INTRACRANIAL MICRODIALYSIS OF SALICYLIC ACID TO DETECT HYDROXYL RADICAL GENERATION BY ANTIDEPRESSANT DRUGS IN THE RAT

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SUMMARY

We examined the effect of the antidepressant drugs nomifensine, imipramine and nortriptyline on the generation of hydroxyl radicals (•OH) in the rat brain using striatal microdialysis. All these drugs are inhibitors of MAO-A or MAO-B, but were more potent inhibitors of MAO-B *in vitro*. When the tricyclic antidepressant drugs nortriptyline and imipramine (1 mM) were directly infused into the rat brain through a microdialysis probe for 60 min, the level of 3,4-dihydroxyphenylacetic acid (DOPAC) in the striatum gradually decreased in a time-dependent manner. When salicylic acid in Ringer's solution (0.5 mM or 0.5 nmol/μl/min) in addition to the tricyclic antidepressants was infused through the microdialysis probe to detect the generation of •OH, as reflected by the formation of dihydroxybenzoic acid (DHBA) in the striatum, marked transient elevations in the dialysate levels of 2,3- and 2,5-DHBA were observed. However, when the noncyclic antidepressant drug nomifensine was used, there was no increase in DHBA. The present results suggest that the dopamine (DA) that accumulates in the extracellular fluid after treatment with tricyclic antidepressant drugs undergoes autooxidation, which in turn leads (possibly by an indirect mechanism) to the formation of cytotoxic •OH free radicals.

KEY WORDS: Antidepressants, monoamine oxidase (MAO), hydroxyl radical, dopamine, rat

INTRODUCTION

Tricyclic antidepressant drugs are considered effective in the treatment of depression because they inhibit the active uptake of amines into presynaptic cells of the brain (1,2). It has been demonstrated (3,4) that tricyclic antidepressant drugs inhibit monoamine oxidase (MAO; EC 1.4.3.4.) *in vitro*. MAO exists in two forms, form A (MAO-A) and form-B (MAO-B), based on the substrate specificity and sensitivity to inhibitors (5-8). MAO-A preferentially deaminates 5-hydroxytryptamine (5-HT), whereas MAO-B deaminates β-phenylethylamine (β-PEA). It has been shown that MAO-A is the major enzyme responsible for the deamination of DA in the rat striatum (9). In addition, a recent report has documented that DA is also oxidized by MAO-B (10). The major catabolic pathways for DA are those catalyzed by MAO and catechol-O-methyl transferase (COMT), which give rise to 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT) and homovanillic acid (HVA). Autooxidation and MAO-dependent oxidation of DA can lead to the formation of reactive, cytotoxic free radicals whose generation could be influenced by the antidepressants used in the study because these antidepressants seem to be both inhibitors of catecholamine uptake and MAO-inhibitors. In dopaminergic nerve cells, free radicals are mainly generated by MAO via deamination of DA and non-enzymatically by the autooxidation of DA (11).

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The hydroxyl radical (•OH) is extremely reactive and readily reacts with a number of compounds, including lipids and proteins (12). This radical also reacts with salicylate and generates 2,3- and 2,5-dihydroxybenzoic acid (DHBA) (13,14), which can be measured electrochemically in picomole quantities by high-performance liquid chromatography (HPLC) (15-18). The formation of DHBA after systemic administration of salicylate is used as an index of •OH generation in the brain (13,16,19-22).

The present study examined the effect of antidepressants, such as the tricyclic drugs imipramine, nortriptyline and the noncyclic drug, nomifensine, on •OH generation in the rat brain by using intracranial microdialysis.

MATERIALS AND METHODS

Animals: Adult male Wistar rats (weighing 250-350 g) were housed in temperature-controlled animal quarters and kept on a circadian cycle with 12-h light and 12-h dark. The rats were anesthetized with chloral hydrate (400 mg/kg ip; Sigma Chemical, St. Louis, MO). This study was approved by the Ethics Committee for Animal Experiments, Oita Medical University.

Assay of MAO activity: The rats were killed by decapitation and the brains were quickly removed and homogenized in 10 ml of 10 mM phosphate buffer, pH 7.4, containing 0.32 M sucrose solution. The mitochondrial fraction was prepared by differential centrifugation (23). All operations were carried out at 4°C. MAO activity was assayed radiochemically as described by Fowler *et al* (24), with [^{14}C]-5-HT or [^{14}C]-ß-PEA after dilution with the respective unlabelled amines as substrate. The standard assay mixture contained 20 µl of mitochondrial suspension, 20 µl of 0.1 M phosphate buffer, pH 7.4, and 140 µl distilled water. The reaction was started 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 an ethyl acetate-benzene mixture (1:1, v/v) saturated with water, and the radioactivity in the extract was measured in a Beckmann LS-9000 scintillation spectrometer. When the effects of the antidepressant drugs on MAO activity *in vitro* were investigated, the enzyme was preincubated for 20 min at 25°C with the antidepressants at concentrations of 1.0 mM to 0.1 µM before the substrates were added. The remaining MAO activity was measured after the substrates were added. Final concentrations of substrates were 100 µM 5-HT and 10 µM β-PEA.

Measurement of protein: The protein concentration of the enzyme preparations was measured according to the method of Lowry *et al* (25), using bovine serum albumin as the standard. The protein concentration of the enzyme preparations was adjusted to 1.0 mg/ml.

Microdialysis: For this purpose, we made a suitable microdialysis probe using a micropipette tip, a silica tube, and a polyethylene tube (26). This brain dialysis probe (0.2 mm diameter, 3 mm exposure, 50,000 Daltons cut off) was washed with Ringer's solution for at least 30 min prior to stereotaxical implantation in the striatum (27) (stereotaxic coordinates AP 10, R/L 2.5, H3). Ringer's solution was infused (1 µl/min) for at least 60 min before switching to the experimental drug solution by means of a liquid switch. The drugs were dissolved in Ringer's solution containing 147 mM NaCl, 2.3 mM $CaCl_2$ and 4 mM KCl (pH 7.0). With a perfusion rate of 1 µl/min, the relative recovery of 10^{-7}M standard solution of DOPAC, 2,3- and 2,5-DHBA was about 30, 11 and 12%, respectively. Thereafter in order to trap •OH radicals (14,16), the striatum was perfused with 0.5 mM sodium salicylate in Ringer's solution (0.5 nmol/µl/min) for 120 min. Brain dialysates were collected every 15 min in tubes containing 15 µl of 0.1 N $HClO_4$ and assayed immediately for DOPAC, 2,3-DHBA and 2,5-DHBA by high-performance liquid chromatography with electrochemical (HPLC-EC) detection (17,18). The HPLC-EC system was equipped with a glassy carbon working electrode (EICOM CORP., Japan) and an analytic reverse-phase column on an Eicompak MA-5ODS column (5 µm 4.6 x 150 mm; EICOM). The working electrode was set at a detector potential of 0.75 V. Each liter of mobile phase contained 1.5 g 1-heptansulfonic acid sodium salt (Sigma), 0.1 g Na_2EDTA, 3 mL triethylamine (Wako Pure Chemical Industries, Japan) and 125 mL acetonitrile (Wako) dissolved in H_2O. The pH of the solution was adjusted to 2.8 with 3 mL phosphoric acid (Wako). The results are reported as the mean ± SE output in a 120-min period.

Drugs: The following drugs, donated by the manufacturers were used in the study: imipramine hydrochloride (Ciba-Geigy, Takarazuka, Japan), nortriptyline hydrochloride (Dainippon, Osaka, Japan), and nomifensine maleate (Hoechst, Frankfurt, Germany). Sodium salicylate and its hydroxylated metabolites were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). The two radiochemical substrates used in this study,

hydroxytryptamine binoxalate (5-[2-^{14}C]-5-HT; 1.48 to 2.22 GBq/nmol) and phenylethylamine hydrochloride (β-[ethyl-1-^{14}C]-(β-PEA; 1.48 to 2.22 GBq/nmol), were purchased from Dupont NEN (New England Nuclear) Products (Boston, MA, USA).

Statistical analysis: All values are expressed as means ± standard error of the mean (S.M.E.). Differences in time course between DOPAC, 2,3-DHBA and 2,5-DHBA levels were statistically evaluated by using the Mann-Whitney *U*-test. AP value of less than 0.05 was considered significant.

RESULTS

1. Effect of antidepressant drugs on MAO activity in vitro.

To determine the mechanism by which MAO activity was inhibited by these drugs, the effects of various concentrations of these reagents on MAO in rat brain mitochondria were studied *in vitro* using 5-HT and β-PEA as substrates. When the noncyclic antidepressant, nomifensine, was used, the residual activity of MAO-A was 75% with 1.0 mM nomifensine and the residual activity of MAO-B was 60% (Fig. 1). All of the tricyclic antidepressant drugs (imipramine, nortriptyline) potently inhibited MAO activity in a dose-dependent manner. With 1.0 mM of each drug, the residual activity of MAO-A and MAO-B was 50% and 5% for imipramine, and 20% and 2% for nortriptyline, respectively (Fig. 2).

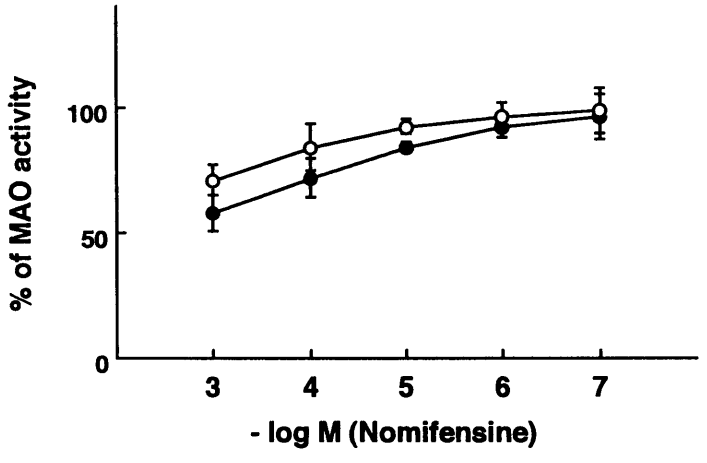

Fig. 1. *In vitro* effect of nomifensine on MAO activity in mitochondria of rat brain. After incubation at 25°C for 20 min with various concentrations of this reagent, MAO activity was determined with 100 μM 5-HT (O—O) or 10 μM β-PEA (●—●) as substrate at 37°C for 20 min. The mean control value for MAO-A was 0.22 ± 0.01 nmol/min/mg protein, and the mean control value for MAO-B was 0.25 ± 0.03 nmol/min/mg protein. The results are means of triplicate assays.

2. Changes in levels of monoamine metabolites in rat striatum.

In an in vivo perfusion system, time-dependent changes in the levels of the DA metabolite DOPAC are shown in Fig. 3. After imipramine ornortriptyline (1 mM; 1(l/min) was directly infused into the rat brain through a microdialysis probe for 60 min, the DOPAC level decreased in a time-dependent manner. However, when nomifensine was used, the peak DOPAC level did not change.

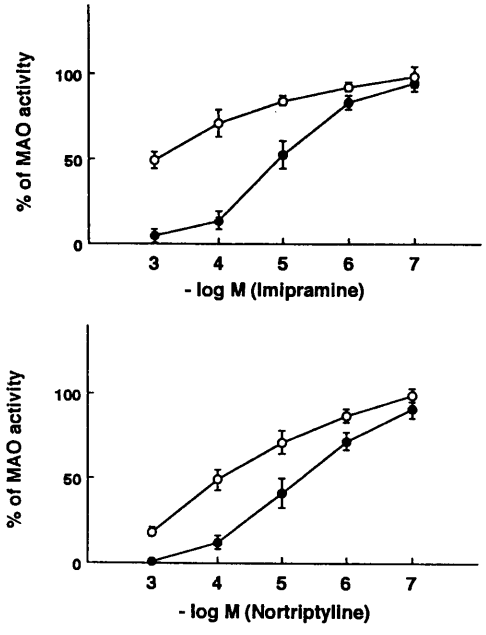

Fig. 2. *In vitro* effects of imipramine and nortriptyline on MAO activity in mitochondria of rat brain. After incubation at 25°C for 20 min with various concentrations of these reagents, MAO activity was determined with 100 μM 5-HT (O—O) or 10 μM β-PEA (●—●) as substrate at 37°C for 20 min. The mean control value for MAO-A was 0.22 ± 0.01 nmol/min/mg protein, and the mean control value for MAO-B was 0.25 ± 0.03 nmol/min/mg protein. The results are means of triplicate assays.

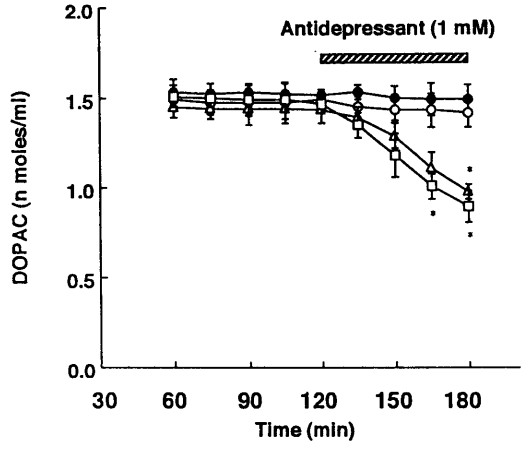

Fig. 3. Time course of dialysate DOPAC in extracellular fluid of caudate nucleus. Each antidepressant drug (1 mM; 1 μl/min) was infused directly through a microdialysis probe placed in the rat striatum for 60 min (diagonal column). Brain dialysate was collected every 15 min into 0.1 N $HClO_4$ and immediately assayed for DOPAC by an HPLC-EC procedure. Extracellular DOPAC levels at each time were compared by using Mann-Whitney *U*-test. Data for three treatment groups (O nomifensine-treated rats, Δ imipramine-treated rats and □ nortriptyline-treated rats) were individually compared with those of non-treated rats (●). Values are expressed as mean ± SE from six rats. Asterisk indicates $p<0.05$.

3. The •OH generation after antidepressant drug treatment.

The present dialysis results demonstrated that the generation of •OH in the rat striatum was increased after treatment with the tricyclic antidepressant drugs imipramine and nortriptyline. Sodium salicylate in Ringer's solution (0.5 mM or 0.5 nmol/μl/min) was infused for 120 min to trap ·OH formed after treatment with the antidepressant drugs. When imipramine or nortriptyline (1 mM; 1μl/min) was directly infused in the rat brain through a microdialysis probe for 60 min, a marked transient elevation in the levels of 2,3- and 2,5-DHBA was observed in the brain dialysate. The level at 120-135 min (or 15 min after administration of imipramine or nortriptyline) was significantly increased in relation to the level at 105-120 min. However, when the noncyclic drug, nomifensine, was used no increase in 2,3- and 2,5-DHBA was observed (Fig. 4).

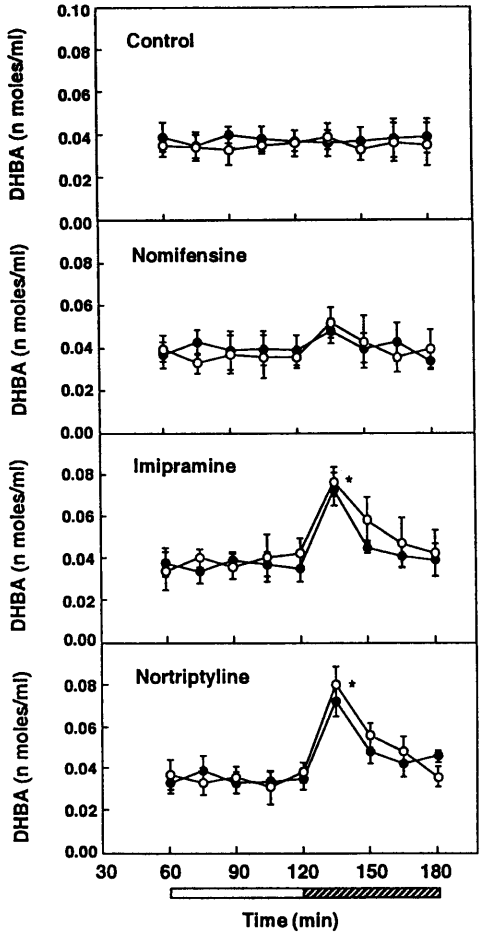

Fig. 4. Effects of antidepressant drugs on the formation of •OH in the striatum of rats. After a 60-min washout with Ringer's solution, sodium salicylate (open column; 0.5 mM or 0.5 nmol/μl/min) was infused for 60 min to trap •OH. Each antidepressant drug (1 mM; 1 μl/min) in salicylic acid solution was infused directly through a microdialysis probe placed in the rat striatum for 60 min (diagonal column; total dose: 60 n mol). Brain dialysate was collected every 15 min into 0.1 N $HClO_4$ and immediately assayed for 2,3- and 2,5-DHBA by an HPLC-EC procedure. Time course of changes in 2,3- and 2,5-DHBA levels was statistically evaluated by using Mann-Whitney *U*-test. Values are expressed as mean±SE from six rats. *$p<0.05$ versus level at 105-120 min. Abscissa, after 60-min washout, infusion of salicylic acid was started.

DISCUSSION

The effect of antidepressants on the generation of •OH was investigated by using striatal microdialysis. The formation of DHBAs following the administration of salicylate is currently used as an index of •OH generation (13,16,19-22). MAO is a brain enzyme that plays a role in the metabolism of various catecholamines. In the present study, MAO-A and MAO-B in the rat brain were inhibited by the antidepressant drugs nomifensine, imipramine and nortriptyline. These drugs are more potent inhibitors of MAO-B (Fig. 2). The kinetics of the inhibition of MAO by these drugs were investigated by using Lineweaver-Burk double-reciprocal plots. All of the drugs were competitive inhibitors of MAO-A and noncompetitive inhibitors of MAO-B (data not shown). It is known that tricyclic antidepressant drugs competitively inhibit MAO and that, *in vitro*, these drugs are more potent inhibitors of MAO-B than of MAO-A (4).

We previously reported (26,28) that the level of the oxidized DA metabolite DOPAC in the dialysate decreased following the administration of a MAO inhibitor. This result indicated that MAO inhibitors cause the accumulation of DA in the extracellular fluid (29). DA is known to undergo autooxidation in the presence of oxygen and transition metals (30-32). Theoretically, •OH may be formed *in vivo* during non-enzymatic oxidation (31, 33-35) and/or enzymatic oxidation of DA, especially in the brain regions (putamen, caudate nucleus and substantia nigra zona compacta) where there are high levels of DA. The sustained autooxidation of DA could lead to excessive accumulation of toxic quinones and potentially cytotoxic oxygen free radicals. Chiueh *et al* reported (15) a linear correlation between the formation of •OH products of salicylate and the efflux/oxidation of DA. When the level of DOPAC decreased after the administration of the tricyclic antidepressants imipramine and nortriptyline, a marked transient elevation in the level of 2,3- and 2,5-DHBA was observed in the brain dialysates. However, when the noncyclic antidepressant drug nomifensine was used, the level of DOPAC did not change, and thus there was no elevation in the level of DHBAs. When the control experiments were performed, a transient increase in •OH as a result of flow or pressure changes in the microdialysis system due to liquid switching was not observed. We cannot explain why there was only a transient increase in both forms of DHBA in the presence of excessive DA following inhibition of MAO activity. Further investigation is necessary to solve this problem. When the same experiment was performed in rat liver, which is known to contain very low levels of catecholamines, no elevation in the levels of DHBA products was observed (data not shown). The of DA that accumulates in the extracellular fluid following administration of antidepressants can undergo autooxidation, which in turn leads (possibly by an indirect mechanism) to the formation of cytotoxic •OH free radicals. The site of •OH formation is such that the antidepressants inhibit MAO *in vitro* and that a similar effect *in vivo* will lead to the accumulation of DA in the extracellular space (29). However, the drugs are also potent inhibitors of DA re-uptake, so regardless of their specific mechanisms of action the consequence is increased extracellular levels of DA. This, in turn, could explain both the observed decrease in DOPAC and the increase in •OH. Both non-enzymatic and enzymatic mechanisms may contribute to free radical formation induced by antidepressant drugs in the striatum *in vivo*. This finding shows that extracellular DA is needed for the observed effect of these tricyclic antidepressant drugs on the hydroxylation of salicylic acid.

The present results demonstrate an increase in 2,3- and 2,5-dihydroxylation of salicylate following the administration of tricyclic antidepressant drugs. In the future, intracranial microdialysis experiments using the hydroxylation of salicylate to monitor •OH generation may be useful in answering some of the fundamental questions concerning the relevance of oxidant damage in the side effects of antidepressant drugs in the clinical setting. Further studies are necessary to determine whether or not these effects are important with regard to the antidepressive action of these antidepressant drugs.

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