD-Tg mice as previously reported.

The increase in lipid peroxidation observed in the present study replicates similar findings reported in a previous study in which one large dose (40 mg/kg) of MDMA was given to rats. In that study, deprenyl which afforded protection against the toxic effects of MDMA also prevented the increases in lipid peroxidation. These observations are consistent with our present demonstration that the MDMA-induced increase in lipid peroxidation is attenuated in SOD-Tg mice. Thus, concordant observations provide conclusive support for the idea that MDMA causes oxidative stress in mammalian brains.

**CONCLUSIONS**

The present paper documents, for the first time, the effects of toxic doses of MDMA on free radical scavenging enzymes in non-Tg and SOD-Tg mice. The effects of MDMA on these antioxidant enzymes and the increase observed in lipid peroxidation provide support for a role of a cascade of toxic events that appear to consist of the generation of superoxide radicals, hydrogen peroxide and hydroxyl radicals.

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**REFERENCES**


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