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Original Article

The Effect of Heavy Metals on Nicotinamide *N*-methyltransferase Activity *In Vitro* Relating to Parkinson's Disease

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Abstract

Objective: The aims of this study were to determine the effects of heavy metals such as manganese on nicotinamide *N*-methyltransferase (EC 2.1.1.1) (NNMT) activity and to consider the possibility of involvement of NNMT activation in the pathogenesis of heavy metal induced Parkinson's disease.

Methods: NNMT activity in supernatants separated from brain, liver and kidney homogenates of 5 elderly male Wistar rats by centrifugation were measured by high performance liquid chromatography system with fluorescence. NNMT activity under the conditon of 0.5 or 5.0 mM Mn^{2+}, Fe^{2+}, Cu^{2+} or Cd^{2+} was compared with control (no metal ion existence).

Results: NNMT activities in rat brain, liver and kidneys were significantly decreased by Cu^{2+}, and those in the liver and kidneys were significantly decreased by Cd^{2+}. Mn^{2+} reduced NNMT activity only in the liver. Fe^{2+} had no effect on NNMT activity.

Conclusions: No metal increased NNMT activity in this study, contrary to our hypothesis. Further study is needed to clarify the reason why the effects of Mn^{2+} and Fe^{2+} which have a high relevance to Parkinson's disease on NNMT activity differ from those of Cu^{2+} and Cd^{2+}.

Key words: Parkinson's disease, nicotinamide *N*-methyltransferase, heavy metal, sulfhydryl group

Introduction

Manganese neurotoxicity was first reported in the early 1800s in workers from a manganese ore crushing plant in France (1). Chronic exposure to manganese produces a Parkinsonian syndrome especially in miners, welders, and ferroalloy and battery manufacture workers. Furthermore, central nervous system disorders have been attributed to manganese toxicity in patients receiving long-term parenteral nutrition (2). Several studies, in addition, indicated that some heavy metals, such as manganese, iron and copper, increased the risk of Parkinson's disease (PD). Gorell et al. found a significant association between PD and exposure to copper, manganese and combinations of lead-copper, lead-iron and iron-copper in workers with more than 20 years' occupational exposure in a population-based case-control study in Detroit (3). A high intake of iron, especially in combination with high manganese intake, might be related to risk for PD (4).

Since 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was discovered to produce symptoms of PD (5), its analogs (e.g. *N*-methylated β-calbolines or tetrahydroisopuinolines) have been proposed as inducers of idiopathic PD (6, 7). MPTP is converted to 1-methyl-4-phenylpyridinium ion (MPP^+) in brains, and it inhibits NADH:ubiquinone oxidoreductase (complex I) of the mitochondrial electron transport chain (8). PD induction by MPP^+ was associated with defects in complex I. Mizuno et al. demonstrated disruption of complex I subunits in brain tissues from patients affected with PD (9), and researchers have described losses in electron transport chain activity in various tissues from patients with PD (9–11). Therefore, mitochondrial dysfunction followed by complex I defects may play an important role in the pathogenesis of idiopathic PD.

1-methylnicotinamide (MNA) is produced via nicotinamide *N*-methyltransferase (EC 2.1.1.1) (NNMT) from nicotinamide, and it is structurally related to MPP^+. Urinary excretion of MNA was increased and the excretion of pyridone, its catabolic product, was reduced in PD patients (12). PD patients had higher levels of NNMT activity and protein in brain tissue than controls (13). MNA destroyed several subunits of cerebral

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complex I, especially 30 kDa protein (14), and a MNA injection in rat substantia nigra pars compacta significantly decreased dopamine content in striatum (15). Therefore, MNA is a suspect agent for idiopathic PD.

Therefore, we hypothesized from these results that metal induced PD was caused by excess production of MNA, and demonstrated the effects of some heavy metals on NNMT activity in rat brains. To investigate organ specificity, the effect of metal ions on NNMT activity in rat brains was compared with that in liver and kidneys.

Methods

Five elderly (about 6 months old) male Wistar rats that were purchased from Japan SLC Co., Ltd were killed by decapitation after concussion from a blow on the head. Their brains, livers and kidneys were removed rapidly and homogenized in four volumes of cold 5.0 mM potassium phosphate buffer (pH 7.5). The homogenates were centrifuged at 9,000×*g* for 20 min at 4°C. The supernatants were concentrated using Amicon Ultra-15 Centrifugal Filter Devices 10K (Millipore Corp.) and stored at –40°C prior to enzyme assay.

The enzyme assay and derivatization of MNA to a fluorescent substance were performed as described by Sano et al. (16) and Musfeld et al. (17), respectively, with some modifications.

The reaction mixture contained 50 mM Tris-HCl buffer (pH 8.6), 0.125 mM dithiothreitol (DTT), 1.0 mM nicotinamide, 1.0 mM S-adenosyl-L-methionine and 7.80, 27.0, 18.8 mg/ml protein derived from brain, liver and kidney tissues, respectively. The effects of metal ions (Mn^{2+}, Fe^{2+} and Cu^{2+}) on NNMT activity were determined by adding chloride salts of each metal to final concentrations of 0.5 and 5.0 mM. Because these concentrations were much higher than physiological levels, the effect of endogenous metals could be negligible. In addition, since cadmium has the potential to accumulate in kidneys and liver and produce toxicity, we also determined if Cd^{2+} altered the activity of these organs. After 40 min incubation at 37°C, the reaction was stopped with ethanol and 100 µM 1-ethylnicotinamide was added as an internal standard. The mixtures were centrifuged at 4,000×*g* for 10 min and the supernatants were used for the fluorometric assay of MNA. In preliminary experiments, we concluded that endogenous metals had no effect on the reaction because 1.0 mM EDTA had no effect on reaction velocity.

An aliquot (400 µl) of the supernatant, 100 µl of 1 M isonicotinamide and 200 µl of 100 mM acetophenone in ethanol were incubated for 10 min in a glass tube that was placed in an ice-water bath. A volume of 400 µl of 6 M NaOH was added and the mixtures were incubated in ice-water for 60 min. After 200 µl of formic acid (about 99%) was added, the mixtures were incubated for another 15 min. The samples were placed in a boiling water bath for 3 min. After cooling, the samples were filtered with 0.45 µm PTFE membranes (Minisart SRP4, Sartorius K.K.) and transferred into vials. Each sample (50 µl) was injected into the high performance liquid chromatography (HPLC) system.

The HPLC system consisted of a Waters 510 HPLC pump, Waters 717 plus autosampler, Inertsil ODS-3V 5 µm column (GL Science, Inc., 4.6×250 mm) with Cartridge guard column GL-cart Inertsil ODS-3 5 µm and Waters 470 scanning fluorescence detector with excitation and emission wavelengths set at 366 and 418 nm, respectively. Chromatogram analysis was carried out with Millenium software 2010. The mobile phase was acetonitrile-water-formic acid (22:80:2, v/v/v). The column

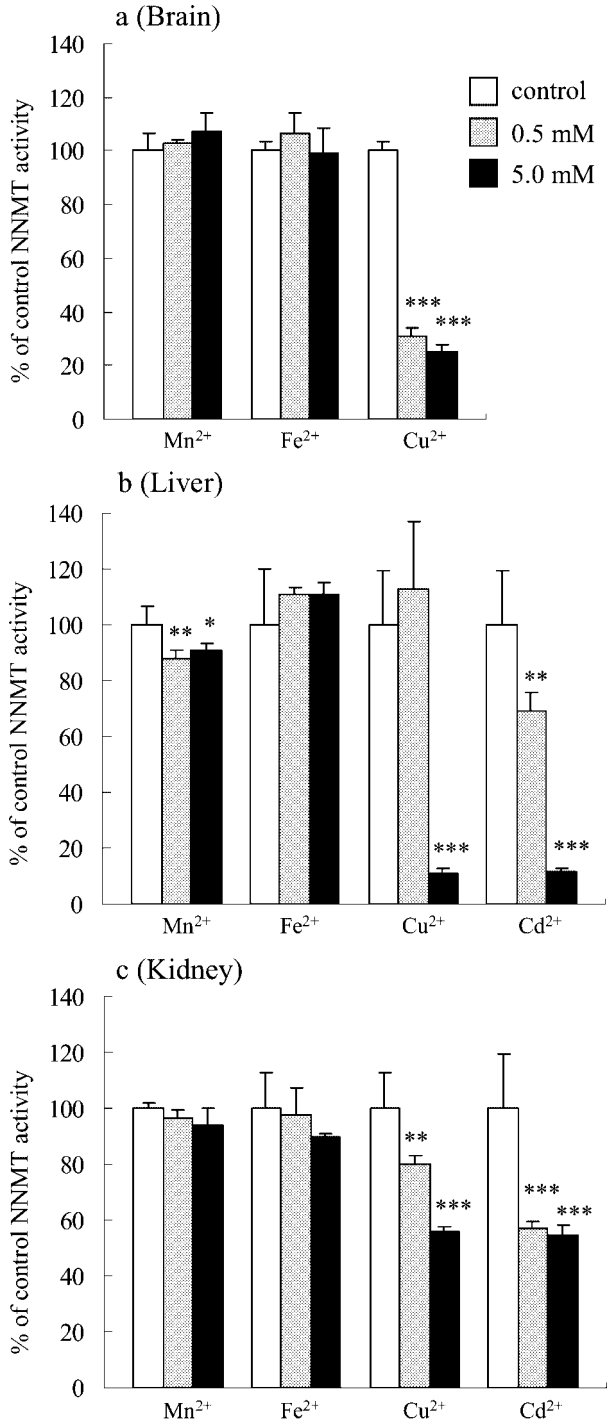

Fig. 1 Effect of metal ions on NNMT activity in rat brain (a), liver (b) and kidneys (c). Activity was expressed as a percentage of activity found in controls. The white bar, spotted bar and black bar indicate control activity and activity in the presence of 0.5 mM and 5.0 mM of each metal ion, respectively. Each value is mean and standard deviation (n=5). *, ** and *** represent significant difference at $p<0.05$, $p<0.01$ and $p<0.001$, respectively, compared with control.

temperature was ambient and the flow rate was 1.0 ml/min.

Trace amounts of NNMT activity in mammalian brains or kidneys are difficult to detect using HPLC with fluorescence. Therefore, centrifugal filtration was used to obtain higher concentrations of the enzyme in sample solutions. A large amount of isonicotinamide was used in the derivatization reaction to block deamination of MNA caused by strong alkaline conditions and prevent non-derivatization. These modifications enabled the detection of NNMT activity in rat brains.

One-way factorial ANOVA followed by Tukey-HSD post-hoc test was used to compare groups.

The experiments presented in this paper were approved by the Animal Research Committee of Fukushima Medical University (No. K03021).

Results

NNMT activities in the rat brain, liver and kidneys were 0.202±0.011, 2.05±0.67 and 0.137±0.018 (mean±SD) nmol/mg protein/h, respectively. NNMT activity in the brain was significantly ($p<0.001$) reduced to 30.6±3.5 and 25.1±2.7% of control in the presence of 0.5 and 5.0 mM Cu^{2+}, respectively (Fig. 1a). In contrast, Mn^{2+} and Fe^{2+} had no effect on activity.

NNMT activity in the liver was significantly decreased by Mn^{2+}, Cu^{2+} and Cd^{2+} (Fig. 1b). NNMT activity was about 90% of control in the presence of both concentrations of Mn^{2+}. Only the high concentration of Cu^{2+} significantly reduced NNMT activity to 11.2±1.4% of control. Cd^{2+} reduced NNMT activity to 69.2±6.4 and 11.7±1.0% of control under 0.5 and 5.0 mM concentration, respectively.

NNMT activity in the kidneys was significantly decreased by Cu^{2+} and Cd^{2+} (80.0±3.0 and 55.7±1.8% of control in the presence of 0.5 and 5.0 mM Cu^{2+}, respectively; 57.2±2.3 and 54.7±3.3% of control in the presence of 0.5 and 5.0 mM Cd^{2+}, respectively) (Fig. 1c). In contrast, Mn^{2+} and Fe^{2+} had no effect on kidney NNMT activity. A similar pattern was observed in the brain.

Discussion

The neurotoxicity of manganese and iron is generally thought to be associated with oxidative stress. The oxidative toxicity of manganese in the brain has been observed in the substantia nigra and dopaminergic neurons (2). Manganese specifically accumulated in mitochondria, inhibited respiratory chain enzymes, produced reactive oxygen species and decreased monoamine oxidase activity in rat livers and brains (18). Therefore, manganese treatment was associated with mitochondrial respiratory chain dysfunction and neuronal death.

Some enzymes require specific metal ions for their activity, and some metal ions activate or inhibit some enzymes. For example, most kinases require magnesium ion for their activity. For example, manganese, nickel and cobalt ions increased the activity of rat brain phosphatase approximately two-fold, while zinc and copper ions reduced the activity (19). A mechanism by which MNA could cause PD was proposed, in that the superoxide anion formed by MNA directly destroys complex I subunits via mitochondria or indirectly destroys it via mitochondrial DNA destruction, resulting in neuronal death (20). Accordingly, we hypothesized in our study that the metals associated with PD increased NNMT activity that resulted in excess MNA.

However, Mn^{2+}, Cu^{2+} and Cd^{2+} had inhibitory effects on NNMT activity in our study. Some heavy metal ions, such as Hg^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+}, have high affinity for sulfhydryl groups (-SH group), and they can inhibit the activity of enzymes with -SH groups (SH-enzyme) (19, 21). A similar enzyme, plant S-adenosyl-L-methionine: nicotinic acid *N*-methyltransferase (EC 2.1.1.7), requires -SH groups for enzyme conformation or catalytic activity (22). Our study showed that Cu^{2+} inhibited NNMT activity in all tissues and Cd^{2+} reduced the activity in both the liver and kidneys. Therefore, NNMT could be a SH-enzyme. Mn^{2+} reduced NNMT activity only in the liver and Fe^{2+} had no effect on all tissues. Since Mn^{2+} has a low affinity for -SH group (23), Mn^{2+} could inhibit the liver NNMT activity in other ways. No metal increased NNMT activity in this experiment. However, it is interesting to note that the effects of Mn^{2+} and Fe^{2+} which have a high relevance to PD on NNMT activity differ from those of Cu^{2+} and Cd^{2+}.

While the reaction mixture contained 0.125 mM DTT to protect enzyme -SH groups, NNMT activity was inhibited strongly by Cu^{2+} and Cd^{2+}. Possibly the power of DTT was insufficient to protect -SH groups and an inhibitory effect of Mn^{2+} on NNMT was observed. Therefore, it is important to consider the experimental conditions and clarify why a different effect of metal ion on NNMT activity was observed between two groups, the Mn^{2+} and Fe^{2+} group and the Cu^{2+} and Cd^{2+} group. Higher concentrations of DTT or lower concentrations of each metal in the reaction mixture could attenuate SH-group-mediated NNMT inhibition, and the effect of these metals on NNMT via an other site would be clear. Furthermore, it may be necessary to evaluate NNMT induction by metal ions *in vivo* to verify our hypothesis.

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