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ENDOGENOUS MONOAMINE OXIDASE INHIBITOR-LIKE SUBSTANCES IN MONKEY BRAIN

TORU EGASHIRA, TOSHIO OBATA, TAKAYUKI NAGAI, YOSHIHIRA KIMBA, RITSUKO TAKANO and YASUMITSU YAMANAKA

Department of Pharmacology, Medical College of Oita, 1–1506, Idaigaoka, Hazama-cho, Oita 879–56, Japan

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Abstract—The extraction and partial purification of endogenous "monoamine oxidase (MAO) inhibitor-like" material from the monkey brain are described. The endogenous material (F-1 and F-2) obtained after Bio-Gel P-2 gel filtration and silica column chromatography inhibited MAO in the monkey brain mitochondria toward 5-hydroxytryptamine (5-HT), β-phenylethylamine (β-PEA), tyramine and dopamine as substrates. The inhibitory effects of F-1 and F-2 were non-linear concentration dependent, and F-1 non-competitively inhibited A-form MAO, while F-2 inhibited A-form MAO competitively and inhibited B-form MAO non-competitively. These substances were more potent inhibitors of A-form than of B-form MAO. F-2 was heat stable but liable to the treatment with pepsin and trypsin. F-1 was not inactivated by heat treatment and digestion with pepsin and trypsin. F-1 may be a low molecular weight (<1350) compound, including certain monoamines or their metabolites or other unidentified compounds, while F-2 was a low molecular weight (about 2500) peptide.

Since the possible physiological relationship between platelet monoamine oxidase (MAO) activity and some psychiatric diseases was reported [1–5], several endogenous MAO modulators have been detected in the human plasma or isolated from it [6–10]. Giambalvo and Becker [8] demonstrated that the addition of plasma to striatum or platelet inhibited MAO-A or MAO-B activities, and these effects were associated with the presence of low molecular weight proteins in plasma. On the contrary, Yu and Boulton [7], using different experimental conditions, found that human plasma increased platelet MAO activity. In addition a further putative endogenous MAO inhibitor has been isolated from human cerebrospinal fluid (CSF) [11]. The endogenous substances in human CSF are present in low molecular weight fractions and act like MAO inhibitor drugs to inhibit both type A and type B MAO. While the materials in dog CSF also possess the action to activate the imipramine binding and 5-HT uptake as well as MAO inhibition [12, 13]. The other endogenous inhibitor of MAO which is capable of inhibiting both MAO-A and MAO-B has been discovered in normal human [14, 15] and rat urine [16]. Significantly high values of this inhibitor have been observed in alcoholics recently withdrawn from alcohol, compared with controls [17]. These investigators indicated that endogenous MAO modulators might be important as physiological regulators. There are many reports on the possible existence of the multiple modulators of MAO being present in the cytosol fractions of various organs of animals [18–21]. These observations have prompted us to search for the endogenous MAO inhibitor in the brain, exhibiting higher sensitivity to MAO enzyme. We demonstrate in this paper the existence of endogenous factors in the monkey brain extract which is a potent inhibitor of the MAO activity *in vitro* in the monkey brain.

MATERIALS AND METHODS

1. *Extraction of an endogenous MAO inhibitor-like substance.* Endogenous MAO inhibitor-like substance was extracted from the brains of adult monkey by a modified method of Rehavi *et al.* [22]. Three monkey brains (*ca.* 300 g) were homogenized by a Teflon homogenizer in 5 vol. of ice-cold 5% trichloroacetic acid. The homogenate (*ca.* 1500 ml) was centrifuged for 10 min at 9000 *g* and the pellets were resuspended in the same solution and was recentrifuged. The pooled supernatants (2000 ml) were extracted with ether to remove the acid and lipid. The residual ether was evaporated from the aqueous phase and a protein-free, lipid-free solution was freeze-dried, dissolved in 20 ml of 0.2 M acetic acid and applied to a Bio-Gel P-2 gel filtration column (2.5 × 95 cm). The column was eluted with 0.2 M acetic acid at the rate of 170 ml/hr and fractions of 4.2 ml were collected. Each of these fractions was tested for its ability to inhibit MAO activity in the monkey brain mitochondria. The potent fractions from the Bio-Gel P-2 column (Fraction No. 115–145) were pooled. After ultrafiltration of the fraction through DIAFLO membrane (YM 30) to remove substances of molecular weight > 30,000, lyophilized and reconstituted in 10 ml of distilled water and applied in it to a silica column (1.8 × 22 cm). The column was eluted stepwise by the pure water and water-acetonitrile (30:70). The fractions, unbound (F-1) and bound (F-2) to silica were collected. After freeze-drying and reconstitution in 5 ml water, samples were tested for their ability to inhibit MAO activity in the monkey brain mitochondria.

2. *MAO activity.* Mitochondrial fractions from

monkey brains were prepared by differential centrifugation as described earlier [23]; the mitochondria suspended in 0.32 M sucrose were used as the enzyme preparation for determination of MAO activity. MAO activity was estimated by radioisotopic assay with [^{14}C]-5-HT (substrate for type A MAO, final concentration at 200 μM), [^{14}C]-β-phenylethylamine (β-PEA) (substrate for type B MAO, final concentration at 50 μM) and [^{14}C]tyramine and [^{14}C]dopamine (substrate for type A and B, final concentration at 200 μM) as described earlier [13]. The incubation medium contained a suitable amount of the enzyme to give a linear reaction for at least 40 min in a total volume of 225 μl of a 0.1 M phosphate buffer, pH 7.2. The reaction was started by adding 25 μl of a labelled substrate; incubation was carried out for 20 min at 37°. Then the reaction was stopped by adding 2 N HCl (200 μl). The reaction products were extracted with ethylacetate–benzene (1:1, v/v). Samples of the extracts were mixed with Triton X-100-toluene scintillation liquid and their radioactivities were measured by liquid scintillation spectrometry.

3. *Heat treatment and digestion with pepsin and trypsin.* For determination of the heat stability of the endogenous MAO inhibitor-like substances, an aliquot of the silica column elute containing the inhibitory activity on MAO was heated at 90° for 20 min prior to its addition to the mitochondrial preparations.

The silica column elute samples were incubated with a 1 mg/ml pepsin or 1 mg/ml trypsin at 37° for 30 min. Then pepsin or trypsin was inactivated by heating at 90° for 10 min. The samples were then added to mitochondrial preparations to determine their effects on MAO activity. Controls containing an equal volume of the buffer instead of the MAO inhibitor-like substances were treated with pepsin or trypsin and then heated in the same manner.

4. *Determination of molecular weight.* The molecular weights of these substances were estimated using chromatography on a Sephadex G-25 column. Silica column elute samples were lyophilized, redissolved in a small volume of water, and loaded into a Sephadex G-25 column (2.5 × 50 cm) that had been calibrated with markers of known molecular weight. The column was eluted with 1 mM phosphate buffer pH 7.4 at the rate of 10 ml/hr and effluent fractions were monitored at 280 nm in a spectrophotometer. The V_e/V_0 for markers were as follows: carbonic anhydrase, (M.W. 29,000) 1.23; cytochrome *c*, (M.W. 12,400) 1.47; approtinin, (M.W. 6500) 1.82; glucagon, (M.W. 3500) 2.23 and cyanocobalamine, (M.W. 1350) 2.50.

5. *Estimation of protein.* Protein concentrations of the preparations were measured by the method of Lowry *et al.* [24] with bovine serum albumin as the standard.

6. *Chemicals.* The radiochemicals substances [^{14}C]β-phenylethylamine HCl (β-PEA) (50 mCi/mmole, 97.5% purity) and [^{14}C]tyramine HCl (50 mCi/mmole, 98.5% purity) were purchased from New England Nuclear (Boston, MA) and [^{14}C]dopamine (46.8 mCi/mmole, 98.0% purity) and [^{14}C]-5-hydroxytryptamine creatinin sulfate (5-HT) (50.7 mCi/mmole, 99.0% purity) were from Amersham Japan Co. Trichloroacetic acid, acetonitrile, acetic acid and silica gel were obtained from Wako Pure Chemical Industries, Ltd. Bio-Gel P-2 was obtained from Bio-Rad Japan Lab.

RESULTS

1. *Separation of endogenous substances*

Fractions 115–145 were found to inhibit MAO activity in the monkey brain with tyramine as substrate following the elution on Bio-Gel P-2 column (Fig. 1). These fractions were pooled, freeze-dried and were further purified by using a silica column. The column was eluted stepwise by the pure water

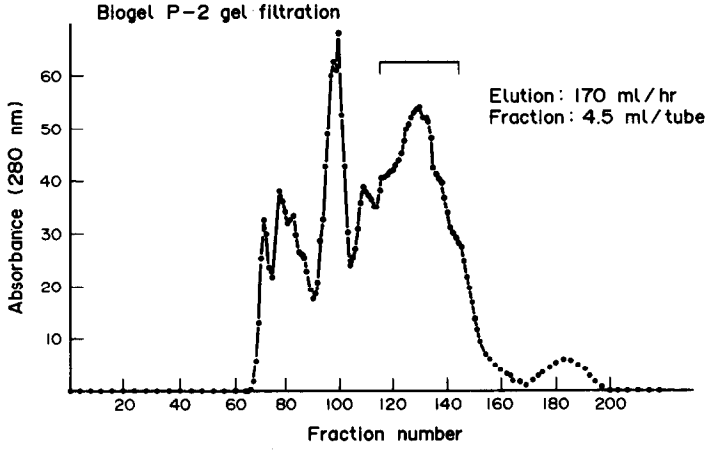

Fig. 1. Gel filtration of monkey brain extract on Bio-Gel P-2 column. The sample of brain extract (*ca.* 20 ml) was chromatographed in 0.2 M acetic acid at a flow rate of 170 ml/hr and fractions of 4.2 ml were collected. Column effluents were monitored by measuring absorbance at 280 nm. An aliquot of each fraction of tested for ability to inhibit MAO activity in the monkey brain mitochondria with tyramine as substrate. Fractions 115–145 were found to inhibit MAO activity. These fractions were pooled and freeze-dried.

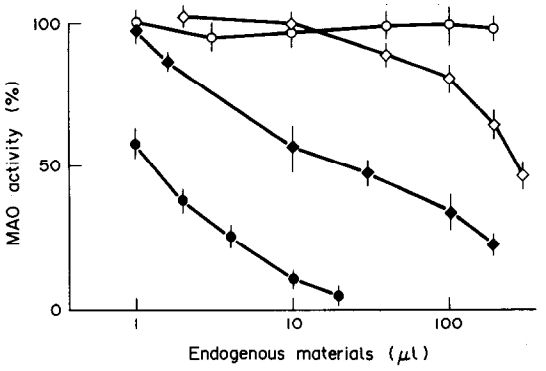

Fig. 2. Inhibition of MAO activity by the endogenous substances. After incubation at 37° for 20 min with various amounts of endogenous substances (F-1 and F-2), MAO activity in monkey brain mitochondria was determined with 200 μM 5-HT (F-1 ●——●, F-2 ◆——◆) and 50 μM β-PEA (F-1 ○——○, F-2 ◇——◇) as substrates at 37° for 20 min. The mean ± SE control values for MAO activities were 0.91 ± 0.03 and 1.32 ± 0.04 nmoles/min/mg protein with 5-HT and β-PEA as substrates. Each point represents the mean percentages (± SE) of the control MAO activity without endogenous substances in triplicate determinations.

Table 1. Effects of F-1 and F-2 on monkey brain mitochondrial MAO activity in the presence of different substrates

	% of MAO activity	
	F-1 (4 μl)	F-2 (60 μl)
5-HT	25.1 ± 3.8	40.3 ± 2.4
Tyramine	83.7 ± 3.2	79.5 ± 5.6
Dopamine	75.2 ± 5.8	74.8 ± 4.1
β-PEA	98.8 ± 3.2	80.1 ± 1.2

After the mitochondrial preparations of monkey brain were incubated at 37° for 20 min in the presence of F-1 (4 μl) or F-2 (60 μl), MAO activity determined with 200 μM 5-HT, tyramine and dopamine and 50 μM β-PEA as substrates at 37° for 20 min. The mean ± SE control values for MAO activities were 0.94 ± 0.02, 4.38 ± 0.15, 2.95 ± 0.21 and 1.58 ± 0.03 nmoles/min/mg protein with 5-HT, tyramine, dopamine and β-PEA as substrates, respectively. The results are the mean of triplicate assays. Values are percentages of the control MAO activity without endogenous substances.

and water–acetonitrile (30:70). The fractions, unbound (F-1) and bound (F-2) to each of the silica column, were collected. After freeze-drying, these samples were reconstituted in 5 ml of water, pH checked to 7.4, and tested for their ability to inhibit MAO activity. Both fractions contained the inhibitory activity against MAO activity. These F-1 and F-2 fractions were used as the preparations of the endogenous MAO inhibitor-like substances in subsequent experiments.

2. *The inhibition of MAO activity by the endogenous substances*

The effects of adding varying amounts of endogenous substances (F-1 and F-2) from monkey brain to monkey brain mitochondrial MAO *in vitro* were studied using 5-HT and β-PEA as substrates. As can be seen in Fig. 2, the inhibition curves of MAO activity by these endogenous substances were non-linear concentration-dependent and IC_{50} values were 23 μl and 270 μl with F-2 using 5-HT and β-PEA as substrates, respectively. In case of F-1, IC_{50} value was 1.3 μl with 5-HT as a substrate.

3. *Effects of* F-1 *and* F-2 *on brain mitochondrial MAO activity in the presence of different substrate*

When mitochondrial MAO from the monkey brain was incubated with different substrates in the presence and absence of F-1 or F-2, the greatest inhibition occurred with 5-HT as a substrate. The next most effective was dopamine followed by tyramine and β-PEA. No effect was observed on the deamination of β-PEA using the F-1 as endogenous MAO inhibitor-like substance (Table 1).

4. *Inhibition by* F-1 *and* F-2 *on MAO activity in the monkey brain mitochondria with* 5-HT *and β-PEA as substrates*

The kinetic studies of the inhibition of MAO by

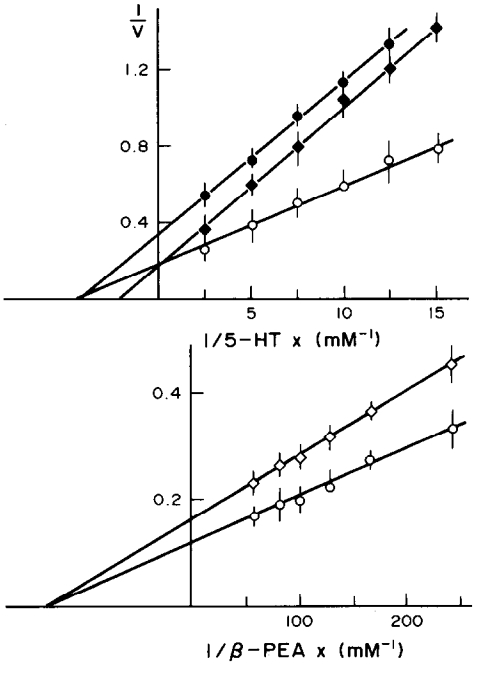

Fig. 3. Effects of endogenous substances on MAO activity in monkey brain mitochondria. Lineweaver–Burk plots of the reciprocal of the initial velocity of 5-HT and β-PEA oxidation against the reciprocal of the substrate concentration in the presence of endogenous substances. Abscissa, 1/substrate concentration in mM; ordinate, 1/initial velocity in nmole/min/mg of protein. Substrates used were 5-HT (upper) and β-PEA (bottom), assayed in the absence and presence of endogenous substances. Each point represents the mean MAO activity (± SE) assayed in triplicate determinations: ○——○, no additional substances; ●——●, 1.2 μl F-1; ◆——◆, 10 μl F-2; ◇——◇, 150 μl F-2.

Table 2. Some properties of the endogenous MAO inhibitor-like substances

	% of maximum MAO activity		
	F-1 (5 μl)	F-2 (50 μl)	
	5-HT	5-HT	β-PEA
No treatment			
F-1 or F-2 only	27.1 ± 1.8	42.5 ± 1.3	80.3 ± 2.6
Heat treatment			
90°, 20 min	30.5 ± 2.0	41.8 ± 5.1	78.5 ± 8.3
Proteolytic degradation			
pepsin	30.3 ± 7.2	52.3 ± 8.8	82.7 ± 3.9
trypsin	30.2 ± 7.6	84.5 ± 5.2	95.1 ± 2.3
Reversibility			
dilute, washing	77.6 ± 2.9	67.7 ± 3.3	87.2 ± 4.1

F-1 (5 μl) and F-2 (50 μl) were tested in various ways, such as heat treatment (90°, 20 min), washing and proteolytic degradation (pepsin 1 mg/ml, trypsin 1 mg/ml). MAO activity was determined with 5-HT and β-PEA as substrates at 37° for 20 min. The means ± SE control values for MAO activity were 0.94 ± 0.01 and 1.56 ± 0.04 nmoles/min/mg protein with 5-HT and β-PEA as substrates. The results are the mean of triplicate assays. Values are percentages of the control MAO activity without F-1 or F-2.

F-1 and F-2 addition were investigated with Lineweaver–Burk double reciprocal plots. The results with 5-HT and β-PEA as substrates were shown in Fig. 3. The inhibition by F-1 was non-competitive at least towards 5-HT, while in case of F-2, the inhibition was competitive (Fig. 3, upper panel). With β-PEA as a substrate, the inhibition by F-2 was non-competitive (Fig. 3, lower panel).

5. *Some properties of endogenous MAO inhibitor-like substances*

F-1 (4 μl) and F-2 (100 μl) were tested with various ways, such as heating in a boiling water bath (90°, 20 min), dilution or washing against distilled water and proteolytic degradation by incubation with crude pepsin (1 mg/ml) and trypsin (1 mg/ml) (Table 2). Heat treatment exhibited no effect on the inhibition. Dialysis removed some of the inhibitive capacities. Digestion with pepsin and trypsin reduced MAO inhibitory activity to about 25 and 50% of that originally present using F-2 as the endogenous MAO inhibitor-like substances. In the case of F-1, digestion had no effect on its inhibition of MAO.

6. *Molecular weight determination*

The molecular weights of these substances (F-1 and F-2) were estimated using chromatography on a Sephadex G-25 column. We found two different peaks: F-2 at the ratio of elution volume to void volume (V_e/V_o) of 2.3 with molecular weight 2500 and F-1 at a V_e/V_o of 2.55, corresponding to a molecular weight less than 1350 (Fig. 4).

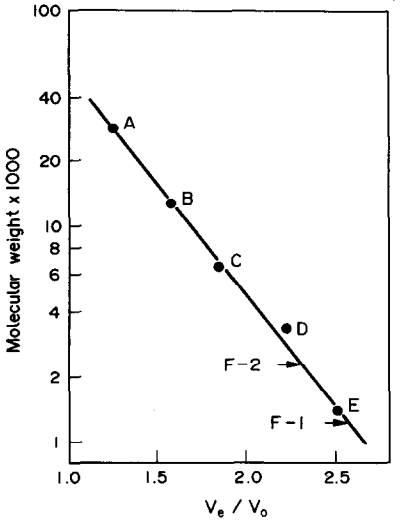

Fig. 4. Estimation of the molecular weight of the endogenous substances by Sephadex G-25 gel chromatography. For the experiment, 1 mM phosphate buffer, pH 7.4 was used. The flow rate was 10 ml/hr and effluent fractions were monitored at 280 nm. The V_e/V_o for markers were as follows: (A) carbonic anhydrase (M.W. 29,000) 1.23; (B) cytochrome *c* (M.W. 12,400) 1.47; (C) approtinin (M.W. 6500) 1.82; (D) glucagon (M.W. 3500) 2.23; and (E) cyanocobalamine (M.W. 1350) 2.50.

DISCUSSION

There have been many reports on the possible presence of MAO modulators in the cytosol of various tissues [18–21], in plasma [6–10], urine [14–16] and CSF [11]. We also have recently demonstrated that addition of dog CSF to the brain mitochondria inhibited A- and B-form MAO activities as measured by the deamination of 5-HT and β-PEA as substrates [12, 13]. However, there have been a few studies on some endogenous MAO modulators isolated from the brain [21, 25]. In this paper, the authors have demonstrated the presence of two types MAO inhibitor-like substances (F-1 and F-2), which inhibited both A- and B-form MAO in the monkey brain mitochondria with 5-HT and β-PEA as substrates. As far as we known, this is the first isolation of the

endogenous inhibitor of MAO from the monkey brain. These materials were more potent inhibitors of A-form MAO than of B-form MAO. The inhibitory effects of these materials were non-linear concentration-dependent. Based on the observations that the significant inhibitory activities persisted after ultrafiltration of these materials through DIAFLO YM 30 membrane, Bio-Gel P-2 gel filtration and fractionation of Sephadex G-25 column, it is likely that these inhibitors may be low molecular weight (>3000) materials. Recently, many investigators reported the isolation of endogenous modulators of one or more proteins with low molecular weight from human CSF [11] (3000 to more than 35,000), human plasma [9, 10] (4000–30,000) and rat heart cytosol [20] (8500–35,000). Berrettini and Vogel [6] indicated that the MAO inhibitory factor from human plasma was destroyed during dialysis, and that it had a low molecular weight (less than 10,000 daltons). In this study, these MAO inhibitors isolated from the monkey brain may also be similar substances.

The F-2 MAO inhibitor had the following properties: it was heat stable but labile to the treatment with pepsin and trypsin, it inhibited A-form MAO competitively at least toward 5-HT, but was non-competitive with B-form MAO. While, the MAO inhibitor-like activity of F-1 was not affected by heat treatment and by digestion with pepsin and trypsin. F-1 also non-competitively inhibited A-form MAO. From these results, it can be concluded that the monkey brain contains structurally different types of low molecular weight materials which act like MAO inhibitor drugs. F-1 may be a low molecular weight compound such as certain monoamines or their metabolites, β-carboline [16, 17] or tribuline [26]. Another, F-2 may be a low-molecular weight peptide of about 2500 M.W. based on the heat-stable but labile to the treatment with pepsin or trypsin.

Although some endogenous MAO modulators have been found in plasma, urine and CSF, there have been few studies on their possible role as physiological regulators of MAO activity. Recently, alterations in activity of catecholaminergic neurons have been suggested in affective disorder and schizophrenia [27, 28]. In addition, there have been many reports which associate platelet MAO activity and depressive symptoms [1–5]. However, the relationship between the plasma MAO modulators and platelet MAO activity has yet to be determined. In this study, our results suggest the possible presence of two types of MAO modulators that inhibit A- and B-form MAO in the brain. We also demonstrate that monkey CSF and plasma contain endogenous substances that act like an MAO-inhibitor to inhibit A- and B-form MAO in the monkey brain mitochondria and also B-form MAO in the monkey platelet [29]. The finding of similar inhibitory substances to MAO in the brain, CSF and plasma indicates a possibility that these MAO inhibitor-like substances produced in the CNS act to modulate MAO activity, while some of these modulators are released to CSF or plasma and inhibit the MAO activity of the platelet. One possibility of this speculation is that the low brain [30, 31] and platelet [1–5, 32, 33] MAO activity in psychiatric illness may be accounted for by an increase in the level of the endogenous MAO modulators.

To support this view, however, the MAO inhibitor-like substances obtained from the brain by us need further purification and more detailed testing for the inhibition of both brain and platelet MAO.

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