— Rapid Communication —

Combined Effects of Manganese, Iron, Copper, and Dopamine on Oxidative DNA Damage

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Manganese (Mn) damages the central nervous system and causes Parkinson's disease (PD)-like syndrome called manganism. Abnormal accumulation of Mn and iron (Fe) is observed in the basal nuclei of patients with PD. Dopamine induces oxidative DNA damage in the presence of Fe or copper (Cu) *in vitro*. Therefore, an Mn-induced neural disorder may involve combined actions of Mn, Fe/Cu, and catecholamines. Here we investigated the combined effects of Mn(II) on the DNA damage induced by dopamine and either Fe(III) or Cu(II). Mn(II) enhanced the formation of 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG) induced by dopamine and Fe(III)/Cu(II), whereas Mn(II) did not induce 8-oxodG formation in the absence of Fe(III)/Cu(II). Mn(II) accelerated hydrogen peroxide (H_2O_2) production in the dopamine-Fe(III)/Cu(II) system. Furthermore, Mn(II) decreased superoxide anion radicals ($O_2^{\bullet-}$) generated in the xanthine-xanthine oxidase system. These results indicate that Mn(II)-mediated enhancement of DNA damage is caused by facilitated conversion of $O_2^{\bullet-}$ to H_2O_2. In addition, Mn(II) accelerated the formation of aminochrome, an oxidized dopamine product, in the presence of Cu(II). This reaction may have involved Mn(III) accompanied by H_2O_2 production from $O_2^{\bullet-}$, suggesting a mechanism for Mn-induced oxidative stress on dopaminergic neurons through the combined actions of Mn, Fe, Cu, and dopamine.

Key words —— oxidative DNA damage, metal ion, dopamine, manganism, Parkinsonism, superoxide anion radical

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INTRODUCTION

Manganese (Mn) is an essential metal in humans that functions in many enzymes, such as pyruvate carboxylase, glutamine synthetase, and numerous peroxidases.[1] In contrast, excessive exposure to Mn results in neurotoxicity.[2] Symptoms of acute Mn toxicity are collectively called manganism, and these symptoms are similar to those seen in patients with Parkinson's disease (PD).[3] Considering that manganism and PD, a dopaminergic cell disorder, are common in many ways and the fact that Mn accumulates in dopaminergic areas, Mn neurotoxicity has been suggested to involve an imbalance in dopaminergic neurotransmission.[4,5] Neurodegenerative loss of substantia nigra dopaminergic neurons is a known characteristic of PD. While the nature of this neuronal loss is unknown, it is hypothesized that accelerated auto-oxidation of dopamine to quinones and semiquinones accompanied by the generation of reactive oxygen species (ROS) causes direct damage leading to the neuronal loss.[6,7] This aberrant oxidation could result from increased concentrations of catalytic metals such as iron [Fe(III)] and copper [Cu(II)].

In the body, Fe is mostly bound to proteins such as transferrin in order to regulate its reduction-oxidation (redox) activity; however, some Fe is loosely bound to small molecules such as amino acids and other biomolecules. Certain chemicals reduce this unstable Fe(III) to elicit the redox activity of Fe(II).[8] Fe(II) reacts with H_2O_2 in the Fenton reaction to produce hydroxyl radicals that attack biomolecules. Cu is found in chromosomes and is closely associated with DNA bases.[9–11] In the presence of Cu(II), catechols such as dopamine are readily oxidized to semiquinones, which are capable of reducing oxygen to produce ROS, such as superoxide anion radicals ($O_2^{\bullet-}$). The resulting Cu(I) and

H_2O_2 reportedly form Cu(I)-OOH, which attacks DNA.[12,13] This Fe/Cu-mediated oxidative damage may be responsible for the neurodegeneration associated with PD and Alzheimer's disease,[14,15] although its pathogenic role has not been elucidated.

Dopamine-induced oxidative DNA damage or cytotoxicity in the presence of Cu(II) is enhanced by Mn(II).[16,17] However, in those studies, the direct detection of ROS ($O_2^{\cdot-}$ and H_2O_2) in the metal-mediated oxidative DNA damage is not performed. In this study, we investigated the combined effects of Mn, Fe, Cu, and dopamine on oxidative DNA damage, focusing on ROS ($O_2^{\cdot-}$ and H_2O_2) generation and dopamine oxidation.

MATERIALS AND METHODS

Materials —— Methanol (99.7%), ethanol (99.5%), diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA), sodium dihydrogen phosphate, disodium hydrogen phosphate, Cu(II) chloride dihydrate ($CuCl_2 \cdot 2\,H_2O$, 99.9%), bovine liver catalase, superoxide dismutase (SOD), xanthine, scopoletin, ferric nitrate nonahydrate [$Fe(NO_3)_3 \cdot 9\,H_2O$], boric acid, sodium hydroxide, nuclease P1, sodium tetraborate decahydrate, sodium acetate, acetic acid, and *N*-(1-naphthyl)-ethylenediamine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Dopamine, calf thymus DNA, 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-oxodG), xanthine oxidase, and 2′-deoxyguanosine (dG) were purchased from Sigma (St. Louis, MO, U.S.A.). Sulfanilic acid and hydroxyl ammonium were obtained from Kanto Chemical Co. (Tokyo, Japan). Calf intestine alkaline phosphatase was obtained from Roche Diagnostics (Mannheim, Germany), Mn(II) chloride ($MnCl_2$, 99.9%) was from Nacalai Tesque (Kyoto, Japan), nitrilotriacetate (NTA) was from Dojindo Chemicals (Kumamoto, Japan), H_2O_2 was from Mitsubishi Gas Chemical Co. (Tokyo, Japan), and horseradish peroxidase (HRP) was obtained from Toyobo Co. (Osaka, Japan).

Measurement of 8-oxodG Formation —— Reaction mixtures consisting of 10 μM dopamine with or without 10 and 20 μM $MnCl_2$ in 400 μl of 4 mM sodium phosphate buffer (pH 7.8) were preincubated for 1 hr at 37°C. Then, calf thymus DNA (100 μM base) and 20 μM $CuCl_2$ or $Fe(NO_3)_3$ plus NTA [$Fe(NO_3)_3$: NTA = 1 : 1] were added to the mixtures, followed by incubation for 1 hr at 37°C. After ethanol precipitation, DNA was digested to nucleosides using nuclease P1 (10 units) and calf intestine alkaline phosphatase (1.3 units); the DNA was then analyzed by high-performance liquid chromatography (HPLC; LC-10 series, Shimadzu, Kyoto, Japan) using an electrochemical detector (ECD; Coulochem II, ESA, Chelmsford, MA, U.S.A.). The HPLC conditions were as follows: column, ODS-80Ts (Tsk-gel, 150 × 4.6 mm *i.d.*; Tosoh, Tokyo, Japan); column temperature, 25°C; flow rate, 1 ml/min; and detection wavelength, 254 nm (for dG). The amount of 8-oxodG was determined by ECD analysis. The ECD conditions were as follows: guard cell, 400 mV; channel 1, E 150 mV/R 100 μA/filter 2/output 1 V; channel 2, E 300 mV/R 200 nA/filter 10/output 1 V.

Determination of H_2O_2 Content —— The amount of H_2O_2 was determined by the scopoletin assay.[18,19] Reaction mixtures consisting of 10 μM dopamine with or without 10 and 20 μM $MnCl_2$ in 396 μl of 4 mM sodium phosphate buffer (pH 7.0) were preincubated for 1 hr at 37°C. Thereafter, 4 μl of 2 mM $CuCl_2$ was added to the reaction mixture, and 80 μl of each reaction mixture was mixed with 50 μl of 42 μM (8 μg/ml) scopoletin and 0.125 units of HRP in 2 ml of 9 mM borate buffer (pH 10.0). Fluorescence intensity was measured using a spectrofluorometer (RF-1500, Shimadzu) with excitation at 397 nm and emission at 460 nm. To measure peroxides other than H_2O_2, the mixture, including 10 units of catalase at room temperature, was incubated for 4 min, and its fluorescence intensity was measured.

Determination of $O_2^{\cdot-}$ Content —— Reaction mixtures consisting of 0.8 mM xanthine; 0.8 mM hydroxyl ammonium; 0.1 unit/ml xanthine oxidase; and 0, 10, or 20 μM $MnCl_2$ with or without 0.1 unit SOD in 0.25 ml of 50 mM sodium phosphate buffer (pH 7.8) were incubated for 10 min at 25°C. Thereafter, 0.25 ml of 20 mM sulfanilic acid was added, and the mixtures were incubated for 20 min at 25°C. Then, 0.25 ml of 10 mM *N*-(1-naphthyl)-ethylenediamine was added and incubated for 20 min at 25°C. Finally, absorbance at 545 nm was measured with a UV/visible (Vis) spectrophotometer (V-550, Jasco, Tokyo, Japan), and the absorbance of mixtures with SOD was subtracted from that of mixtures without SOD. The $O_2^{\cdot-}$ concentration was calculated from the subtracted absorbance using a standard curve.

Measurement of Aminochrome Formation —— UV spectra of dopamine were recorded using a UV/Vis spectrophotometer (Jasco) at room temper-

ature in a measurement cell with a 10-mm path length. Reaction mixtures consisting of 10 μM dopamine, with or without 10 and 20 μM $MnCl_2$ in 1 ml of 4 mM sodium phosphate buffer (pH 7.8), were preincubated for 1 hr at 37°C, and their UV spectra were recorded at 20-min intervals. After adding 20 μM $CuCl_2$ to the reaction mixtures, their UV spectra were obtained at 5-min intervals for 30 min and then at 10-min intervals for another 150 min.

Statistical Analysis —— Student's *t*-test was performed to evaluate the significance between the two groups. A value of $p < 0.05$ was considered significant.

RESULTS

Mn(II)-dependent Enhancement of 8-oxodG Formation Induced by Fe(III)-NTA/Cu(II) and Dopamine

First, the effect of Mn(II) on 8-oxodG formation induced by Fe(III)-NTA/Cu(II) and dopamine was investigated. 8-oxodG formation increased significantly in the presence of Fe(III)-NTA/Cu(II) and dopamine; however, it did not increase in the presence of Fe(III)-NTA, Cu(II), or dopamine alone (Fig. 1). Although Mn(II) did not induce 8-oxodG formation in the presence or absence of dopamine, the Fe(III)-NTA/Cu(II)-mediated oxidative DNA damage by dopamine increased significantly after adding Mn(II). The increasing effect of Mn(II) on the Cu(II)-mediated oxidative DNA damage was more effective than that on the Fe(III)-NTA-mediated damage.

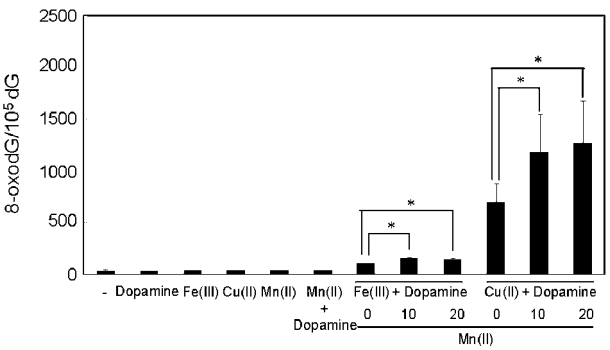

Fig. 1. Enhanced Fe(III)/Cu(II)-dopamine-mediated Oxidative DNA Damage Caused by Mn(II)

Concentrations: 100 μM base (DNA), 10 μM (dopamine), 20 μM (metals, unless otherwise indicated). $^{*}p > 0.05$, *vs.* sample consisting of Fe(III)/Cu(II) and dopamine.

Effect of Mn(II) on the Time Course of H_2O_2 Production in the DNA-damaging System

The effect of Mn(II) was investigated on the time course of H_2O_2 production in the DNA-damaging system consisting of dopamine and Cu(II). In the absence of Mn(II), H_2O_2 production was detected 20 min after addition of Cu(II) and reached a maximum at 45 min (Fig. 2). However, in the presence of 10 or 20 μM Mn(II), H_2O_2 production was detected at 15 or 10 min, respectively, and was maximum at 20 min.

Mn(II)-dependent Decrease in $O_2^{\cdot-}$

The effect of Mn(II) on $O_2^{\cdot-}$ was determined by the nitrous acid method. A xanthine-xanthine oxidase system was used for $O_2^{\cdot-}$ formation. The amount of $O_2^{\cdot-}$ decreased with an increasing Mn(II) concentration (Fig. 3). Mn(II) did not inhibit xanthine oxidase activity based on the production of uric acid, a final oxidized product of xanthine

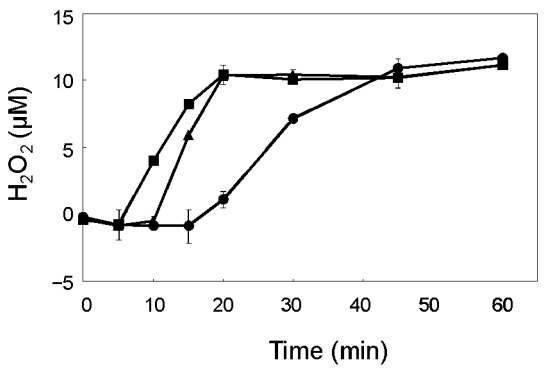

Fig. 2. Increased H_2O_2 Generation by Mn(II) in the Reaction of Dopamine with Cu(II)

Mn concentrations: 0 μM (circles), 10 μM (triangles), and 20 μM (squares) in the presence of Cu (20 μM) and dopamine (10 μM). Data are presented as means ± S.D. of at least three independent reactions.

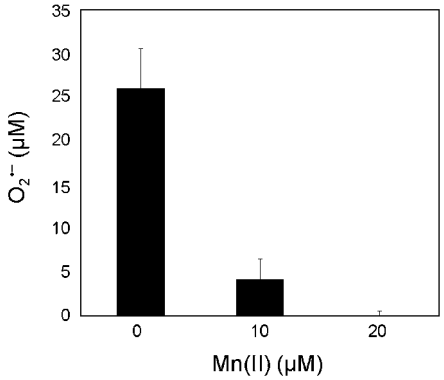

Fig. 3. Decrease in $O_2^{\cdot-}$ by Mn(II) in the Xanthine-xanthine Oxidase System

Data are presented as means ± S.D. of at least three independent reactions.

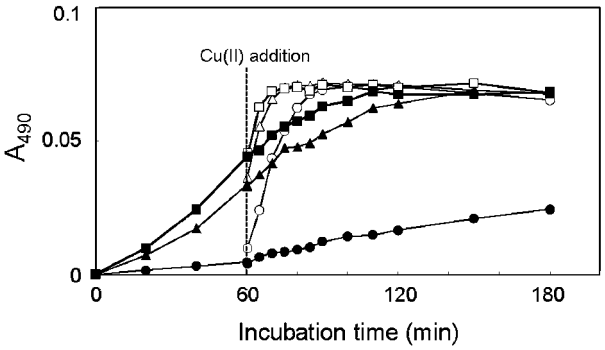

Fig. 4. Enhanced Cu(II)-mediated Dopamine Oxidation by Mn(II)

Mn concentrations: 0 μM (circles), 10 μM (triangles), and 20 μM (squares) in the presence (white symbols) or absence (black symbols) of 20 μM Cu.

(data not shown). $O_2^{\cdot-}$ decreased with increasing Mn(II) concentration in the DNA-damaging system of dopamine-Cu(II) (data not shown).

Mn(II)-dependent Promotion of Cu(II)-mediated Dopamine Oxidation

The effect of Mn(II) on the dopamine redox reaction with Cu(II) was determined by UV spectrophotometry. Dopamine is oxidized to aminochrome, which shows maximum absorption at 490 nm.[20] Absorption at 490 nm increased with increasing Mn(II) concentration (Fig. 4). Mn(II)-dependent aminochrome formation was dramatically promoted in the presence of Cu(II).

DISCUSSION

We demonstrated that Mn(II) enhances 8-oxodG formation and accelerates H_2O_2 production and provided with the first evidence that Mn(II) decreases the $O_2^{\cdot-}$ generated in the DNA damaging system. These data strongly suggest that Mn(II) enhances oxidative DNA damage through SOD-like activity that converts $O_2^{\cdot-}$ to H_2O_2, which is involved in the DNA damage mediated by Fe(III)/Cu(II) and catechol compounds. The enhancing effect of SOD on the DNA damage induced by Cu(II) and H_2O_2 has been reported.[21] Mn(II) probably exerts SOD activity without protein, although its activity would be restricted. Mn(II) is oxidized to Mn(III) coupled with the conversion of $O_2^{\cdot-}$ to H_2O_2.[22] Mn(III) may accelerate dopamine oxidation because it oxidizes hydroquinones.[23] In addition, the decomposition of $O_2^{\cdot-}$ by Mn(II) would also promote dopamine oxidation because reduction of $O_2^{\cdot-}$ coupled with dopamine oxidation enhances oxygen composition.[16]

We showed for the first time that Mn(II) enhanced the oxidative DNA damage induced by Cu(II) more effectively than that induced by Fe(III)-NTA in the presence of dopamine. Cu(II) is closely located to DNA, and its reduced form Cu(I) tightly binds to DNA and introduces a conformational change.[24] The bound Cu(I) anchors the H_2O_2 that attacks DNA by forming a DNA-Cu(I)-OOH complex.[16] In contrast, Fe(III)-NTA provides hydroxyl radicals far from DNA. Therefore, the accelerated supply of H_2O_2 by Mn(II) may have enhanced the DNA damage in the Cu(II)-dopamine system compared to that in the Fe(III)-NTA.

Some crosstalk through transporters occurs during metal metabolism. Divalent metal transporter 1 (DMT1) is one of the transporter proteins for divalent metals including Mn(II), Cu(II), and Fe(II). Exposure to Mn(II) induces DMT1 upregulation and increases intracellular Fe concentration.[4,25] Increased DMT1 expression and Fe accumulation were observed in an animal model of PD treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). A mutation that impairs DMT1 function protects animals from the neurotoxins MPTP and 6-hydroxydopamine.[26] Increased Cu and Mn contents were observed in rat corpus striatum after quinolinate intrastriatal injection.[27] Together with these findings, the combined action of Mn with Fe/Cu demonstrated in the present study may contribute to Mn-induced neurodegenerative Parkinsonism.

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