Protective Effects of *N*-acetyl-L-cysteine on the Reduction of Dopamine Transporters in the Striatum of Monkeys Treated with Methamphetamine

Kenji Hashimoto^{*1}, Hideo Tsukada², Shingo Nishiyama², Dai Fukumoto², Takeharu Kakiuchi², Eiji Shimizu¹ and Masaomi Iyo¹

¹Department of Psychiatry, Chiba University Graduate School of Medicine, Chiba, Japan; ²Central Research Laboratory, Hamamatsu Photonics K.K., Hamakita, Shizuoka, Japan

Several lines of evidence suggest that oxidative stress might contribute to neurotoxicity in the dopaminergic nerve terminals after administration of methamphetamine (MAP). We undertook the present study to determine whether intravenous administration of *N*-acetyl-L-cysteine (NAC), a potent antioxidant drug, could attenuate the reduction of dopamine transporter (DAT) in the striatum of monkey brain after administration of MAP. Positron emission tomography studies demonstrated that repeated administration of MAP (2 mg/kg as a salt, four times at 2-h intervals) significantly decreased the accumulation of radioactivity in the striatum after intravenous administration of [¹¹C]β-CFT. In contrast, the binding of [¹¹C]SCH 23390 to dopamine D₁ receptors in the monkey striatum was not altered after the administration of MAP. A bolus injection of NAC (150 mg/kg, i.v.) 30 min before MAP administration and a subsequent continuous infusion of NAC (12 mg/kg/h, i.v.) over 8.5 h significantly attenuated the reduction of DAT in the monkey striatum 3 weeks after the administration of MAP. These results suggest that NAC could attenuate the reduction of DAT in the monkey striatum after repeated administration of MAP. Therefore, it is likely that NAC would be a suitable drug for treatment of neurotoxicity in dopaminergic nerve terminals related to chronic use of MAP in humans.

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INTRODUCTION

The abuse of methamphetamine (MAP), a potent psychostimulant, is an extremely serious and growing problem in the world. The action of MAP is thought to involve rapid entry into the brain, followed by influx into monoaminergic terminals, interaction with vesicular monoaminergic transporter, entry into monoaminergic vesicles and displacement of monoamines into the cytoplasm of the terminals, and subsequent release of the monoamines into the synaptic cleft (Cadet *et al*, 2003). Recent studies using positron emission tomography (PET) suggest that chronic use of MAP causes the reduction of dopamine transporter (DAT) in the human brain (McCann *et al*, 1998; Sekine *et al*, 2001,

2003; Volkow *et al*, 2001a,b), suggesting the neurotoxic effects of MAP in the human brain. These findings are supported by a report demonstrating that the densities of DAT are significantly decreased in the postmortem brain striatum of chronic MAP users (Wilson *et al*, 1996). However, the precise mechanisms underlying MAP-induced neurotoxicity in dopaminergic nerve terminals of the brain are currently not known (Davidson *et al*, 2001; Cadet *et al*, 2003).

Oxidative stress generated by an imbalance between reactive oxygen species (ROS; hydrogen peroxide, superoxide radical, and hydroxyl radical) and antioxidants might contribute to the neurotoxicity of MAP in the brain (Imam *et al*, 2001; Davidson *et al*, 2001; Cadet *et al*, 2003). *N*-acetyl-L-cysteine (NAC), the acetylated variant of the amino acid L-cysteine, is an excellent source of sulfhydryl (SH) groups and is converted in the body into metabolites capable of stimulating glutathione synthesis, promoting detoxification, and acting directly as free radical scavengers (Kelly, 1998). In addition to its mucolytic action, NAC has been studied and utilized to treat conditions characterized by decreased glutathione or oxidative stress such as HIV infection,

*Correspondence: Dr K Hashimoto, Department of Psychiatry, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chiba 260-8670, Japan, Tel: +81 43 226 2147, Fax: +81 43 226 2150, E-mail: hashimoto@faculty.chiba-u.jp

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cancer, and heart disease (Kelly, 1998). Owing to its known characteristics, NAC has been used as a tool for investigating the role of ROS in numerous biological and pathological processes (Kelly, 1998; Zafarullah *et al*, 2003). Recently, we reported that NAC significantly attenuated 6-hydroxydopamine-induced apoptotic neuronal cell death in human neuroblastoma SK-N-SH cells, suggesting that NAC could work as a beneficial dopaminergic neuron-survival factor (Shimizu *et al*, 2002). In addition, NAC directly modifies the activity of several proteins by its reducing activity (Zafarullah *et al*, 2003). Based on the role of oxidative stress in the neurotoxicity of MAP in the brain (Imam *et al*, 2001; Davidson *et al*, 2001; Cadet *et al*, 2003), it is interesting to study the effects of the antioxidant NAC on MAP-induced neurotoxicity in the brain.

We reported recently that pretreatment with NAC significantly attenuates hyperlocomotion, development of sensitization, and neurotoxicity after the administration of MAP in rats, suggesting that NAC might be a suitable drug for treatment of MAP abuse (Fukami *et al*, 2004). The purpose of the present study was to discover therapeutic drugs to prevent or protect against neurotoxicity in dopaminergic terminals in chronic MAP abusers. We performed the present PET study to determine whether intravenous administration of NAC could attenuate the reduction of DAT in the striatum of monkey brain after administration of MAP. To minimize the effects of anesthetics on the behavior of a labeled compound *in vivo*, we performed PET scans of monkeys in the conscious state (Onoe *et al*, 1994; Tsukada *et al*, 1999, 2000, 2001, 2002).

MATERIALS AND METHODS

Subjects

Six young-adult male rhesus monkeys (*Macaca mulatta*) weighing from 4 to 6 kg were used for PET measurements. Monkeys were maintained and handled in accordance with the recommendations of the US National Institutes of Health and also the guidelines of the Central Research Laboratory, Hamamatsu Photonics (Hamakita, Shizuoka, Japan). Over the course of 3 months, the monkeys were trained to sit on a chair twice a week. The magnetic resonance images (MRIs) of all monkeys were obtained with a Toshiba MRT-50A/II (0.5 T) under anesthesia with pentobarbital. The stereotactic coordinates of PET and MRI were adjusted based on the orbitomeatal (OM) line with monkeys secured in a specially designed head holder (Takechi *et al*, 1994). At least 1 month before the PET study, an acrylic plate, with which the monkey was fixed to the monkey chair, was attached to the head under pentobarbital anesthesia as described previously (Onoe *et al*, 1994).

Drug Administration

MAP hydrochloride (Dainippon Pharmaceuticals Ltd, Osaka, Japan, 2 mg/kg as a salt, four times at 2-h intervals) was administered intramuscularly into each monkey (Figure 1). We used that dose because it is reported to produce long-term neurotoxic effects on the brain of baboons (Villemagne *et al*, 1998). Also, such a dose regimen closely approximates the binge use of MAP by some humans

Figure 1 Schedule of treatment of MAP and/or NAC in monkeys.

(20–40 mg every 2–3 h) (Konuma, 1994). For administration of NAC, subjects received a bolus of NAC (Wako Pure Chemicals Ltd, Tokyo, Japan, 150 mg/kg, i.v.) 30 min before administration of MAP and a subsequent continuous infusion of NAC (12 mg/kg/h, i.v.) over 8.5 h, with a slight modification of the method reported previously (Molnar *et al*, 1999; Rank *et al*, 2000) (Figure 1).

Synthesis of [^{11}C]-Labeled Compounds

Carbon-11 (^{11}C) was produced by ^{14}N (p,α) ^{11}C nuclear reaction using the cyclotron (HM-18, Sumitomo Heavy Industry, Tokyo, Japan) at the Hamamatsu Photonics PET Center and obtained as [^{11}C]CO₂, which was converted to [^{11}C]methyl iodide. [^{11}C]2 β -carbomethoxy-3 β -(4-fluorophenyl)tropane (β -CFT) (for DAT) and [^{11}C]SCH 23390 (for DA D₁ receptors) were synthesized as previously reported (Tsukada *et al*, 2001; Harada *et al*, 2002). The radiochemical and chemical purities of labeled compounds were greater than 98 and 99%, respectively. After analysis for identification, the solution was passed through a 0.22- μm pore size filter before intravenous administration to the monkey.

PET Scans

PET data were collected before and 3 weeks after administration of MAP or MAP/NAC. Data were collected on a high-resolution PET scanner (SHR-7700, Hamamatsu Photonics KK, Hamamatsu, Japan) with a transaxial resolution of 2.6 mm full-width at half-maximum (FWHM) and a center-to-center distance of 3.6 mm (Watanabe *et al*, 1997). The PET camera allowed 31 slices for imaging to be recorded simultaneously. After an overnight fast, animals were fixed to the monkey chair with stereotactic coordinates aligned parallel to the OM line. A cannula was implanted in the posterior tibial vein of the monkey for administration of [^{11}C]-labeled compounds. [^{11}C] β -CFT or [^{11}C]SCH 23390 was injected through the posterior tibial vein cannula. For [^{11}C]SCH 23390, a PET scan was performed for 64 min with six time frames at 10-s intervals, six time frames at 30-s intervals, 12 time frames at 1-min intervals, followed by 16 time frames at 3-min intervals. For [^{11}C] β -CFT, additional scans of nine time frames at 3-min intervals were carried out to collect data for 91 min total. After completion of the first scan with [^{11}C] β -CFT, scans with [^{11}C]SCH 23390 were continuously performed at 3-h intervals. Due to the very short half-life of ^{11}C (20.4 min), a time lag of at least 3 h between scans provided a sufficient decay time of radioactivity in monkeys (approximately 1/400 of the injected dose). Therefore, the level of radioactivity associated with

the previous injection of labeled compound would not interfere with the next scan.

Data Analysis and Statistical Analysis

For quantitative analysis, time-activity curves of radioactivity in the cerebellum, used as an input function because of the much lower density of dopamine receptors (Creese *et al*, 1975), and each region of interest (ROI) were fitted to a two-compartment model using the least-squares fitting method to estimate the kinetic parameters, and the binding potential in each ROI was calculated as described previously (Lammertsma and Hume, 1996). The differences between the control (pre-MAP) monkeys and the MAP or NAC plus MAP (post MAP) monkeys were determined using a paired two-tailed *t*-test. The difference between monkeys in the MAP-treated group and those in the NAC plus MAP-treated group was determined using an unpaired two-tailed *t*-test. Significance was set at $p < 0.05$.

RESULTS

PET studies using [^{11}C] β -CFT (for DAT) or [^{11}C]SCH 23390 (for DA D₁ receptor) were performed before and 3 weeks after repeated administration of MAP. High accumulation of radioactivity in the striatum after intravenous administration of [^{11}C] β -CFT or [^{11}C]SCH 23390 was detected in the control monkeys, whereas levels of radioactivity in the cerebellum were much lower compared to those in the striatum (Figure 2). At 3 weeks after administration of MAP (2 mg/kg $\times$ 4, 2-h intervals), the binding of [^{11}C] β -CFT in the striatum was significantly ($t = 10.01$, $p = 0.010$) decreased, whereas the binding of [^{11}C]SCH 23390 to DA D₁

Figure 2 PET images of [^{11}C]SCH 23390 and [^{11}C] β -CFT in the brains of a rhesus monkey (*Macaca mulatta*). PET data were collected on an animal PET scanner (Hamamatsu SHR-7700) with a transaxial resolution of 2.6 mm (FWHM). The PET image of [^{11}C]SCH23390 was generated by the summation of data from 37 to 64 min after injection. PET images for [^{11}C] β -CFT were generated by the summation of data from 61 to 91 min after injection. The stereotactic coordinates of PET were adjusted based on the OM line. These PET images were from the same monkey.

Table 1 Effects of NAC on Reduction of DAT and DA D₁ Receptors in the Monkey Striatum after Administration of MAP

	Control	MAP
Monkey-1		
DAT	3.563 (100%)	1.022 (28.7%)
DA D ₁	1.824 (100%)	1.678 (92.0%)
Monkey-2		
DAT	3.255 (100%)	1.025 (31.5%)
DA D ₁	1.836 (100%)	1.927 (105%)
Monkey-3		
DAT	4.827 (100%)	1.700 (35.2%)
DA D ₁	2.272 (100%)	2.171 (95.6%)
DAT (mean $\pm$ SEM)	3.882 $\pm$ 0.481 (100%)	1.249 $\pm$ 0.226 (31.8 $\pm$ 1.88%)*
DA D ₁ (mean $\pm$ SEM)	1.977 $\pm$ 0.147 (100%)	1.925 $\pm$ 0.142 (97.5 $\pm$ 3.88%)
	Control	NAC+MAP
Monkey-4		
DAT	4.269 (100%)	2.648 (62.0%)
DA D ₁	2.780 (100%)	2.587 (93.1%)
Monkey-5		
DAT	2.649 (100%)	1.696 (64.0%)
DA D ₁	1.680 (100%)	1.788 (106%)
Monkey-6		
DAT	2.524 (100%)	1.743 (69.1%)
DA D ₁	1.742 (100%)	1.667 (95.7%)
DAT (mean $\pm$ SEM)	3.147 $\pm$ 0.562 (100%)	2.029 $\pm$ 0.310 (65.0 $\pm$ 2.11%)*.#
DA D ₁ (mean $\pm$ SEM)	2.067 $\pm$ 0.357 (100%)	2.014 $\pm$ 0.289 (98.3 $\pm$ 3.94%)

Values of binding potential are shown.

* $P < 0.05$ as compared with the control group (a paired *t*-test).

$P < 0.001$ as compared with the MAP-treated group (a unpaired *t*-test).

receptor in the striatum was not altered ($t = 0.716$, $p = 0.549$) (Figure 2 and Table 1). Time-activity curves of radioactivity after intravenous administration of [^{11}C] β -CFT indicated a high accumulation of radioactivity in the striatum, with a low level of radioactivity in the cerebellum, of the control monkeys (Figure 3). At 3 weeks after administration of MAP (2 mg/kg $\times$ 4, 2-h intervals), time-activity curves of radioactivity in the striatum after intravenous administration of [^{11}C] β -CFT were markedly decreased, whereas those of radioactivity in the cerebellum were not altered (Figure 3).

As shown in Figure 4, the accumulation of radioactivity in the striatum of monkeys treated with NAC plus MAP after administration of [^{11}C] β -CFT was higher than that of radioactivity in the striatum of monkeys treated with MAP alone (Figure 4). The binding potential in the striatum of monkeys treated with NAC plus MAP after administration of [^{11}C] β -CFT was significantly ($t = 11.74$, $p < 0.001$) higher than that of the striatum of monkeys treated with MAP alone, although the binding potential in the striatum of monkeys treated with NAC plus MAP was significantly

($t=4.367$, $p=0.049$) lower than that of the striatum of control monkeys (Table 1).

DISCUSSION

The major finding of the present study is that infusion of the antioxidant NAC could attenuate the reduction of DAT in the monkey striatum after administration of MAP. Repeated administration of MAP caused a marked reduction of DAT in the monkey striatum, which is consistent with previous reports (Melega *et al*, 1998, 2000; Villemagne *et al*, 1998). In contrast, we found that the binding of [^{11}C]SCH 23390 to DA D₁ receptors in the monkey striatum was not altered by administration of MAP, suggesting that administration of MAP could damage the dopaminergic nerve terminals but not the postsynaptic neurons. In addition, it seems that the drug schedule (2 mg/kg $\times$ 4, 2-h intervals) used in this study is neurotoxic to DAT in the monkey brain. As described in Introduction, the marked release of DA produced by MAP could be implicated in the

neurotoxic effects on dopaminergic terminals in the brain after administration of MAP (Cadet *et al*, 2003; LaVoie and Hastings, 1999; Stokes *et al*, 1999). Two metabolic routes seem possible: (1) formation of 3,4-dihydroxyphenyl acetic acid (DOPAC) and hydrogen peroxide (H_2O_2) by monoamine oxidase (MAO), or (2) formation of reactive DA-quinones and free radicals by auto-oxidation (Asanuma *et al*, 2003). As DA-induced modifications of protein structure and function may result in cellular toxicity, it is likely that DA quinones produced by auto-oxidation contribute to MAP-induced neurotoxicity to dopaminergic nerve terminals, supporting the evidence of oxidative stress in this model (LaVoie and Hastings, 1999; Stokes *et al*, 1999). In addition, high ROS, including hydrogen peroxide, superoxide radical, and hydroxyl radical, are generated not only during the oxidation of DA but also during the decay of redox-active DA quinones, suggesting that superoxide radicals, hydrogen peroxide, and nitric oxide might be involved in the neurotoxicity of MAP (Davidson *et al*, 2001; Imam *et al*, 2001; Cadet *et al*, 2003).

It is well known that NAC can act as a precursor for glutathione synthesis, as well as a stimulator of the cytosolic enzymes involved in glutathione regeneration. Furthermore, NAC can act by direct reaction between its reducing thiol group and ROS. It has been shown that NAC can prevent programmed cell death in cultured neuronal cells and that NAC increases mitochondrial complex I and IV specific activities both *in vitro* and *in vivo* in synaptic mitochondrial preparations from aged mice (Banaclocha, 2001). Therefore, it should be noted that a potent antioxidant NAC could attenuate the reduction of DAT in the monkey striatum after the repeated administration of MAP.

DAT knockout mice are resistant to MAP-induced neurotoxicity of dopaminergic nerve terminals, suggesting the role of DAT in the MAP-induced neurotoxicity in these nerve terminals (Fumagalli *et al*, 1998). Nevertheless, it is possible that MAP-induced DA released within the cytoplasm of dopaminergic terminals might be a more critical trigger of MAP-induced neurotoxicity in the dopaminergic terminals, since vesicular monoamine transporter 2

Figure 3 Time-activity curves of radioactivity in the striatum and cerebellum of the monkey before and 3 weeks after administration of MAP. PET data were collected for 91 min. The radioactivity in the striatum and cerebellum was plotted against time after intravenous administration of [^{11}C] β -CFT into a rhesus monkey.

Figure 4 PET images of [^{11}C] β -CFT in the brains of a rhesus monkey 3 weeks after administration of MAP or NAC plus MAP. Each PET image was generated by the summation of data from 61 to 91 min after injection of [^{11}C] β -CFT.

(VMAT2) knockout mice are more susceptible to the toxic manifestations of MAP (Fumagalli *et al*, 1999). These findings are supported by the report that MAP-induced neurotoxicity might be related to the formation of DA quinones, a process dependent on increased DA levels within dopaminergic nerve terminals (LaVoie and Hastings, 1999). It is likely that NAC binds to reactive DA quinones and ROS by auto-oxidation, resulting in the protection of the neurotoxicity by DA quinones.

The frequency of emergency room visits due to acute MAP intoxication has increased dramatically in the past few years (Lan *et al*, 1998; Cadet *et al*, 2003). In toxic doses, MAP can cause agitation, anxiety, hallucinations, delirium, psychosis, cognitive and psychomotor impairment, seizures, and death (Lan *et al*, 1998; Cadet *et al*, 2003). NAC is currently the 'gold-standard' treatment approach for management of acetaminophen-induced hepatotoxicity. Furthermore, NAC appears to have some clinical usefulness as a chelating agent in the treatment of acute heavy-metal poisoning, both as an agent capable of protecting the liver and kidney from damage and as an intervention to enhance elimination of the metals (Kelly, 1998). Given these two advantages, it is likely that NAC is a useful drug for treatment of neurotoxicity in dopaminergic nerve terminals in the human brain caused by chronic use of MAP.

In conclusion, our findings demonstrate that the potent antioxidant NAC could attenuate the reduction of DAT in the monkey striatum after repeated administration of MAP. Therefore, it is possible that NAC would be a suitable drug for treatment of MAP abusers, since NAC has been widely used as a therapeutic drug or a nutritional supplement.

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