

Multipoint analysis of reduced ^{125}I -*meta*-iodobenzylguanidine uptake and norepinephrine turnover in the hearts of mice with MPTP-induced Parkinsonism

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Abstract

Introduction: ^{125}I -Meta-iodobenzylguanidine (MIBG) cardiac uptake is reduced in mice with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinsonism, although the cause of disturbance of norepinephrine (NE) turnover is unclear.

Methods: C57BL6 mice (15-week-old) were divided into six groups (n=14 each) according to the timing of MPTP injection (40 mg/kg) before ^{125}I -MIBG: A, control (no MPTP injection); B, 1 day; C, 4 days; D, 7 days; E, 21 days; F, 7, 14, and 21 days. ^{125}I -MIBG (0.185 MBq) was injected and the cardiac percentage injected dose per gram of tissue (%ID/g), dopamine (DA), and NE concentrations were measured. The cardiac maximal binding potential (B_{max}) of NE transporter (NET) was also calculated in 20 mice per group.

Results: The %ID/g of B, C, D, E, and F mice were significantly lower than in A, those of C, D, and E were significantly higher than in B, and that of F was significantly lower than in E. The DA concentrations were similar among all groups. The NE concentrations of B, C, and F mice were significantly lower than in A, while those of C, D, E, and F were

significantly higher than in B, and that of F was significantly lower than in E. The B_{\max} of NET in B was significantly lower than in A.

Conclusions: Thus, MPTP causes rapid reductions in cardiac ^{125}I -MIBG uptake and B_{\max} of NET, followed by partial recovery of ^{125}I -MIBG uptake. Changes in cardiac ^{125}I -MIBG uptake and NE turnover were closely related in postganglionic cardiac sympathetic nerve terminals in mice with MPTP-induced Parkinsonism.

1. Introduction

Meta-iodobenzylguanidine (MIBG) is a physiological analogue of norepinephrine (NE) that is actively transported into NE granules of sympathetic nerve terminals by the norepinephrine transporter (NET) [1,2].

^{123}I -MIBG is a radiolabeled version used to evaluate myocardial sympathetic nerve dysfunction in heart disease and to diagnose ischemic heart disease, cardiomyopathy, and heart failure.

Patients with Parkinson's disease show sympathetic nerve dysfunction in both the central and peripheral nervous systems [3-5], which is reflected by a reduced cardiac uptake of ^{123}I -MIBG. This finding in patients with Parkinson's disease has attracted recent attention, with the reduction detectable before other autonomic disturbances and clinical features become evident including bradykinesia, resting tremor, rigidity, and response to levodopa. Thus, cardiac sympathetic nerve dysfunction might be a major pathology in Parkinson's disease, and ^{123}I -MIBG uptake is used increasingly in clinical examinations of patients with the disease [6-10]. However, changes in NE turnover observed in hearts with reduced ^{123}I -MIBG uptake remain unresolved, particularly as ^{123}I -MIBG has been associated with neuropathological findings in postmortem human heart [11-13]. Clarifying

these changes in the hearts of patients with Parkinson disease might therefore help to elucidate the pathology of sympathetic nerve dysfunction.

The activity of the neurotoxic chemical agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) involves several steps. First, MPTP readily enters the brain and is converted, possibly in the glia, by monoamine oxidase B (MAO-B) to MPP⁺, which is most likely the toxic agent. The second step is the fairly selective uptake of MPP⁺ by dopaminergic terminals in the striatum via a monoamine transporter and subsequent intraterminal vesicular storage [14,15]. MPTP produces symptoms resembling those of Parkinson's disease in humans and monkeys [16,17], and induces an almost complete, permanent, and selective degeneration of nigrostriatal dopaminergic neurons in a specific strain of mice [18,19]. Several studies have demonstrated reduced cardiac NE concentrations in mice treated with MPTP [20-23]. Furthermore, pretreatment of mice with MPTP significantly reduces cardiac ¹²⁵I-MIBG uptake [24-27], similar to the effects observed in patients with Parkinson's disease.

Investigating the mechanism of reduced cardiac ¹²³I-MIBG uptake and NE turnover using an MPTP-induced mouse model of Parkinson's disease may help clarify the mechanism of sympathetic nerve dysfunction in human patients. However, previous preclinical mice studies were designed using a

one-point, acute-phase analysis after MPTP administration [24-27]. A multipoint analysis with a long-term observation period is necessary to investigate chronic neurodegenerative diseases like Parkinsonism.

The present experimental study investigated the long-term changes in cardiac ^{125}I -MIBG uptake and NE turnover by multipoint analysis in an MPTP-induced mouse model of Parkinson's disease.

2. Materials and methods

All experimental animal protocols were pre-approved by the Animal Experiment Committee of the University of Tsukuba and were conducted in accordance with the "Principles of Laboratory Animal Care" (NIH Publication No. 86-23, Revised 1985).

2.1. Cardiac ^{125}I -MIBG uptake and NE synthesis pathway

Experiments were conducted in 84 C57BL6 mice (age, 15 weeks, mean weight, 23.1 ± 1.6 g, \pm standard deviation (SD)). The mice were fed standard laboratory chow and given free access to water. They were divided into six groups of 14 mice each according to the timing of the last intraperitoneal (i.p.) injection of 40 mg/kg of MPTP (Sigma-Aldrich, St. Louis, MO; dissolved in 0.9% saline, divided into four injections given at 12-h intervals)

prior to the injection of ^{125}I -MIBG: group A: control, group B: 1 day before, group C: 4 days before, group D: 7 days before, group E: 21 days before, and group F: unlike groups B-E, mice of this group received 3 injections at 7, 14 and 21 days before injection of ^{125}I -MIBG.

A total of 0.185 MBq of ^{125}I -MIBG (Fujifilm RI Pharma Co, Tokyo, Japan) with a specific activity of 9.25 MBq/mg was injected through the lateral tail vein. The mice were sacrificed by cervical dislocation 4 h later, blood was collected, and the hearts were dissected out immediately. The hearts were weighed, the radioactivity was measured using a gamma counter to calculate the percentage injected dose per gram of tissue (%ID/g) of ^{125}I -MIBG.

The concentrations of NE and dopamine (DA) in the heart as well as NE levels in plasma were measured in all mice. A mixture of 0.3 mL of deproteinized solution (6% perchloric acid solution/3.5 M sodium acetate, 5:1 v/v) and 0.6 mL of heart tissue or 0.6 mL of plasma was stirred and centrifuged (1200 x *g*) at 4°C for 15 min. The clear supernatant was subjected to high-performance liquid chromatography (HPLC) via an autosampler. Free NE and DA concentrations were measured in 250 μl of the supernatant automatically injected into the analyzer. Using a microprocessor-controlled column-switching device, each sample was

delivered to a precolumn (TSK precolumn CA1, 7.5 x 7.5 mm; Tosoh, Tokyo) equilibrated with eluant A (0.06 M sodium acetate, anhydrous buffer containing 4.8% acetonitrile). The pass fraction was delivered to a second precolumn (TSK precolumn CA2, 4 x 60 mm; Tosoh) also equilibrated with eluant A. The absorbed catecholamines (CAs) were eluted with eluant B (0.32 M ammonium nitrate buffer) and delivered to an analytical column (Wacasil-II 5C18RS, 4.6 x 150 mm; Wako Industries, Osaka, Japan) equilibrated with eluant B. Each separated CA was delivered to a reaction coil (60°C) with fluorogenic reagent C (diphenylethylenediamine in a 50% ethanol solution) and converted to diphenylethylenediamine derivatives. The fluorescent intensity of each eluate from the reaction unit was measured at a wavelength of 483 nm with an excitation wavelength of 347 nm.

Tyrosine (TYR) concentrations in the heart were measured in mice of groups A, B, D, and F using 0.6 ml of heart tissue mixed with 0.3 ml of deproteinized solution (6% perchloric acid solution/3.5 M sodium acetate, 5:1 v/v) before centrifugation (1200 x g) at 4°C for 15 min. The amino acid concentrations in the heart tissue samples were measured using an automatic amino acid analyzer (L-8500, L-8500A; Hitachi High-Technologies Corporation, Tokyo) with cation-exchange chromatography and

spectrophotometric detection of the separated amino acids after a postcolumn reaction with ninhydrin reagent.

2.2. Cardiac NET density and striatum DA concentration

In these experiments, 120 C57BL6 mice (age, 15 weeks, weight, 25.5 ± 1.7 g) were divided into six groups of 20 mice each, similar to the groups used in experiment 2.1. The mice were sacrificed, and their hearts and brains were dissected out immediately.

The maximal specific binding (B_{\max}) of cardiac NET, whose function is NE reuptake, was calculated. Membrane preparations for the binding assays were made as described previously [28,29]. Briefly, mouse hearts were homogenized in 20 volumes of ice-cold 250-mM sucrose buffer (5 mM Tris, 1 mM $MgCl_2$, and 250 mM sucrose) with a 30-s burst in a tissue homogenizer (Kinematica Polytron PT 10/35; Brinkmann Instruments, Westbury, NY) set at speed 6. Homogenates were centrifuged at $750 \times g$ for 10 min. The pellets were discarded, and the supernatant was recentrifuged at $20,000 \times g$ for 20 min. Pellets were resuspended in 10 volumes of ice-cold 50 mM Tris-HCl buffer (50 mM Tris, 5 mM KCl, and 120 mM NaCl, pH 7.4) and recentrifuged to obtain a pellet. The obtained pellets were resuspended in 5 volumes of Tris-HCl buffer and stored at $-80^\circ C$ until use.

The protein concentrations were measured using the method described by Lowry [30]. Binding assays were performed as described previously, with minor modifications [28,29]. Briefly, [³H]desipramine binding was determined by incubating aliquots of membrane suspension with [³H]desipramine (0.25–0.30 nM) in a final volume of 250 µl for 30 min at 25°C. The binding was terminated by adding 5 ml of ice-cold 50 mM Tris–HCl buffer (pH 7.4) and filtering through glass microfiber filters (GF/B; Whatman, Maidstone, Kent, UK) with a 24-channel cell harvester (M-48; Brandel, Gaithersburg, MD). Finally, each filter was rinsed three times with 5 ml of ice-cold 50-mM Tris–HCl buffer (pH 7.4). The radioactivity on the filters was determined using a liquid scintillation counter (LSC-5300, Aloka, Tokyo, JAPAN). Nonspecific binding was defined using 100 µM nisoxetine. The B_{\max} was calculated from the specific binding values using Scatchard analyses. The B_{\max} was calculated in three independent experiments.

The brain was placed in ice-cold physiological saline and allowed to cool for 1 min. A 1-mm slice passing through the striatum was transferred to a thin metal plate and placed on dry ice. After freezing, samples from the striatum (2 mm in diameter) were punched out. Pooled samples were assayed for DA concentration, and the measurement repeated 3 times in the same manner as for the heart.

2.3. Statistical analysis

All data were expressed as mean \pm SD. All results were analyzed using a one-factor analysis of variance with the Bonferroni/Dunn correction for intergroup comparisons. The adjusted *P* values were calculated as the raw *P* value \times (the number of groups $- 1$). An adjusted *P* value < 0.05 was considered statistically significant.

3. Results

3.1. Striatum DA concentration

The striatum DA concentrations were 49.8 ± 0.8 ($\times 10^5$ pg/wet g) in group A, 2.2 ± 0.0 in group B, 19.0 ± 0.1 in group C, 18.7 ± 0.1 in group D, 16.6 ± 0.5 in group E, and 3.3 ± 0.0 in group F. The striatum DA concentrations of groups B, C, D, E, and F were significantly lower than that of group A ($P < 0.001$, for all comparisons); those of groups C, D, and E were significantly higher than that of group B ($P < 0.001$, for all comparisons); those of groups E and F were significantly lower than that of group C ($P < 0.001$, for both comparisons) and group D ($P < 0.001$, for both comparisons); and, that of group F was significantly lower than that of group E ($P < 0.001$). Thus, group A had an extremely high striatum DA

concentration, groups B and F had extremely low concentrations, and groups C, D, and E had similar, intermediate values (Figure 1).

3.2. Cardiac ^{125}I -MIBG uptake

The %ID/g values of cardiac ^{125}I -MIBG uptake were 18.4 ± 3.5 (%/g) in group A, 5.2 ± 0.7 in group B, 7.8 ± 1.0 in group C, 9.3 ± 1.8 in group D, 9.7 ± 1.6 in group E, and 7.3 ± 1.1 in group F. The %ID/g values of groups B, C, D, E, and F were significantly lower than that of group A ($P < 0.001$, for all comparisons), while those of groups C, D, and E were significantly higher than that of group B ($P < 0.005$, < 0.001 , < 0.001 , respectively) and that of group F was significantly lower than that of group E ($P < 0.005$). Thus, group A had extremely high %ID/g values and group B values were extremely low, while groups B, C, D, and E had gradually increasing values (Figure 2).

3.3. B_{max} of Cardiac NET

The B_{max} values of cardiac NET were 320.0 ± 34.9 (fmol/mg protein) in group A, 246.8 ± 16.7 in group B, 267.4 ± 11.9 in group C, 267.1 ± 14.8 in group D, 294.5 ± 5.7 in group E, and 281.3 ± 10.5 in group F. The B_{max} value of cardiac NET in group B was significantly lower than that in group A ($P < 0.005$) (Figure 3).

3.4. Cardiac TYR concentration

The cardiac TYR concentrations were 68.8 ± 10.9 (pg/wet g) in group A, 80.3 ± 21.1 in group B, 74.1 ± 14.7 in group D, and 80.3 ± 36.3 in group F. No significant differences in cardiac TYR concentrations were observed among the groups (Figure 4).

3.5. Cardiac DA concentration

The cardiac DA concentrations were 2.3 ± 0.8 ($\times 10^4$ pg/wet g) in group A, 1.5 ± 0.4 in group B, 1.8 ± 0.6 in group C, 1.8 ± 1.1 in group D, 1.9 ± 0.4 in group E, and 1.7 ± 0.7 in group F. The mean cardiac DA concentration in group B was slightly lower than that in group A, but the difference was not significant (Figure 5).

3.6. Cardiac NE concentration

The cardiac NE concentrations were 6.8 ± 0.6 ($\times 10^5$ pg/wet g) in group A, 2.2 ± 0.5 in group B, 4.9 ± 0.6 in group C, 5.9 ± 1.1 in group D, 6.5 ± 0.7 in group E, and 5.0 ± 1.2 in group F. The cardiac NE concentrations of groups B, C, and F were significantly lower than that of group A ($P < 0.001$, for all comparisons), while those of groups C, D, E, and F were significantly higher

than that of group B ($P < 0.001$, for all comparisons), and those of groups D and E were significantly higher than that of group C ($P < 0.05$, < 0.001 , respectively). The cardiac NE concentration of group F was significantly lower than that of group E ($P < 0.001$). Thus, group A had an extremely high mean cardiac NE concentration and group B values were extremely low, while groups B, C, D, and E had gradually increasing concentrations (Figure 6).

3.7. NE plasma concentration

The NE plasma concentrations were 5.0 ± 2.0 ($\times 10^3$ pg/wet g) in group A, 2.3 ± 1.5 in group B, 5.1 ± 2.1 in group C, 4.3 ± 2.3 in group D, 3.3 ± 1.8 in group E, and 4.0 ± 3.5 in group F. The NE plasma concentration of group B was significantly lower than that of group A ($P < 0.05$), while that of group C was significantly higher than that of group B ($P < 0.01$) (Figure 7).

4. Discussion

The C57BL6 mouse has been used in many studies to investigate various aspects of Parkinson's disease [18-23]. This mouse is highly sensitive to MPTP, which induces dose-dependent neurotoxic effects [31-34]. This study used the modified protocol of Marien et al. [34], in which 40 mg/kg of

MPTP was administered (10 mg/kg, i.p., 4 times at 2-h intervals). The reduced striatum DA concentrations in group D mice (7 days after MPTP administration) were similar to the results of previous reports, confirming that the model used was suitable to represent MPTP-induced Parkinson's disease. The striatum DA concentration results also showed a rapid and large decrease after 1 day, followed by a partial recovery to a plateau value 4–7 days after MPTP administration, with long-term administration inducing further reductions. Indeed, the decreased mean striatum DA concentration of 93% in group F was significantly larger than any reported previously [31-34]. The slightly lower striatum DA concentrations in mice at 21 days after MPTP administration compared with 7 days could represent subacute damage in the striatum. We hypothesized that group B represents an extremely acute-stage model of Parkinson's disease, while groups C and D represent acute-stage models, group E represents a subacute-stage model, and group F represents a long-term repeated neuronal damage model.

The cardiac ^{125}I -MIBG uptake pattern closely followed the changes in striatum DA concentration, suggesting that cardiac ^{125}I -MIBG uptake may reflect the changes and severity of MPTP-induced Parkinsonism. The time course of cardiac ^{125}I -MIBG uptake indicates that cardiac sympathetic nerves are rapidly and severely damaged on day 1 and then gradually

recover from 4 to 21 days after MPTP administration. Further, the nerves are damaged to an even greater extent after long-term repeated MPTP administration. The only discrepancy between the cardiac ^{125}I -MIBG uptake and striatum DA concentration patterns lay in the delayed recovery in cardiac ^{125}I -MIBG uptake in the acute stage compared to the striatum DA concentrations. Thus, the sensitivity of the striatum to MPTP may differ slightly from that of cardiac sympathetic nerves.

The B_{\max} values of cardiac NET suggested a rapid decrease in cardiac NET density on day 1 after MPTP administration. The minimal recovery after that further indicated that damage to the cardiac sympathetic nerve terminal is probably almost completely irreversible on day 1 after MPTP administration. Although the patterns of B_{\max} of cardiac NET and cardiac ^{125}I -MIBG uptake were similar, the change in B_{\max} of cardiac NET was not as dramatic. Consequently, ^{125}I -MIBG would probably more reliably and readily detect small changes in NET in the cardiac sympathetic nerve terminals. The B_{\max} values of NET are an index of NET expression but it does not directly represent the expression of NET protein. Therefore, the estimation of the expression of NET protein with Western blot analysis and the expression of NET mRNA with quantitative RT-PCR analysis would

match the changes in ^{125}I -MIBG uptake. Further studies of expression of NET protein and mRNA are required.

The cardiac TYR, DA, and NE concentrations shown here suggested that among the components of the NE synthesis pathway, only cardiac NE concentration is rapidly reduced on day 1 after MPTP administration, followed by a gradual recovery over the subsequent 4–21 days. In fact, the cardiac NE concentration reduced even further after long-term repeated MPTP administration. The cardiac DA concentration exhibited a pattern similar to the cardiac NE concentration, although with only minimal and non-significant differences among the groups. The cardiac TYR concentrations of groups A, B, D, and F were also similar. A reduction in TYR hydroxylase, an important enzyme in the synthesis of DA from TYR, is often associated with cardiac sympathetic nerve disturbances in patients with Parkinson's disease [13,35,36]. However, the difference between the cardiac DA and NE concentration patterns here was more marked than that between the TYR and DA concentrations. In addition, the mean cardiac NE concentration in group B was reduced to 33% of that in group A. According to the process of NE metabolism described by Eisenhofer et al. [37], most of the NE released from vesicles at the sympathetic nerve terminal is taken up again by sympathetic nerves. The axoplasmic NE is either sequestered into

vesicles or metabolized to dihydroxyphenylglycol in the normal human heart at rest. Because axoplasmic NE represents 90% of the NE that leaks from vesicles and only 10% of the NE that is released by sympathetic nerves and then taken up again by NET, NE synthesis is probably already severely damaged on day 1 after MPTP administration. However, these previous data represented the situation at rest, and the current study did not recapitulate the same circumstance. Thus, it added no data about the ratio of NE that is released and taken up in cardiac sympathetic nerve terminals immediately after MPTP administration, nor can it speak to the degree of cardiac NE synthesis obstruction after MPTP administration. It is therefore difficult to explain the recovery in cardiac NE concentration and the reduction after long-term repeated MPTP administration based on only the cardiac NET density, which showed little change. The cardiac NE concentration is likely to be greatly influenced by the pathway of NE synthesis in sympathetic nerves, with cardiac NET contributing significantly to the cardiac NE concentration on day 1 after MPTP administration, then to a lesser degree thereafter and after long-term repeated MPTP administration.

The plasma concentration of NE decreases rapidly and then recovers after MPTP administration [38]. In support of this, the present results showed a reduced NE plasma concentration on day 1 and subsequent

recovery over a period of 4 days. This pattern of temporary decrease in plasma NE might reflect an acute adrenal gland insufficiency caused by MPTP. The absence of significant difference among groups C, D, E, and F further suggested that the NE plasma concentration does not contribute to cardiac NE turnover after 7–21 days and after long-term repeated MPTP administration.

We clarified the long-term change of reduced cardiac ^{125}I -MIBG uptake and NE turnover using Parkinson's disease model mice in this study. In the next step, we plan to identify the molecules that are crucial for the control of cardiac NE turnover in Parkinson's disease. Ligands that reflect the activity of the molecules could provide more detailed information of sympathetic nerve dysfunction and make a breakthrough in the treatment for the sympathetic nerve dysfunction of patients with Parkinson's disease.

The multipoint analyses of cardiac ^{125}I -MIBG uptake and NE turnover using the same time scale after MPTP administration clearly showed that cardiac ^{125}I -MIBG uptake and NET density are rapidly reduced after MPTP administration, followed by the gradual recovery of ^{125}I -MIBG uptake. There was an even greater reduction of ^{125}I -MIBG after long-term repeated MPTP administration, coincident with NE synthesis in the mice heart.

5. Conclusion

Changes in cardiac ^{125}I -MIBG uptake and NE turnover are closely related in postganglionic cardiac sympathetic nerve terminals in the hearts of mice with MPTP-induced Parkinsonism.

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Figure legends

Fig. 1. Striatum DA concentrations in the six experimental groups. Data are mean \pm SD of 3 repeated measurement of homogenized sample from 20 mice in each group. Note that injection of MPTP reduced striatum DA concentrations. **** $P < 0.001$. Group A: control mice (no MPTP injection), group B: last injection of four 12-hourly injections of MPTP at 1 day before injection of ^{125}I -MIBG, group C: 4 days before, group D: 7 days before, group E: 21 days before, and group F: unlike groups B-E, mice of this group received 3 injections at 7, 14 and 21 days before injection of ^{125}I -MIBG.

Fig. 2. Cardiac ^{125}I -MIBG uptake. Uptake is expressed by (%ID/g). Data are mean \pm SD of 14 mice in each group. *** $P < 0.005$, **** $P < 0.001$. See Figure 1 for definitions of the six groups of mice.

Fig. 3. B_{\max} of cardiac NET. The B_{\max} value of cardiac NET in group B was significantly lower than that in group A. Data are mean \pm SD of 3 repeated measurement of homogenized sample from 20 mice in each group.

*** $P < 0.005$. See Figure 1 for definitions of the six groups of mice.

Fig. 4. Cardiac TYR concentrations. Data are mean \pm SD of 14 mice in each group. No significant differences were observed among groups A, B, D, and F. See Figure 1 for definitions of the four groups of mice.

Fig. 5. Cardiac DA concentrations. Data are mean \pm SD of 14 mice in each group. No significant differences among the groups were observed. See Figure 1 for definitions of the six groups of mice.

Fig. 6. Cardiac NE concentrations. Data are mean \pm SD of 14 mice in each group. * P <0.05, **** P <0.001. See Figure 1 for definitions of the six groups of mice.

Fig. 7. NE plasma concentrations. Data are mean \pm SD of 14 mice in each group. * P <0.05, ** P <0.01. See Figure 1 for definitions of the six groups of mice.