Effect of imipramine on 1-methyl-4-phenylpyridinium ion-induced hydroxyl radical generation in rat striatum

Toshio Obata *, Toru Egashira
Department of Pharmacology and Therapeutics, Oita Medical University, 1-1, Idaigaoka, Hasama-machi, Oita 879-5593, Japan
Received 8 April 2002; received in revised form 20 June 2002; accepted 26 June 2002

Abstract

We examined the effect of imipramine (a tricyclic antidepressant drug) on hydroxyl radical (•OH) generation induced by 1-methyl-4-phenylpyridinium ion (MPP+) in extracellular fluid of rat striatum, using a microdialysis technique. Imipramine enhanced the formation of •OH trapped as 2,3-dihydroxybenzoic acid (DHBA) induced by MPP+ (5 mM). Introduction of imipramine (0.1, 0.5 and 1.0 mM) dose-dependently increased the level of dopamine (DA) release. Concomitantly, imipramine enhanced DA efflux and the level of DHBA induced by MPP+, as compared with MPP+ -treated control. When corresponding experiments were performed with reserpinized rats, there were small increases in the levels of DA and nonsignificant increase in the formation of DHBA. When iron (II) was administered to imipramine (1 mM)-treated animals, a marked elevation of DHBA was observed, compared with MPP+ -only treated animals. A positive linear correlation was observed between iron (II) and DHBA ($R^2 = 0.985$) in the dialysate. These results indicate that imipramine enhances generation of •OH induced by MPP+ during enhanced DA overflow.

© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Imipramine; 1-Methyl-4-phenylpyridinium ion (MPP+); Dopamine; Hydroxyl radical; Parkinson’s disease

1. Introduction

Various antidepressant drugs have been developed and their antidepressive effects have been observed in animal model [1]. It has been demonstrated that tricyclic antidepressant drugs inhibit monoamine oxidase (MAO; EC 1.4.3.4) in vitro [2–4]. MAO exists in two forms, form A (MAO-A) and form B (MAO-B), based on its substrate specificity and sensitivity to inhibitors [5,6]. MAO-A preferentially deaminates 5-hydroxytryptamine (5-HT), whereas MAO-B deaminates β-phenylethylamine (β-PEA). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces a parkinsonian syndrome after its conversion to 1-methyl-4-phenylpyridinium ion (MPP+) by MAO-B in the brain [7,8]. It has been shown that MAO-A is the major enzyme responsible for the deamination of dopamine (DA) in the rat striatum [9]. Although there are many papers showing that DA autoxidation and oxidative stress may be involved in Parkinson’s disease [10–12], the etiology of Parkinson’s disease still remains obscure.

Autoxidation and MAO-dependent oxidation of DA can lead to the formation of reactive cytotoxic free radicals whose generation could be influenced by the antidepressants because these antidepressants could inhibit catecholamine uptake and MAO activities. We as well as others reported that MPP+ induces a massive release of DA in the striatum, which leads to increased free radicals [11,13,14]. In dopaminergic nerve cells, free radicals are mainly generated by MAO via deamination of DA and nonenzymatically by the autoxidation of DA [15]. The present study focuses on the effect of imipramine (tricyclic antidepressant drug) on hydroxyl radical (•OH) generation induced by MPP+ in rat striatum.

2. Materials and methods

2.1. Animals

Adult female Wistar rats weighing 300–400 g were kept in an environmentally controlled room (20–23 °C, 50–60%
humidity, illuminated from 07:00 to 19:00) and fed food and water ad libitum. At the end of the experiments the rats were sacrificed using an overdose of anesthetic. All procedures in dealing with the experimental animals met the guideline principles stipulated by the Physiological Society of Japan and the Animal Ethics Committee of the Oita Medical University.

### 2.2. Chemicals

Imipramine hydrochloride was purchased from Ciba-Geigy (Takarazuka, Japan). MPP⁺ was purchased from Research Biochemicals Inc., MA, USA. Sodium salicylate and its hydroxylated metabolites were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan). The two radiochemical substrates used in this study, 5-hydroxytryptamine binoxalate ([2-14C]-5-HT; 1.48–2.22 Gbq/mmol) and β-phenylethylamine hydrochloride ([ethyl-1-14C]-β-PEA, 1.48–2.22 Gbq/mmol), were purchased from Dupont NEN (New England Nuclear) Products (Boston, MA, USA). In the case of reserpinized rats, reserpin (5 mg/kg; Daiichi Pharmaceutical, Japan) was injected intravenously into the rats 24 h before the experiments.

### 2.3. Experimental protocol

#### 2.3.1. Assay of MAO activity

The rats were killed by decapitation and the brains were quickly removed and homogenated in 10 ml of 10 mM phosphate buffer, pH 7.4, containing 0.32 mM sucrose solution. To examine the effects of imipramine on MAO activity in vitro, the enzyme was preincubated for 20 min at 25 °C with imipramine (0.001 to 1.0 mM) before adding the substrates. Final concentrations of substrate (5-HT for MAO-A, and β-PEA for MAO-B) were 100 and 10 μM, respectively. The remaining MAO activity was measured by adding 20 μl of substrate solution and the mixture was incubated for 20 min at 37 °C. The reaction was stopped by adding 2 N HCl (200 μl). The reaction products were extracted with ethyl acetate–benzene mixture (1:1, v/v) saturated with water, and the radioactivity in the extract was measured using Beckmann LS-9000 scintillation counter. The protein concentrations of the enzyme preparations were measured according to the method of Lowry et al. [16] using bovine serum albumin as a standard. The data were corrected using protein concentration, and expressed as percent, compared to control (100%). One hundred percent of MAO-A and -B activities were 0.072 ± 0.03 and 1.02 ± 0.05 nmol/min/mg protein, respectively.

#### 2.3.2. Microdialysis experiments

The rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.), and the level of anaesthesia was maintained by intraperitoneal injection of chloral hydrate (20 mg/kg). Ringer’s solution containing 147 mM NaCl, 2.3 mM CaCl₂ and 4 mM KCl, pH 7.0, was used for perfusion (1 μl/min) through a microdialysis probe into the striatum. The recovery rate of 0.1 μM DA was 20.8 ± 0.9% at flow rate of 1 μl/min. The microdialysis probe was pre-washed with Ringer’s solution for at least 30 min prior to stereotaxical implantation in the striatum (stereotaxic coordinates: AP: 1.0, R/L: 2.5, H: - 7 mm from dura matter) [17]. Thereafter, sodium salicylate in Ringer’s solution (0.5 nmol/μl/min) was perfused by a microinjection pump (Carnegie Medicine CMA/100, Stockholm, Sweden) to trap OH radicals [18,19] in the striatum, and basal levels of dihydroxybenzoic acid (DHBA) were determined during a definite period. Brain dialysate (1 μl/min) was collected every 15 min in small tubes containing 15 μl of 0.1 N HClO₄ to prevent amine oxidation and assayed immediately for DHBA by high-performance liquid chromatography with electrochemical (HPLC-EC) procedure [18–20]. The dialysate samples were promptly injected into an HPLC-EC system equipped with a glassy carbon working electrode (Eicom, Kyoto, Japan) and an analytic reverse-phase column on an Eicompak MA-5ODS column (5 μm, 4.6 ± 150 mm; Eicom). The working electrode was set at a detection potential of 0.75 V.

### 2.4. Statistical analysis

All values are presented as means ± S.E. The significance of difference was determined by using ANOVA with Fisher’s post-hoc test. A P value of less than 0.05 was regarded as being statistically significant.

### 3. Results

#### 3.1. Effect of imipramine on MAO activity in vitro

To determine the mechanism by which MAO activity was inhibited by imipramine, the effects of various concen-
trations of imipramine on MAO in rat brain homogenates were studied in vitro using 5-HT and \( \beta \)-PEA as substrates. Imipramine (0.001, 0.01, 0.1 and 1 mM) inhibited both MAO-A and -B activities in a dose-dependent manner. When the brain homogenates were preincubated at 25 °C for 20 min with imipramine, the residual activity of MAO-A and MAO-B activities with 100 \( \mu \)M imipramine were 63% and 4%, respectively (Fig. 1). However, 1 mM imipramine completely inhibited both MAO activities.

3.2. Effect of imipramine on •OH generation induced by MPP⁺

After a 60-min washout with pH 7.4 Ringer’s solution, the striatum was infused with MPP⁺ (5 mM) for 15 min (total dose 75 nmol). When 1 mM imipramine was introduced into the dialysate, time-dependent changes in the level of DA and the formation of DHBA from •OH were monitored in the dialysates from rat brain after MPP⁺ treatment. Although imipramine (1 mM) alone did not induced DA efflux, imipramine (1 mM) enhanced the level of DA induced by MPP⁺ (Fig. 2A). Introduction of imipramine drastically enhanced MPP⁺-induced DA release with concomitant increase in •OH formation trapped as DHBA in the brain dialysate, compared with MPP⁺-only treated animals. At 90 and 105 min, imipramine significantly increased DHBA formation as shown in Fig. 2B (\( P<0.05 \)). When corresponding experiments were performed with reserpinized rats, small increases in the level of DA (Fig. 3A) and nonsignificant increase in the formation of DHBA were

![Fig. 2. Effect of MPP⁺ on the release of DA and the formation of •OH after imipramine treatment in the striatum of rats. After a 60-min washout with Ringer’s solution (pH 7.4), striatum was infused with MPP⁺ (5 mM) for 15 min (solid bat; total dose, 75 nmol). Then, at 60 min after probe implantation, sodium salicylate (hatched bat; 0.5 nmol/\( \mu \)l/min) was infused through the microdialysis probe for 90 min to trap •OH (B). Brain dialysates were collected every 15 min in 0.1 N HClO₄ and immediately assayed by an HPLC-EC. MPP⁺ (open circle), MPP⁺ and 1 mM imipramine (closed circle), and no MPP⁺ (rectangle) were compared. Values are means ± S.E. for five animals. Both the total DA efflux and DHBA formation elicited by imipramine were significantly elevated over that of MPP⁺ alone group (\( P<0.05 \) for MPP⁺ alone vs. MPP⁺ and 1 mM imipramine: ANOVA and Fisher’s test).

![Fig. 3. Effect of MPP⁺ on the release of DA and the formation of •OH after imipramine treatment by reserpinized rats. Experimental conditions and symbols are the same as for Fig. 2.]

![Fig. 4. Dose-dependent effects of imipramine on the level of DA in the MPP⁺-treated rat striatum. The level of DA measured at 30–45 min after application of various concentrations of imipramine (as indicated abscissa) (hatched column) are given as a percentage of the value measured just before application of imipramine. Each column and vertical bar indicates mean ± S.E. The number in the parentheses indicates the number of animal. *\( P<0.05 \) versus the level of DA immediately before application of imipramine (ANOVA and Fisher’s test).]
imipramine increased the level of DA. MAO is one of the
administration of imipramine. This result indicates that
of the oxidized DA metabolite DOPAC decreased following
extracellular fluid. We previously reported[3] that the level

observed (Fig. 3B). As shown in Fig. 4, imipramine (0.01,
0.1 and 1.0 mM) dose-dependently increased the level of
DA release. At 1 mM of imipramine, the increase in DA was
statistically significant (P < 0.05, n = 5). To confirm whether the •OH generation was based on a Fenton-type reaction,
iron (II) was infused through the dialysis probe. When iron
(II) (2, 5 and 10 μM) was administered to MPP + -pretreated
animals, iron (II) clearly produced a dose-dependent
increase in the levels of DHBA, showing a positive linear
correlation between iron (II) and •OH formation trapped as
DHBA (R² = 0.985) in the dialysate, as compared with the
iron (II)-only treated group (Fig. 5). The data suggest that
the effect of imipramine on •OH formation was based on the
Fenton-type reaction.

4. Discussion

We have demonstrated that imipramine (a tricyclic anti-
depressant drug) enhances generation of •OH induced by
MPP + in the extracellular space of the striatum. It is known
that tricyclic antidepressant drugs inhibited MAO activities
and that, in vitro, these drugs are more potent inhibitors of
MAO-B than MAO-A [3,4]. MAO is a brain enzyme that
plays a role in the metabolism of various catecholamine.
Both MAO-A and MAO-B activities in rat brain were
inhibited by imipramine (Fig. 1). Kato et al. [21] reported
that MAO inhibitor caused the accumulation of DA in the
extracellular fluid. We previously reported [3] that the level
of the oxidized DA metabolite DOPAC decreased following
the administration of imipramine. This result indicates that
imipramine increased the level of DA. MAO is one of the
enzymes metabolizing various neurotransmitter monoamines
[14]. If MPP + or its related compound(s) is responsible for
inducing Parkinson disease, inhibition of MAO-B may lead
to induce protective effect against •OH formation [22]. It
was reported that the MAO-B inhibitors can diminish neuronal
cell death [23]. In rat brain, MPP + is a more potent
MAO-A inhibitor than MAO-B [24], and MAO-A is pre-
dominantly responsible for striatal DA oxidation [21]. On the
contrary, in human brain, MAO-B is responsible for DA
oxidation [25,26]. The DA that accumulates in the extra-
cellular fluid following administration of antidepressants can
undergo autoxidation, which in turn leads (possibly by an
indirect mechanism) to the formation of cytotoxic •OH free
radicals. As shown in the present study, the antidepressant
inhibited MAO activities in vitro. Antidepressant drugs have
heretofore been considered effective in the treatment of
depression because they inhibit the active uptake of amines
in the presynaptic cells of the brain [27,28]. Imipramine is
also potent inhibitors of DA reuptake. Regardless of their
specific mechanisms of action, the consequence is increased
extracellular levels of DA. This may explain the observed
increase in •OH. Both nonenzymatic and enzymatic mech-
nisms may contribute to free radical formation induced by
antidepressant drugs in the striatum in vivo.

The concentration profile of the administered compounds
in the surrounding interstitial space is unknown: in general,
the extracellular concentration of a compound given through
the probe would never reach the concentration in the
dialysis probe. This is an unavoidable limitation of the
microdialysis technique that should be kept in mind when
interpreting the experimental data. Introduction of imipra-
mine (1 mM) drastically increased the DA efflux and the
level of DHBA induced by MPP + (Fig. 2). Although there
are many reports about the effect of MPP + on •OH genera-
tion, the mechanism of •OH generation by MPP + is obscure. Free radical formation enhanced by MPP + in the
striatum appeared to be dose-dependent and positively
correlated with amounts of sustained DA overflow [13].
DA is known to be autoxidated in the presence of oxygen
and transition metal [12,29]. When imipramine concentra-
cion in the perfusate was increased, DA efflux was enhanced
with concomitant increase in •OH formation. Our obtained
data demonstrated that sustained DA overflow elicited by
imipramine in the striatum also led to enhanced MPP + -
induced •OH formation. These data are consistent with the
notion that DA autoxidation and sustained DA turnover can
lead to free radical formation, which in turn causes oxidative
damage in the iron-enriched nigral neuron during senes-
cence and Parkinson’s diseases [12,13]. This evoked DA
overflow was enhanced by imipramine. The levels of DA
and DHBA in reserpinned rats were drastically reduced, as
compared with that of imipramine and MPP + -treated
groups (Fig. 3). The results also suggest that the mechanism
of imipramine and MPP + -induced •OH formation was via
DA efflux. The present study demonstrated that imipramine
increased •OH formation in the extracellular space of the
Amine increased the striatum with concomitant DA release. Therefore, imipramine increased the \( \cdot \text{OH} \) formation trapped as DHBA. Moreover, to confirm whether the imipramine-evoked \( \cdot \text{OH} \) generation in the MPP\(^+\)-treated rats was based on the Fenton-type reaction, the DHBA formation was measured in the presence of iron in imipramine- and MPP\(^+\)-treated rats. Iron (II) clearly produced a dose-dependent increase in the levels of DHBA (Fig. 5). This finding indicates that extracellular DA is needed for the observed effect of Fenton-type reaction.

MPP\(^+\) causes a sustained DA release into extraneuronal space, generating oxygen free radicals with extracellular DA autoxidation [11]. This could lead to the formation of \( \cdot \text{OH} \) radicals, which may induce lipid peroxidation, protein cross-linking and DNA damage, mediated by base pair mutation [30]. If toxic species are being produced on Parkinson's disease, they would normally be inactivated by a variety of protective mechanisms, but the mechanisms, however, may be impaired in the substantia nigra of patients with Parkinson's disease; reduced levels of, e.g., catalase and glutathione (GSH) [31]. The enzyme MAO is one of the enzymes metabolizing various neurotransmitter monoamines [14]. Among MAO-B inhibitors, \( \cdot \text{deprenyl} \) is widely used for the treatment of patients with Parkinson's disease. Inhibition of MAO-B may be involved in its protective effect [22], if MPP\(^+\) or related compound(s) is responsible for inducing Parkinson's disease. It is clear that MAO-B is responsible for the production of MPP\(^+\). Therefore, the MAO-B inhibitors can diminish neuronal cell death [23].

DA is known to be autoxidized in the presence of oxygen and iron (II), and to be converted to semiquinone, quinone, zwitteric 5,6-dihydroxyindoles and melanin [11]. The DA efflux was enhanced imipramine and MPP\(^+\) administration. DA is known to undergo autoxidation in the presence of oxygen and iron (II) [13,29]. Normally, iron (III) is bound to endogenous chelators such as ADP and, in the absence of a significant amount of ferritin in the substantia nigra, could be chelated by melanin. Increased concentrations of DA could serve as the catalyst for the conversion of iron (III) to iron (II) by melanin and, in the presence of \( \text{H}_2\text{O}_2 \), results in further formation of \( \cdot \text{OH} \), depending on the environmental conditions [32]. The production of \( \cdot \text{OH} \) in the presence of melanin is significantly greater when iron (III) is predominant [32], and is further demonstrated by the greater lipid peroxidation of rat cerebral cortex in the presence of iron (II) and higher DA melanin concentrations [33]. An enhanced generation of cytotoxic \( \cdot \text{OH} \) radicals through DA accumulation could accelerate the MPP\(^+\)-induced degeneration of nigral neurons. Accordingly, imipramine enhances generation of \( \cdot \text{OH} \) induced by MPP\(^+\) during enhanced DA overflow.

In conclusion, a tricyclic antidepressant drug may play a key role in \( \cdot \text{OH} \) formation by the release of DA from nigrostriatal nerves in the brain by the action of MPP\(^+\). The results in the experiment may be useful for elucidating the actual mechanism of free radical formation in the pathogenesis of neurodegenerative brain disorders including Parkinson's disease, Alzheimer's disease and traumatic brain injuries.

Acknowledgements

We are thankful to Prof. Lars Oreland (Department of Neuroscience, Section of Pharmacology, Box 593 Biomedical Centre, Uppsala University, 751-24, Uppsala, Sweden) for valuable discussions.

References