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Life Sciences 69 (2001) 879–889

Life Sciences

Inhibition of brain monoamine oxidase activity by the generation of hydroxyl radicals Potential implications in relation to oxidative stress

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Received 20 October 2000; accepted 5 January 2001

Abstract

Monoamine oxidase (MAO) is an enzyme involved in brain catabolism of monoamine neurotransmitters whose oxidative deamination results in the production of hydrogen peroxide. It has been documented that hydrogen peroxide derived from MAO activity represents a special source of oxidative stress in the brain. In this study we investigated the potential effects of the production of hydroxyl radicals (•OH) on MAO-A and MAO-B activities using mitochondrial preparations obtained from rat brain. Ascorbic acid (100 μM) and Fe^{2+} (0.2, 0.4, 0.8, and 1.6 μM) were used to induce the production of •OH. Results showed that the generation of •OH significantly reduced both MAO-A (85–53%) and MAO-B (77–39%) activities, exhibiting a linear correlation between both MAO-A and MAO-B activities and the amount of •OH produced. The reported inhibition was found to be irreversible for both MAO-A and MAO-B. Assuming the proven contribution of MAO activity to brain oxidative stress, this inhibition appears to reduce this contribution when an overproduction of •OH occurs.

Keywords: Monoamine oxidase; Hydroxyl radical; Enzyme inhibition; Oxidative stress; Brain mitochondria; Rat

Introduction

Monoamine oxidase (MAO) is an FAD-dependent enzyme localized in the outer membrane of the mitochondria which plays an essential role in the turnover of monoamine neu-

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0024-3205/01/$ – see front matter
PII: S0024-3205(01)01178-X

rotransmitters such as dopamine, serotonine and noradrenaline. It occurs in at least two forms, MAO-A and MAO-B, with different specificities for substrates and inhibitors. The cloning of cDNAs for MAO-A and MAO-B has demonstrated that both isoenzyme forms are encoded by different genes, associating their different specificities for substrates and inhibitors to their corresponding primary structures [1]. However, the potential contribution of the membrane lipid environments on MAO-A and MAO-B specificities remain unsolved [2,3]. It catalyzes the oxidative deamination of biogenic amines to their corresponding aldehydes, which is accompained by the reduction of molecular oxygen to hydrogen peroxide (H_2O_2) [4,5].

As is well-known, H_2O_2 is a reactive oxygen species (ROS) which, through the Fenton reaction, can generate a hydroxyl radical ($^{\bullet}OH$), this being considered the most damaging free radical for living cells due to its high reactivity [6]. The involvement of $^{\bullet}OH$ in neuronal loss has been postulated in cerebral ischemia [7], in aging [8], in Parkinson's disease [9], and in Alzheimer's disease [10].

Although, H_2O_2 is also formed during mitochondria respiration, the amount of H_2O_2 generated by MAO activity greatly exceeds the amount produced during electron flow [5], which identifies the activity of this enzyme as a process with a considerable toxic potential. This suggestion is corroborated *in vivo* by the diminution in brain production of H_2O_2 observed in rats by the inhibition of MAO activity with pargyline [11]. In spite of the existence of two enzymatic scavenging systems to protect cells from the presence of H_2O_2, catalase and glutathione peroxidase, the brain levels of these two enzymes are very low compared to those found in other tissues [12].

The aim of the present study was to investigate the potential *in vitro* effects of the production of $^{\bullet}OH$ on both MAO-A and MAO-B activities. MAO activity was determined in crude mitochondrial fractions obtained from rat brain and $^{\bullet}OH$ was generated using a mixture of ascorbic acid (AA) and ferrous iron (Fe^{2+}).

Methods

Chemicals

Kynuramine dihydrobromide, AA, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Clorgyline hydrochloride and R(-)-deprenyl hydrochloride was obtained from Research Biochemicals International (Natick, MA, USA). Ferrous chloride tetrahydrate was purchased from Fluka Chemie AG (Buchs, Switzerland). Terephthalic acid (THA), disodium salt was from Aldrich Chemical Co. (Milwaukee, WI, USA). The water used for the preparations of solutions was of Milli-RiOs/Q-A10 grade (Millipore Corp., Bedford, MA, USA). All remaining chemicals used were of analytical grade and were purchased from Fluka Chemie AG (Buchs, Switzerland). Fresh stock solutions of AA and Fe^{2+} were prepared immediately before each experiment in water and a buffer solution (Na_2PO_4/KH_2PO_4 isotonized with KCl, pH 7.4), respectively.

Preparation of brain mitochondria

Male Sprague-Dawley rats weighing 200–250 g were used. The rats were received from the breeder at least four days before sacrifice, and were kept on a 12:12 light-dark schedule

with *ad libitum* access to food and water. Animals were stunned with carbon dioxide and killed by decapitation. Brains were immediately removed and washed in ice-cold isolation medium (pH 7.4, Na_2PO_4/KH_2PO_4 isotonized with sucrose). Brain mitochondrias were then obtained by diferential centrifugation with minor modifications to a previously published method [13]. Briefly, after removing blood vessels and pial membranes, the brains were manually homogenized with four volumes (w/v) of the isolation medium. Then, the homogenate was centrifuged at 900 *g* for 5 min at 4 °C. The supernatant was centrifuged at 12,500 *g* for 15 min. The mitochondria pellet was then washed once with isolation medium and recentrifuged under the same conditions. Finally, the mitochondrial pellet was reconstituted in a buffer solution (Na_2PO_4/KH_2PO_4 isotonized with KCl, pH 7.4) and stored in aliquots under liquid nitrogen.

The protein concentration of mitochondrial suspensions was determined according to the method of Markwell et al. [14], using bovine serum albumin as the standard.

Determination of MAO activity

MAO activity was measured by a spectrophotometric assay based on the original procedure of Weissbach et al. [15], as previously reported [13]. A Ultrospec III spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) with a cuvette holder thermostatized at 37 °C was used. (-)-Deprenyl (250 nM) and clorgyline (250 nM) were used as irreversible and selective inhibitor to assay MAO-A and MAO-B activity, respectively. Mitochondrial incubations were performed in a buffer solution (Na_2PO_4/KH_2PO_4 isotonized with KCl, pH 7.4) at a final protein concentration of 1 mg/ml. A 5 minute preincubation of the irreversible inhibitor and the mitochondria preparation was made, followed by the concurrent addition of AA (100 μM) and Fe^{2+} (0.2, 0.4, 0.8 or 1.6 μM). After 5 min of incubation, kynuramine was added as a non selective substrate at concentrations equal to the corresponding K_M value (90 μM for MAO-A and 60 μM for MAO-B). All concentrations are final concentrations. The formation of 4-hydroxyquinoline (4-OHQ) was then followed at 314 nm for 5 min.

The reversibility of the inhibition was determined by dialysis using a Biodialyser® (Sigma Chemical Co.) with an ultrafiltration membrane of a nominal molecular weight limit of 10,000 [16]. Mitochondrial preparations were preincubated at 37 °C for 15 min in the absence (control) or presence of both AA (100 μM) and Fe^{2+} (0.8 μM). The resulting mixtures were then dialysed at 4 °C using 250 ml of outer buffer (Na_2PO_4/KH_2PO_4 isotonized with KCl, pH 7.4). The outer buffer was replaced with fresh buffer every 2 hours for a time period of 10 h. Finally, the dialysed mixtures were then assayed for MAO-A and MAO-B activity.

Monitoring of ·OH formation

The generation of ·OH was fluorimetrically monitored using a modification to a previously published method [17] in which THA is used as a chemical dosimeter of ·OH. A luminiscence spectrometer Model LS50B (Perkin-Elmer, Norwalk, CT, USA) was used. The cuvette holder was thermostatically maintained at 37 °C and a magnetic stirrer was used for a continous mixing of the sample. For each assay, 2000 µl of a buffer solution (Na_2PO_4/KH_2PO_4 isotonized with KCl, pH 7.4) containing 10 mM THA (final concentration) were incubated in a quartz cuvette for 5 min to reach the temperature. An aliquot of phosphate buffer (pH 7.4) was added to complete the final volumen of the incubation to 2.5 ml. Then, 100 µl of AA

(100 μ*M*) and 20 μl of varying concentrations of Fe^{2+} (0.2, 0.4, 0.8 or 1.6 μ*M*) were added. All the concentrations are final concentrations. The monitoring of ·OH formation was immediately initiated and maintained for the subsequent 5 min, using 312 nm and 426 nm as excitation and emission wavelengths, respectively.

Statistical analysis

All results are expressed as means±SEM. Data were tested for significant differences between means by a two-way Student's *t*-test. Significance was indicated when *p* was equal to or less than 0.05. Data analysis was aided by use of the computer program Origin® v. 6.0 (Microcal Software Inc., Northampton, MA, USA).

Results

In this study we investigated the *in vitro* effects of the generation of ·OH on MAO-A and MAO-B activities using crude mitochondrial fractions obtained from rat brain. In order to optimize the analytical assay for MAO-A and MAO-B determination, we previously studied the effects of the concentration of both clorgyline and (-)-deprenyl on MAO-A and MAO-B activities, respectively. Fig. 1 shows the results obtained in this study. From the reported data, we selected the concentration of 250 nM because it represents the lowest concentration of in-

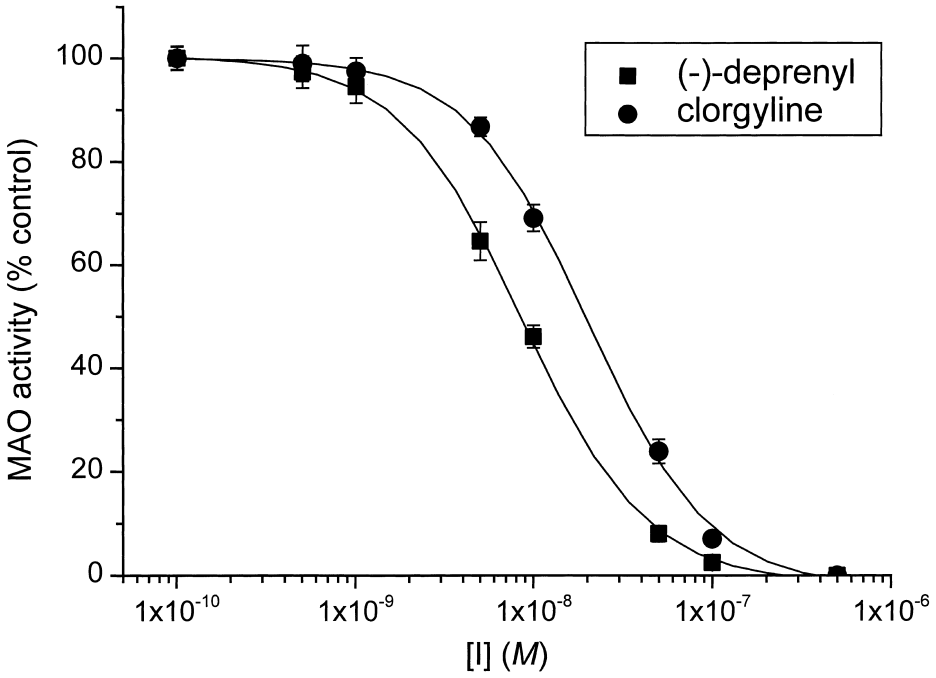

Fig. 1. Effects of clorgyline on MAO-A activity and (-)-deprenyl on MAO-B activity after preincubation of mitochondrial preparations with 1μ*M* of (-)-deprenyl or clorgyline, respectively. Each point represents the mean±S.E.M. from three determinations.

hibitor which guarantees a 100% inhibition of the corresponding MAO isoform. After preincubation of mitochondrial preparations with clorgyline (250 n*M*) or (-)-deprenyl (250 n*M*) for 5 min, K_M values for MAO-A and MAO-B were estimated by linear regression analysis of the corresponding Lineweaver-Burk plots using the following concentrations of kynuramine: 20, 40, 80, and 140 μ*M* for MAO-A and 20, 40, 60, 80, and 100 μ*M* for MAO-B. Under the here reported experimental conditions, MAO-A and MAO-B activities (controls) were of 1.521±0.083 nmol 4-OHQ/mg protein·min and 4.562±0.037 nmol 4-OHQ/mg protein·min, respectively.

As shown in Fig. 2, the production of ·OH was achieved by the use of the combined action of AA (100 μ*M*) and Fe^{2+} (0.2, 0.4, 0.8, and 1.6 μ*M*), with ·OH formation being dependent on the Fe^{2+} concentration used. To assess the production of ·OH, the area under the curve (AUC) was used as an "impregnation factor", and was calculated using the graph package Origin®.

The effect of the concurrent preincubation of AA and Fe^{2+} with the mitochondrial preparations for 5 min was a significant reduction in both MAO-A and MAO-B activities, which was dependent on Fe^{2+} concentration used and consequently on the ·OH production achieved. Fig. 3 illustrates the MAO activity found under the different experimental conditions assayed, and shows that MAO activities ranged from 85% to 53% (of control) for MAO-A and from 77% to 39% (of control) for MAO-B. As can be seen, the reduction observed in both MAO-A and

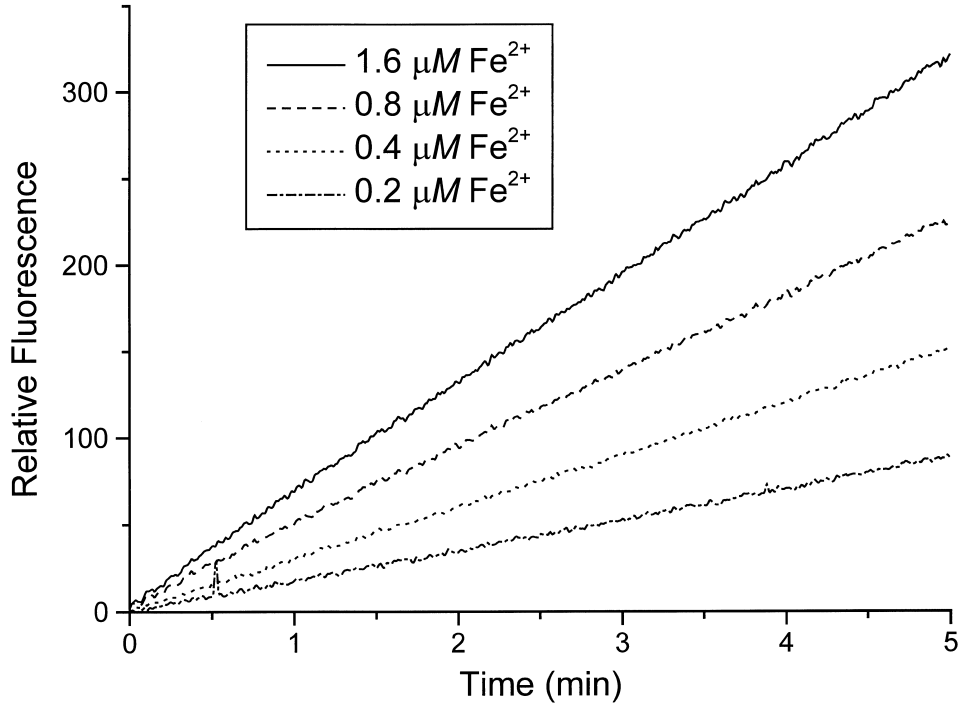

Fig. 2. Representative fluorimetric recording of the production of ·OH from the oxygen disolved in the incubation medium (pH 7.4, Na_2PO_4/KH_2PO_4 isotonized with KCl) in the presence of AA (100 μ*M*) and different concentrations of Fe^{2+}. THA (10 m*M*) was used to detect the production of ·OH and the wavelenghts of excitation and emission were of 312 nm and 426 nm, respectively.

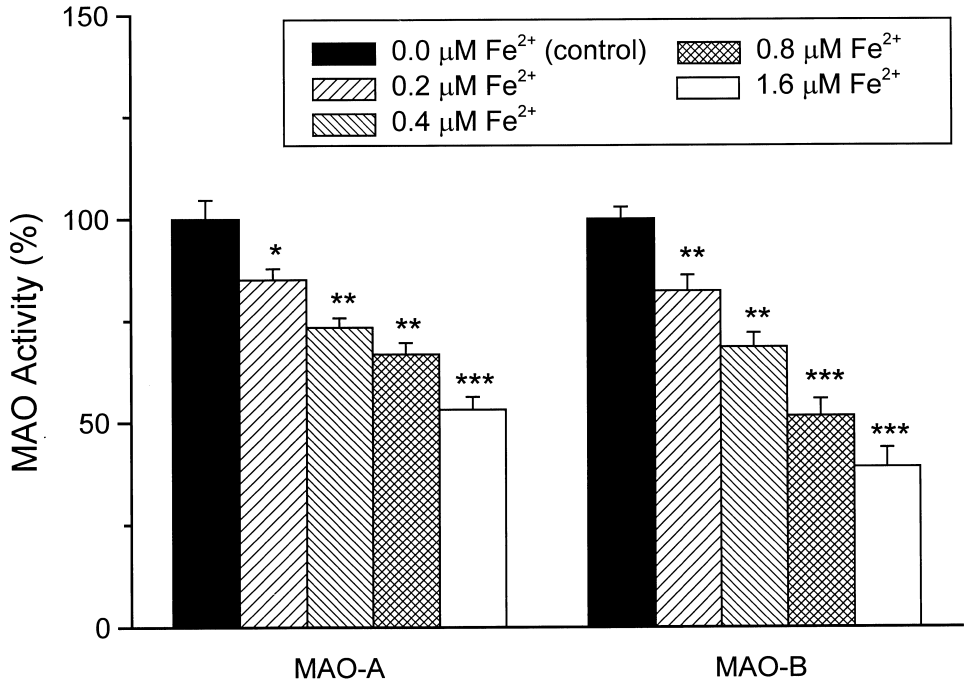

Fig. 3. Effects of the production of ·OH on MAO-A and MAO-B activities. MAO activity was assayed after 15 min of incubation of the mitochondrial preparation with AA (100 μ*M*) and the corresponding concentration of Fe^{2+}. Controls for MAO-A and MAO-B activities were obtained in the absence of AA and Fe^{2+}. Data are mean ± SEM derived from 4 separate experiments. Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ as compared to the corresponding control (Student's *t*-test).

MAO-B activities were in all cases statistically significative. In addition, we verified that the reported inhibition is dependent on the preincubation time (data not shown), which is clearly related to the amount of ·OH generated.

In this study we attempted to correlate the MAO inhibition found with the amount of ·OH generated by the system AA+Fe^{2+}. For this reason we submited the data of MAO activities versus ·OH accumulation expressed as AUC to a lineal regression analysis. As shown in Fig. 4, the correlation coefficients obtained were r = 0.894 for MAO-A and r = 0.953 for MAO-B, which confirms the existence of a direct correlation between MAO inhibition and the production of ·OH.

For reversibility studies, mitochondrial preparations were preincubated in the absence (control) or presence of the both AA (100 μ*M*) and Fe^{2+} (0.8 μ*M*). As shown in Table 1, after dialysis of the mitochondrial preparations, neither MAO-A nor MAO-B activity were recovered, compared to the corresponding controls obtained in the absence of AA+Fe^{2+}. Furthermore, as shown in Table 1, the inhibition found was very similar to that found with non-dialysed samples preincubated with AA+Fe^{2+}.

Although, we performed some mitochondria incubations in the presence of Fe^{2+} (0.8 μ*M*) alone, we did not found statistically significative differences in both MAO-A and MAO-B activities when compared with their corresponding controls (in the absence of Fe^{2+}).

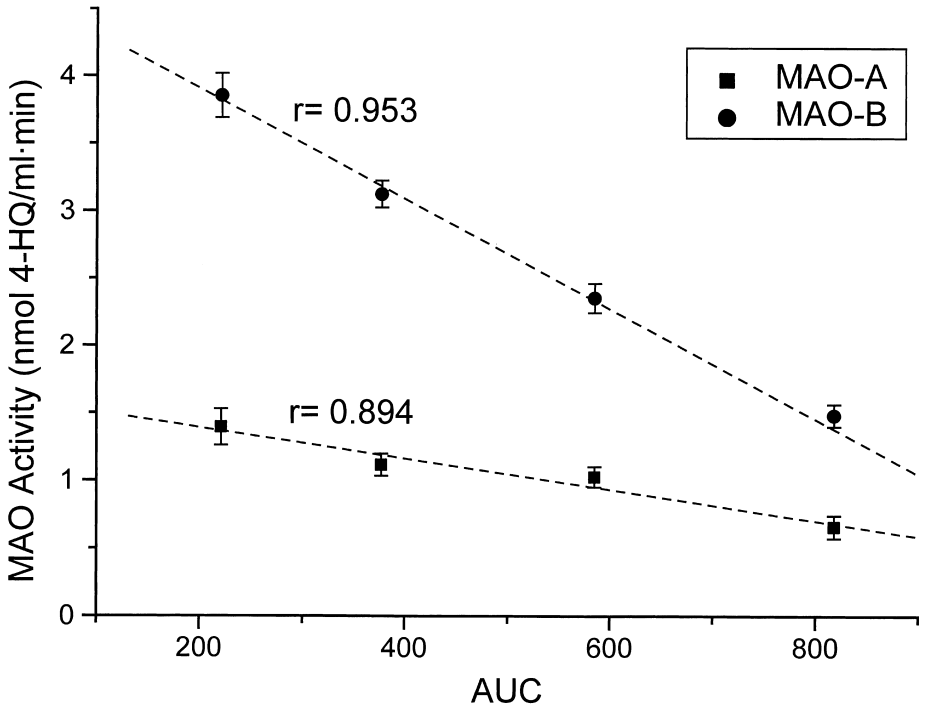

Fig. 4. Variations of MAO-A and MAO-B activities with the generation of ·OH. The production of ·OH is represented by the area under the curve (AUC) from the corresponding fluorimetric recording obtained in the conditions used for the determination of MAO activity. Values are means ± SEM of four experiments. The linear regresion lines for each set of data are shown.

Discussion

MAO activity in the brain is involved in the catabolism of several neurotransmitters such as dopamine, noradrenaline, and serotonine. Obviously, the catabolism of these neurotransmitters by MAO involves the formation of H_2O_2 which in the presence of iron generates ·OH through the Fenton reaction. It has been reported that the main mechanism of the cytotoxicity produced by H_2O_2 is precisely via the generation of ·OH [18]. Despite the existence of two enzymatic scavenging systems, catalase and glutathione peroxidase, to protect cells from the presence of H_2O_2, it is well-known that the brain levels of these two enzymes are low compared to other tissue [12]. In addition, the H_2O_2 generated by mitochondrial monoamine oxidase does not easily reach the catalase compartment [4]. Evidently, these facts make catecholaminergic and serotonergic neurons particularly vulnerable to the oxidative stress caused by MAO activity. In addition, it has been suggested that there are two other factors that may contribute to enhance the damaging potential of this metabolic pathway: a) the reported increase of MAO activity with age in human brain [19,20], which facilitates increased production of H_2O_2 and b) the capacity shown by neuromelanine to bind iron [21], which promotes the generation of specific-sites for the formation of ·OH. The involvement of MAO activity in the pathogenesis and progresion of Parkinson's disease has been previously postulated [22].

Table 1
Reversibility of MAO-A and MAO-B inhibition by ·OH production

	Activity[b] (% Inhibition)	
Preparation[a]	MAO-A	MAO-B
Control	1.521±0.0083 (0%)	4.562±0.037 (0%)
Non-dialysed	1.016±0.010 (33%)	2.354±0.072 (48%)
Dialysed	1.009±0.013 (33%)	2.311±0.095 (49%)

[a] Biological preparations were incubated in the absence (control) or in the presence of AA (100 μ*M*) + Fe^{2+} (0.8 μ*M*) at 37 °C for 15 min and then dialysed or not at 4 °C for 10 hours.

[b] Values represent means ± SEM (n= 4) and are expresed as nmol 4-OHQ/mg protein·min.

The results of the present study show that both MAO-A and MAO-B are inhibited by the generation of ·OH. As previously described, the generation of ·OH was achieved with the combined action of AA+Fe^{2+}. However, no changes in either MAO-A or MAO-B activities were found when MAO activity was determined in the presence of Fe^{2+} alone. Evidently, under these conditions, the H_2O_2 produced by MAO activity gives ·OH (Fenton reaction). For this reason, the reported data shows that very probably the amount of ·OH produced during MAO activity is insufficient to cause MAO inhibition. Nevertheless, additional studies will be required to further evaluate the potential contribution of long term MAO activity in the presence of Fe^{2+} to MAO inhibition. It is important to point out that the concentrations used of both AA and Fe^{2+} are close to those considered physiological [23,24] and lower than those used by other authors to induce lipid peroxidation [25]. In addition, the concentrations of AA and Fe^{2+} used in this study were found necessary to guarantee different rates of ·OH production, which enable us to prove the existence of a direct correlation between ·OH production and MAO activity. It has been reported that copper, zinc superoxide dismutase (Cu,Zn-SOD) is able to catalyze the formation of ·OH from H_2O_2 by a mechanism which involves the participation of free copper released from the oxidatively damaged enzyme [26,27]. However, as only Mn-SOD is present in the mitochondria, and given that this form does not catalyze this reaction [26], then its potential involvement on this present study may be discarded.

The reversibility studies showed that the reported inhibition was irreversible for both MAO-A and MAO-B. However, it was not possible to know if this inhibition is due to the oxidation of the enzyme by ·OH or to a modification of the lipid environment of MAO in the mitochondria membrane caused by a potential peroxidation process [25]. Evidently, further studies of the molecular mechanism of this inhibition will be of great interest.

Taking into consideration the important role attributed to MAO activity in the generation of ROS [5,11], the here reported inhibition might be regarded as a mechanism which reduces the contribution of MAO activity to oxidative stress when an overproduction of ·OH was reached. Thus, the results of this study seem to show that MAO activity does not contribute greatly to sustained ·OH production , which thus limits its suggested involvement in neurodegenerative processes to the initiation of lipid peroxidation on biological membranes. Evidently, the start of lipid peroxidation is sufficient to trigger a cascade of reactions leading to cell damage.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a well-known dopaminergic proneurotoxin widely used to investigate the pathogenesis and progression of Parkinson's disease [28]. This drug is bioactivated in the astrocytes by the action of MAO-B to give the 1-methyl-4-phenylpyridinium ion (MPP^+) [29,30], which is actively taken up by the presynaptic dopamine uptake system and accumulated within these nerve terminals [31]. Then, MPP^+ acts causing inhibition on the mitochondrial oxidation of NAD^+-linked substrates in dopaminergic neurons, and thus leads to a depletion of ATP and consequently causes cell death [32]. However, the molecular mechanism of MPTP neurotoxicity has been also associated with the capacity shown by this drug to produce oxidative stress through the generation of $^{\bullet}OH$ [33,34]. Although, MPTP affects the nigrostriatal dopaminergic system in a wide varity of animal species [35], there are notorious differences in reaction among the different animal species to this compound. Thus, primates [36] and mice [37] are sensitive to MPTP to different degrees while rats are practically insensitive [38]. Assuming that MPTP is activated by MAO and MPTP induced the generation of $^{\bullet}OH$, the inhibition of MAO activity by $^{\bullet}OH$ production might be involved in the different sensitivity of the different species to this proneurotoxin. Evidently, to corroborate this hypothesis it might be particularly useful to investigate the inhibitory properties of $^{\bullet}OH$ production against MAO activity from different animal species.

Acknowledgments

This research has been supported by Grant MP97-0033 from the Dirección General de Enseñanza Superior e Investigación Científica, Madrid, Spain.

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