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Autoxidation and MAO-mediated metabolism of dopamine as a potential cause of oxidative stress: role of ferrous and ferric ions

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Abstract

The autoxidation and monoamine oxidase (MAO)-mediated metabolism of dopamine (3-hydroxytyramine; DA) cause a continuous production of hydroxyl radical ($^{\bullet}OH$), which is further enhanced by the presence of iron (ferrous iron, Fe^{2+} and ferric ion, Fe^{3+}). The accumulation of hydrogen peroxide (H_2O_2) in the presence of Fe^{2+} appears to discard the involvement of the Fenton reaction in this process. It has been found that the presence of DA significantly reduces the formation of thiobarbituric acid reagent substances (TBARS), which under physiological conditions takes place in mitochondrial preparations. The presence of DA is also able to reduce TBARS formation in mitochondrial preparations even in the presence of iron (Fe^{2+} and Fe^{3+}). However, DA boosted the carbonyl content of mitochondrial proteins, which was further increased in the presence of iron (Fe^{2+} and Fe^{3+}). This latter effect is also accompanied by a significant reduction in thiol content of mitochondrial proteins. It has also been observed how the pre-incubation of mitochondria with pargyline, an acetylenic MAO inhibitor, reduces the production of $^{\bullet}OH$ and increases the formation of TBARS. Although, the MAO-mediated metabolism of DA increases MAO-B activity, the presence of iron inhibits both MAO-A and MAO-B activities. Consequently, DA has been shown to be a double-edged sword, because it displays antioxidant properties in relation to both the Fenton reaction and lipid peroxidation and exhibits pro-oxidant properties by causing both generation $^{\bullet}OH$ and oxidation of mitochondrial proteins. Evidently, these pro-oxidant properties of DA help explain the long-term side effects derived from L-DOPA treatment of Parkinson's disease and its exacerbation by the concomitant use of DA metabolism inhibitors.

Keywords: Dopamine autoxidation; Dopamine metabolism; Hydroxyl radical; Lipid peroxidation; Protein oxidation; Mitochondrial preparations; Parkinson's disease.

1. Introduction

Dopamine (3-hydroxytyramine; DA) is a catechol neurotransmitter widely distributed throughout the brain. However, most of the DA in the brain is found in the striatum (Palkovits and Brownstein, 1989) and is synthesized by the dopaminergic neurons which make up the nigrostriatal system. It is well-known that the biosynthesis of DA occurs preferentially in the nerve terminals from tyrosine and through the sequential action of tyrosine hydroxylase and aromatic L-amino acid decarboxylase (L-DOPA decarboxylase). The nigrostriatal system constitutes a set of dopaminergic neurons whose cell bodies are located in the pars compacta of substantia nigra and whose axons are projected towards the striatum (caudate nucleus and putamen). It is assumed that the main function of this neuronal system is the modulation of voluntary movements. Several studies have shown a continuous loss of dopaminergic neurons during normal aging (McGeer et al., 1977; Severson et al., 1982; Mann and Yates, 1983). On the other hand, it is known that Parkinson's disease occurs when a loss of 70–80% of nigrostriatal dopaminergic neurons has been reached (Bernheimer et al., 1973). For both reasons, it has been hypothesized that this disorder may be secondary to subclinical, environmen-

Abbreviations: DA, dopamine; MAO, monoamine oxidase; COMT, catechol-*O*-methyltransferase; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; BSA, bovine serum albumin; SDS, sodium dodecylsulfate; TBARS, thiobarbituric acid reagent substances; MDA, malonodialdehyde; QH_2, reduced form of DA; *o*Q, *o*-quinone of DA; $^{\bullet}$sQH, semiquinone radical of DA; $HO_2^{\bullet}$, hydroperoxyl radical; LOOH, lipid hydroperoxide; $L^{\bullet}$, lipid radical; ParH, reduced form of pargyline; $Par^{\bullet}$, pargylinyl radical; $RO^{\bullet}$, alkoxyl radical; $ROO^{\bullet}$, peroxyl radical; $R–S^{\bullet}$, thiyl radical; $R–SOO^{\bullet}$, sulfonyl radical; $R–SO_2OO^{\bullet}$, sulfonyl peroxyl radical

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tally induced damage to the substantia nigra, followed by the continued age-related attrition of nigral neurons (Langston, 1990). It has also been postulated that the loss of dopaminergic neurons which takes place in both normal aging and Parkinson's disease are closely related to the particular capacity of dopaminergic neurons to generate oxidative stress (Cohen, 1990; Fornstedt, 1990; Zhang and Dryhurst, 1993).

Under physiological conditions, DA is non-enzymatically oxidized by molecular oxygen to form hydrogen peroxide (H_2O_2) and the corresponding *o*-quinone (*o*Q). Then, the *o*Q undergoes an intramolecular cyclization which is immediately followed by a cascade of oxidative reactions resulting in the final formation of a black, insoluble polymeric pigment known as neuromelanin (Graham, 1978; Graham et al., 1978). In addition, DA is also enzymatically deaminated by monoamine oxidase (MAO) to form H_2O_2 and 3,4-dihydroxyphenylacetaldehyde. This latter compound is then oxidized by aldehyde dehydrogenase to gives 3,4-dihydroxyphenylacetic acid (DOPAC), which subsequently is methylated by catechol-*O*-methyltransferase (COMT) to form homovanillic acid (HVA). Therefore, both the autoxidation and the MAO-mediated metabolism of DA involve the formation of H_2O_2, a compound that can easily be reduced in the presence of ferrous iron (Fe^{2+}) to form, through the Fenton reaction, the hydroxyl radical ($^{\bullet}OH$), which is considered the most damaging free radical for living cells. Taking into account that the loss of dopaminergic neurons boosts both DA biosynthesis and turnover in the surviving neurons (Fornstedt et al., 1990), it seems evident that the subsequent excessive autoxidation and metabolism of DA in these cells increases the oxidative stress, and thus contributes to the progressive loss of dopaminergic neurons observed with age and in Parkinson's disease. Furthermore, these phenomena are enhanced by the presence of neuromelanin in dopaminergic neurons, due to its reported ability to accumulate iron (Enoch et al., 1994) and consequently may act by promoting the Fenton reaction.

Although, several studies have shown the neurotoxicity of high concentrations of DA using both in vivo experiments (Filloux and Townsend, 1993; Hastings et al., 1996; Ravinovic et al., 2000) and neuronal cultures (Michel and Hefti, 1990; Tanaka et al., 1991; Pedrosa and Soares-da-Silva, 2002), the precise molecular mechanism involved in this process remains unclear. The first studies on this subject have attributed the neurotoxicity of DA to the oxidative stress derived from its autoxidation (Graham et al., 1978). However, Spencer et al. (1996) reported that DA is able to enhance the production of $^{\bullet}OH$ by H_2O_2/Fe^{3+}-EDTA, but also inhibits the lipid peroxidation induced by iron ion/ascorbate in liposomes, which is interpreted by these authors as a balance between the reported capacity of DA to stimulate $^{\bullet}OH$ production and its ability to scavenge $^{\bullet}OH$ (Richter and Waddell, 1983; Slivka and Cohen, 1985). Later, Li et al. (1995) also found that DA is able to suppress the lipid peroxidation caused by iron-ADP in brain homogenates. These results were corroborated by Yen and Hsieh (1997) who reported antioxidant properties for DA. However, the neurotoxicity of high doses of DA have also been related to the formation of cysteinyl–catechols derivatives and the subsequent depletion caused in the brain levels of glutathione (Ravinovic et al., 2000). Very recently, it has been also suggested that DA neurotoxicity may be a consequence of an apoptotic phenomenon independent of the oxidative stress (Pedrosa and Soares-da-Silva, 2002).

Therefore, in an attempt to assess the potential of DA to cause oxidative stress in brain mitochondria and to shed some light on the molecular mechanisms involved in the reported neurotoxicity of DA, we have performed an exhaustive investigation into oxidative stress caused by the autoxidation and the MAO-mediated metabolism of DA, using mitochondrial preparations obtained from rat brain. This study includes an investigation of the capacity of DA to generate $^{\bullet}OH$ and to accumulate H_2O_2 in the presence and absence of MAO metabolism. It also involves the study of both the capacity of DA to affect the lipid peroxidation observed in mitochondrial preparations and its ability to cause a protein oxidation of mitochondrial proteins (increase in carbonyl content and decrease in thiol content). Finally, the potential of the metabolism and autoxidation of DA to modify MAO activity has been also assessed. In all these cases, the effects provoked by the presence of both ferrous and ferric ions on both the oxidative stress caused by DA and its consequences have also been investigated.

2. Experimental procedures

2.1. Chemicals

DA hydrochloride, DOPAC, thiobarbituric acid, butylated hydroxytoluene crystalline, 2,4-dinitrophenylhydrazine hydrochloride, desferrioxamine, 1,1,3,3-tetraethoxypropane, 5,5′-dithiobis-(2-nitrobenzoic acid), kynuramine dihydrobromide, pargyline hydrochloride, catalase, sodium dodecylsulfate (SDS), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, USA). Clorgyline hydrochloride and *R*(−)-deprenyl hydrochloride was obtained from Research Biochemicals International (Natick, USA). Ferrous chloride tetrahydrate and ferric chloride hexahydrate were purchased from Fluka Chemie AG (Buchs, Switzerland). Guanidine hydrochloride and terephthalic acid disodium salt was from Aldrich Chemical Co. (Milwaukee, USA). The water used for the preparations of solutions was of Milli-RiOs/Q-A10 grade (Millipore Corp., Bedford, USA). All remaining chemicals used were of analytical grade and were purchased from Fluka Chemie AG (Buchs, Switzerland). Stock solutions of DA were prepared in 1 mM KCl (pH 2.0) to prevent its immediate autoxidation. Fresh stock solutions of Fe^{2+} and Fe^{3+} were prepared in water immediately before each experiment.

2.2. Preparation of brain mitochondria

Male Sprague–Dawley rats weighing 250–300 g were used. The rats were received from the breeder (Animalario de la USC, Santiago de Compostela, Spain) at least 2 days before sacrifice, and were kept at 22 °C, on a 12:12 light–dark schedule, and 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 mitochondria were then obtained by differential centrifugation with minor modifications to a previously published method (Méndez-Álvarez et al., 1997). 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 in a Avanti J-25 centrifuge (Beckman Instruments, Palo Alto, USA) at $1000 \times g$ for 5 min at 4 °C. The supernatant was centrifuged at $12{,}500 \times g$ for 15 min. The mitochondrial 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 preparations was determined according to the method of Markwell et al. (1978), using BSA as the standard.

2.3. Monitoring of $^{\bullet}OH$ formation

The $^{\bullet}OH$ formation was monitored fluorimetrically using a previously reported method (Méndez-Álvarez et al., 2001) in which terephthalic acid is used as a chemical dosimeter of $^{\bullet}OH$. A luminescence spectrometer Model LS50B (Perkin-Elmer, Norwalk, USA) was used. The cuvette holder was thermostatically maintained at 37 °C and a magnetic stirrer was used for a continuous mixing of the sample. Brain mitochondria (0.75 mg protein/ml) were incubated in a buffer solution (Na_2PO_4/KH_2PO_4 isotonized with KCl, pH 7.4) containing 10 mM terephthalic acid for 5 min to reach the working temperature. In some cases, pargyline (10 μM) was previously added to the incubation. Then, 100 μM iron (Fe^{2+} or Fe^{3+}) or water was incorporated to the incubation followed by DA (400 μM or 1 mM) or 1 mM KCl (pH 2.0). The monitoring of $^{\bullet}OH$ formation was immediately initiated and maintained for the subsequent 20 min. All the concentrations are final concentrations in the incubation. The excitation and emission wavelengths used were 312 and 426 nm, respectively. Fluorescence measurements were all expressed relative to its initial reading.

2.4. Monitoring of O_2 consumption and H_2O_2 accumulation

The O_2 consumption and H_2O_2 accumulation during DA autoxidation was monitored polarographically according to a previously published method (Soto-Otero et al., 2000). A Clark-type O_2-electrode (Digital Oxygen System Model 10, Rank Brothers, Cambridge, UK) equipped with an amplifier, an A/D converter and the appropriate software for data acquisition and control (AM-Systems, Jaén, Spain) were used. The electrode was assembled in a 5 ml chamber surrounded by a thermostatic water jacket set at 37 °C. A polarizing voltage of +0.60 V was used. For each assay, 25 mM phosphate buffer (pH 7.4) was incubated in the electrode chamber for 5 min in order to reach the working temperature and the saturating O_2 concentration. Then, 100 μM iron (Fe^{2+} or Fe^{3+}) or water was injected to incubation followed 1 min later by 20 mM DA. Concentrations of DA and iron are final concentrations in the incubation. The solution in the electrode chamber was maintained continuously under vigorous stirring by a magnetic agitator. The accumulation of H_2O_2 was estimated from the production of O_2 after the addition of catalase (2000 units). For O_2-electrode calibration, the saturating O_2 concentration was taken as 237 μM and the zero was established in the presence of sodium dithionite (Clark, 1993).

2.5. Determination of thiobarbituric acid reagent substances (TBARS)

The TBARS determination was performed spectrophotometrically using a previously published method (Soto-Otero et al., 2002). Brain mitochondria (1 mg protein/ml) were incubated at 37 °C for 5 min to reach the working temperature. In some cases, pargyline (10 μM) was previously added to the incubation. Then, 100 μM iron (Fe^{2+} or Fe^{3+}) or water was incorporated to the incubation followed by DA (400 μM or 1 mM) or 1 mM KCl (pH 2.0) and the mixture incubated for exactly 20 min. Immediately, butylated hydroxytoluene (1 mM) and desferrioxamine (1 mM) were added to prevent the amplification of the lipid peroxidation during the assay. All concentrations given up to this point are final concentrations in the incubation. Acetic acid (20%) followed by SDS (8%, w/v) were added and the mixture vortexed for 1 min. Then, thiobarbituric acid (0.8%) was added and the resulting mixture incubated at 95 °C for 60 min. After cooling to room temperature, 3 ml of *n*-butanol were added and the mixture shaken vigorously. After centrifugation at $2500 \times g$ for 5 min, the absorbance of the supernatant (organic layer) was measured at 532 nm using an Ultrospec III spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). For calibration, a standard curve (5–150 nM) was generated using the malonodialdehyde (MDA) derived by the acid hydrolysis (SO_4H_2; 1.5%, v/v) of 1,1,3,3-tetraethoxypropane and the TBARS results expressed as nmol MDA/mg protein.

2.6. Estimation of protein carbonyl content

The protein carbonyl content was assessed spectrophotometrically by a modification to the procedure described by Schild et al. (1997). Briefly, brain mitochondria (1 mg

protein/ml) were incubated at 37 °C for 5 min to reach the working temperature. In some cases, pargyline (10 μM) was previously added to the incubation. Then, 100 μM iron (Fe^{2+} or Fe^{3+}) or water was incorporated to the incubation followed by DA (400 μM or 1 mM) or 1 mM KCl (pH 2.0), and the mixture incubated for exactly 20 min. Immediately, protein precipitation was accomplished with the addition of trichloroacetic acid (1 M) followed consecutively by sonication (Branson Sonic Corp., Danbury, USA) and centrifugation in a microcentrifuge (model E, Beckman Instruments) at 12,000 × *g* for 5 min. The resulting pellet was reconstituted in NaOH (0.5 M) with vigorous vortexing for 3 min. Then, 10 mM 2,4-dinitrophenylhydrazine in 2 M chloric acid was added and the mixture incubated at room temperature for 1 h, in darkness, and with continuous agitation. After the addition of trichloroacetic acid (1 M), the resulting mixture was centrifuged at 12,000 × *g* for 5 min. The resulting pellet was washed twice with ethyl acetate:ethanol (1:1, v/v). Then, the washed pellet was reconstituted with 6 M guanidine in a 20 mM KH_2PO_4 buffer (pH 2.3) and the absorbance of the resulting solution measured at 370 nm. The carbonyl content was calculated from the absorbance data using as absorption coefficient for dinitrophenylhydrazone $\varepsilon = 22{,}000\,M^{-1}\,cm^{-1}$ and expressing this parameter as nmol carbonyls/mg protein. Because of the numerous washing steps, protein content in the final pellet was estimated on an HCl blank pellet processed simultaneously, using a BSA standard curve in 6 M guanidine, and reading the absorbance at 280 nm (Reznick and Packer, 1994).

2.7. Estimation of protein thiol groups

The free thiol content of proteins was estimated spectrophotometrically using a modification introduced to a standard assay (Habeeb, 1972). Brain mitochondria (1 mg protein/ml) were incubated at 37 °C for 5 min to reach the working temperature. In some cases, pargyline (10 μM) was previously added to the incubation. Then, 100 μM iron (Fe^{2+} or Fe^{3+}) or water was incorporated to the incubation followed by DA (400 μM or 1 mM) or 1 mM KCl (pH 2.0), and the mixture incubated for exactly 20 min. Immediately, protein precipitation was accomplished with the addition of trichloroacetic acid (0.5 M) followed by vortexing and centrifugation at 12,000 × *g* for 5 min. The resulting pellet was reconstituted in 80 mM Na_3PO_4 and 2 mM EDTA buffer (pH 8.0) containing 70 mM SDS. Then, 100 μM 5,5′-dithiobis-(2-nitrobenzoic acid) in 100 mM Na_3PO_4 buffer (pH 8.0) was added and the resulting mixture incubated at room temperature for 20 min with continuous mixing. The absorbance of the resulting solution was measured at 412 nm and the thiol content calculated from this data using as absorption coefficient for 2-nitro-5-mercaptobenzoic acid $\varepsilon = 13{,}600\,M^{-1}\,cm^{-1}$ and expressing the corresponding parameter as nmol thiols/mg protein.

2.8. Determination of MAO activity

MAO activity was spectrophotometrically measured by a previously reported procedure (Méndez-Álvarez et al., 1997), using an Ultrospec III spectrophotometer equipped with a cuvette holder thermostatized. (−)-Deprenyl and clorgyline were used as irreversible and selective inhibitor to assay MAO-A and MAO-B activity, respectively. Brain mitochondria (1 mg protein/ml) were pre-incubated at 37 °C for 5 min with the corresponding irreversible inhibitor. Then, 100 μM iron (Fe^{2+} or Fe^{3+}) or water was incorporated to the incubation followed by DA (400 μM or 1 mM) or 1 mM KCl (pH 2.0) for exactly 20 min. MAO activity was assessed using kynuramine as a non selective substrate at a concentration equivalent to its K_M (90 μM for MAO-A and 60 μM for MAO-B). All concentrations are final concentrations in the incubation. Finally, the formation of 4-hydroxyquinoline was followed at 314 nm for 5 min.

2.9. Statistical analysis

Data are expressed as mean ± S.E.M. Differences between means were statistically evaluated using the one-way ANOVA followed by the Bonferroni test. Normality of populations and homogeneity of variances was tested before each ANOVA. The accepted level of significance in all cases was $P < 0.05$.

3. Results

3.1. Generation of •OH by DA metabolism and autoxidation

The formation of •OH was monitored fluorimetrically and the peak of relative fluorescence (ΔF_{max}) used as a parameter indicative of the amount of •OH produced. Fig. 1A illustrates a typical recording of •OH formation during DA (1 mM) autoxidation in mitochondrial preparations (0.5 mg protein/ml) incubated under physiological conditions of pH (7.4) and temperature (37 °C). As can be seen in Fig. 1A, a continuous production of •OH was observed during DA autoxidation. In addition, the amount of •OH generated proved to be dependent of the DA concentration used (Table 1). When the metabolism of DA by MAO was inhibited with pargyline (10 μM), a significant reduction (−52% for 400 μM DA and −56% for 1 mM DA) in •OH formation was observed (Table 1). The presence of Fe^{2+} (100 μM) in the incubation caused a pronounced increase (+450% for 400 μM DA and +495% for 1 mM DA) in •OH generation. However, the increase in •OH production was more remarkable (+840% for 400 μM DA and +919% for 1 mM DA) when the incubation was performed in the presence of Fe^{3+} (100 μM). As illustrated in Fig. 1A and shown in Table 1, in the presence of both Fe^{2+}, the inhibition of DA metabolism by pargyline caused a significant diminution (−13% for

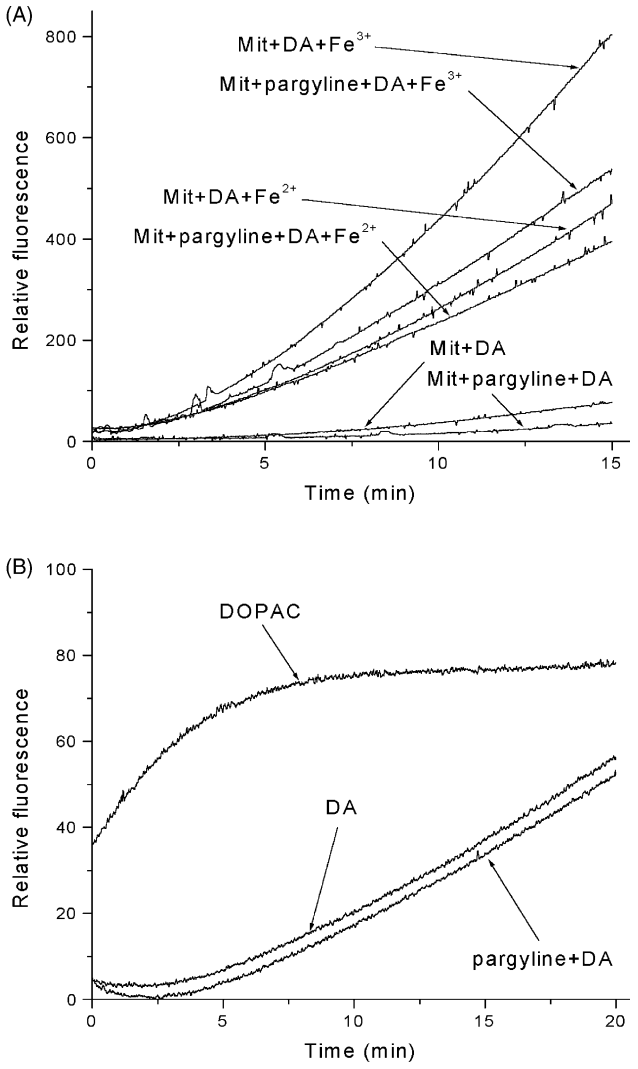


Fig. 1. (A) Representative recording of •OH formation during the incubation of DA (1 mM) in mitochondrial preparations (0.5 mg protein/ml) obtained from rat brain: effects of the presence of Fe^{2+} and Fe^{3+}. Pargyline (10 μM) was used to inhibit the metabolism of DA by MAO. The concentration used of Fe^{2+} and Fe^{3+} was 100 μM. Incubations were performed at 37 °C for 20 min with only the last 15 min recorded. (B) Representative recording of •OH formation during the autoxidation of DA, (DA + pargyline), and DOPAC in a phosphate buffer (pH 7.4). Incubations were performed at 37 °C for 20 min. The concentration used were: 800 μM for DA and DOPAC, and 20 μM for pargiline. The •OH formation is indicated by the fluorescence detected using terephthalic acid as a chemical dosimeter.

400 μM DA and −17% for 1 mM DA) in •OH production when compared with the production found without MAO inhibition. However, the reduction was more pronounced in the presence of Fe^{3+} (−35% for 400 μM DA and −33% for 1 mM DA). In order to understand the reduction in •OH formation observed with the addition of pargyline, the generation of •OH by the autoxidation DA, DOPAC, and (DA + pargyline) was studied. As illustrated in Fig. 1B, the rate of •OH production by DOPAC (800 μM) autoxidation was remarkably faster at the beginning of the reaction than that found in DA (800 μM) autoxidation. Furthermore, the presence of pargyline (20 μM) slightly reduced the production of •OH (Fig. 1B).

Table 1
Production of •OH during the metabolism and autoxidation of DA in mitochondrial preparations from rat brain: effects of Fe^{2+} and Fe^{3+}

Incubation[a]	ΔF_{max}[b]	
	400 μM DA	1 mM DA
DA	33.1 ± 3.5	79.4 ± 5.7
DA + pargyline	15.9 ± 2.2 a	34.9 ± 3.1 a
DA + Fe^{2+}	183.0 ± 5.4 a	472.5 ± 7.8 a
DA + pargyline + Fe^{2+}	159.8 ± 7.5 b	390.2 ± 7.1 b
DA + Fe^{3+}	311.1 ± 11.2 a	809.5 ± 12.0 a
DA + pargyline + Fe^{3+}	203.0 ± 9.6 c	539.3 ± 5.3 c

Values are mean ± S.D. from four independent experiments. Statistical significance at $P < 0.05$ (one-way ANOVA and Bonferroni test) was established in comparison with the following controls (denoted by letters a, b, c; not as superscript): a, DA; b, (DA + Fe^{2+}); c, (DA + Fe^{3+}).

[a] Mitochondrial preparations (1 mg protein/ml) were incubated at 37 °C for 20 min. The concentrations of pargyline and Fe^{2}/Fe^{3+} were 10 and 100 μM, respectively. Reagents were added in the following order: pargyline, Fe^{2+}or Fe^{3+}, and DA.

[b] The value of the relative fluorescence after 20 min of reaction was used to assess the production of •OH.

3.2. O_2 consumption and H_2O_2 accumulation during DA autoxidation

Fig. 2 illustrates a typical polarographic recording of O_2 consumption during DA (20 mM) incubation at 37 °C in a phosphate buffer (pH 7.4), which shows a continuous, slow autoxidation for DA under the reported physiological conditions of pH and temperature. In addition, the expected for-

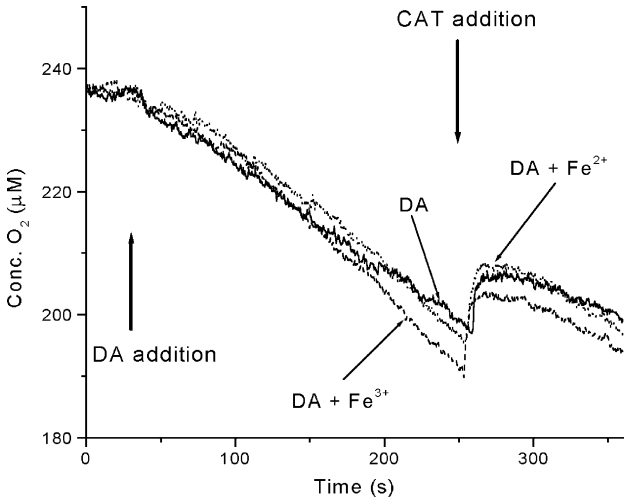


Fig. 2. Representative polarographic recording of O_2 consumption and H_2O_2 accumulation during the autoxidation of DA in a phosphate buffer (pH 7.4): effects of the presence of Fe^{2+} and Fe^{3+}. Incubations were carried out at 37 °C for 6 min and the final reagent concentrations were: DA, 20 mM; Fe^{2+} and Fe^{3+}, 100 μM. The H_2O_2 accumulated after 216 s of reaction was assessed by the addition of 2000 units of catalase (CAT).

Table 2
Accumulation of H_2O_2 during the autoxidation of DA under physiological conditions of pH and temperature: effects of Fe^{2+} and Fe^{3+}

Incubation[a]	H_2O_2 (nmol)[b]
DA	52.0 ± 5.0
DA + Fe^{2+}	73.6 ± 2.6 a
DA + Fe^{3+}	79.0 ± 2.2 a,b

Each value is the mean ± S.D. from four independent experiments. Statistical significance at $P < 0.05$ (one-way ANOVA and Bonferroni test) was established in comparison with the following controls (denoted by letters a, b, c; not as superscript): a, DA; b, (DA + Fe^{2+}).

[a] Incubations were performed at 37 °C in a phosphate buffer pH 7.4. Reagent concentrations in the incubation were: DA, 20 mM; Fe^{2+} and Fe^{3+}, 100 μM.

[b] Data represent the amount of H_2O_2 accumulated after 260 s of reaction which was assessed by the addition of 2000 units of catalase.

mation and accumulation of H_2O_2 during DA autoxidation has been also confirmed. It is particularly interesting to note that the presence of Fe^{2+} (100 μM) in the incubation of DA caused a slight augmentation in O_2 consumption which was followed by a significant increase (+42%) in H_2O_2 accumulation (Table 2). Similar behavior was achieved with the pre-addition of Fe^{3+} (100 μM) to the DA incubation, in which case the observed increase in H_2O_2 accumulation was of +52%.

3.3. Effects of DA metabolism and autoxidation on lipid peroxidation (TBARS)

As shown in Fig. 3, the analysis of the presence of TBARS in mitochondrial preparations (1 mg/ml protein) after incubation at 37 °C for 20 min revealed that lipid peroxidation occurred in a significant proportion. Under these experimental conditions, the presence of DA caused a significant diminution (−66% for 400 μM DA and −61% for 1 mM DA) in TBARS content. When mitochondrial preparations were pre-incubated with pargyline (10 μM) to inhibit DA metabolism by MAO, the observed effect was a significant increase (+86% for 400 μM DA and +131% for 1 mM DA) of TBARS in comparison with the values obtained in the incubation with DA (control). The addition of Fe^{2+} (100 μM) to mitochondrial preparations provoked a remarkable increase (+689%) in the TBARS content found after the corresponding incubation. Again, the presence of DA in the incubation of mitochondria with Fe^{2+} caused a significant reduction (−34% for 400 μM DA and −50% for 1 mM DA) in TBARS content. In this case, the pre-incubation of mitochondrial preparations with pargyline (10 μM) produced a significant increase (+15% for 400 μM DA and +16% for 1 mM DA) in TBARS when compared with the values obtained in the incubation of mitochondria with (DA + Fe^{2+}) (control). However, the presence of Fe^{3+} in the incubation of mitochondria caused less increase in TBARS than that found with the presence of Fe^{2+}. As illustrated in Fig. 3, the presence of Fe^{3+} (100 μM) in the incubation caused a

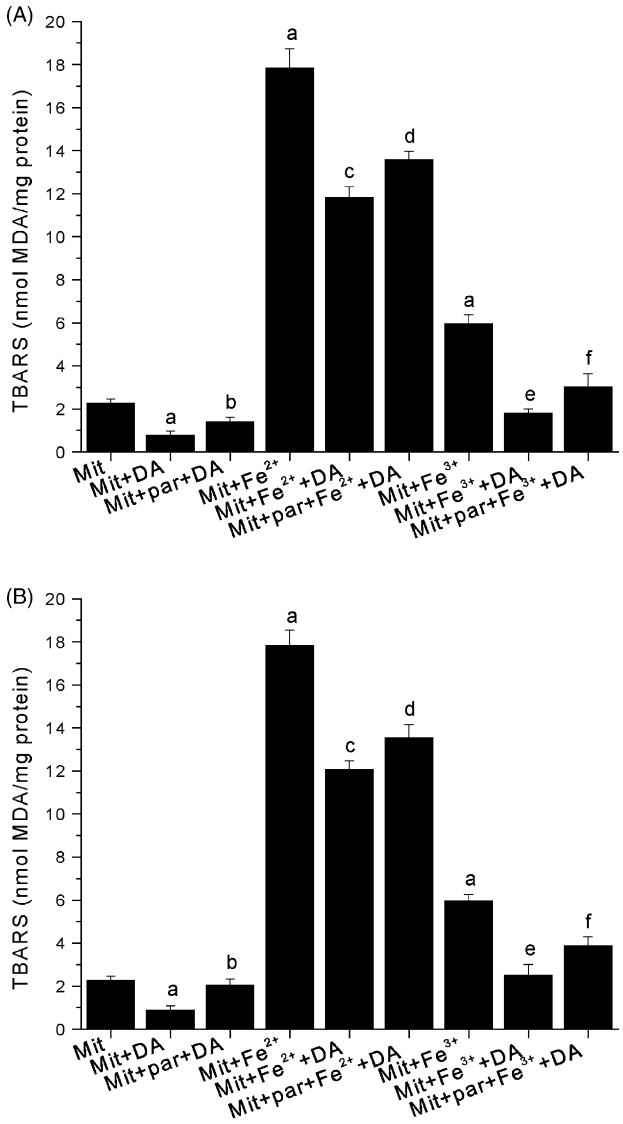


Fig. 3. DA effects on the lipid peroxidation detected after the incubation of mitochondrial preparations (1 mg protein/ml) from rat brain at 37 °C for 20 min: effects caused by the presence of Fe^{2+} and Fe^{3+}. The lipid peroxidation was assessed by the formation of TBARS. DA concentration in the incubation was (A) 400 μM and (B) 1 mM. Pargyline (10 μM) was used to inhibit DA metabolism by MAO. The concentration used of Fe^{2+} and Fe^{3+} was 100 μM. Data are mean ± S.E.M. from four independent experiments. Statistical significance at $P < 0.05$ (one-way ANOVA and Bonferroni test) was established in comparison with the following controls: a, Mit; b, (Mit + DA); c, (Mit + Fe^{2+}); d, (Mit + DA + Fe^{2+}); e, (Mit + Fe^{3+}); f, (Mit + DA + Fe^{3+}).

increase in TBARS content of +163% in relation to that found in the absence of this metal ion (control). Once again, the addition of DA to the incubation of mitochondria with Fe^{3+} provoked a diminution (−68% for 400 μM DA and −58% for 1 mM DA) in TBARS content. However, in this latter case, the pre-incubation of the mitochondrial preparation with pargyline (10 μM) led to an increase (+68% for 400 μM DA and +56% for 1 mM DA) in TBARS in relation to the amount found with (DA + Fe^{3+}) (control).

3.4. Effects of DA metabolism and autoxidation on protein carbonyl content

As illustrated in Fig. 4, the incubation of mitochondrial preparations (1 mg protein/ml) with DA at 37 °C for 20 min caused an increase in carbonyl content of mitochondrial proteins. The reported increase was dependent of the concentration of DA used (+20% for 400 μM DA and +57% for 1 mM DA). However, a slight reduction (−8% for 400 μM DA and −12% for 1 mM DA) in protein carbonyl content was observed when mitochondrial preparations were previously incubated with pargyline (10 μM). Also, the addition of Fe^{2+} (100 μM) to mitochondrial incubations caused a significant increase (+39%) in carbonyl content of mitochondrial proteins. As can be seen in Fig. 4, the combined action of Fe^{2+} and DA significatively augmented (+77% for 400 μM DA and +84% for 1 mM DA) the carbonyl content obtained in the presence of Fe^{2+} alone. In this case, the inhibition of DA metabolism by MAO with the pre-incubation of mitochondrial preparations with pargyline (10 μM) provoked a decrease (−12% for 400 μM DA and −13% for 1 mM DA) in carbonyl content. In contrast to the results obtained with Fe^{2+}, the addition of Fe^{3+} (100 μM) to mitochondrial incubations did not cause any significant change in protein carbonyl content (Fig. 4). Curiously, the augmentation observed with the combined action of Fe^{3+} and DA was greater than that obtained with (Fe^{2+} + DA), at least with 400 μM DA (+77%). However, the increase was less spectacular with 1 mM DA (+84%). In the presence of Fe^{3+} (100 μM), when the metabolism of DA by MAO was inhibited with pargyline, a significant reduction (−25%) in carbonyl content was detected using 400 μM DA in relation to the value found without MAO inhibition. However, no significant changes were observed with 1 mM DA.

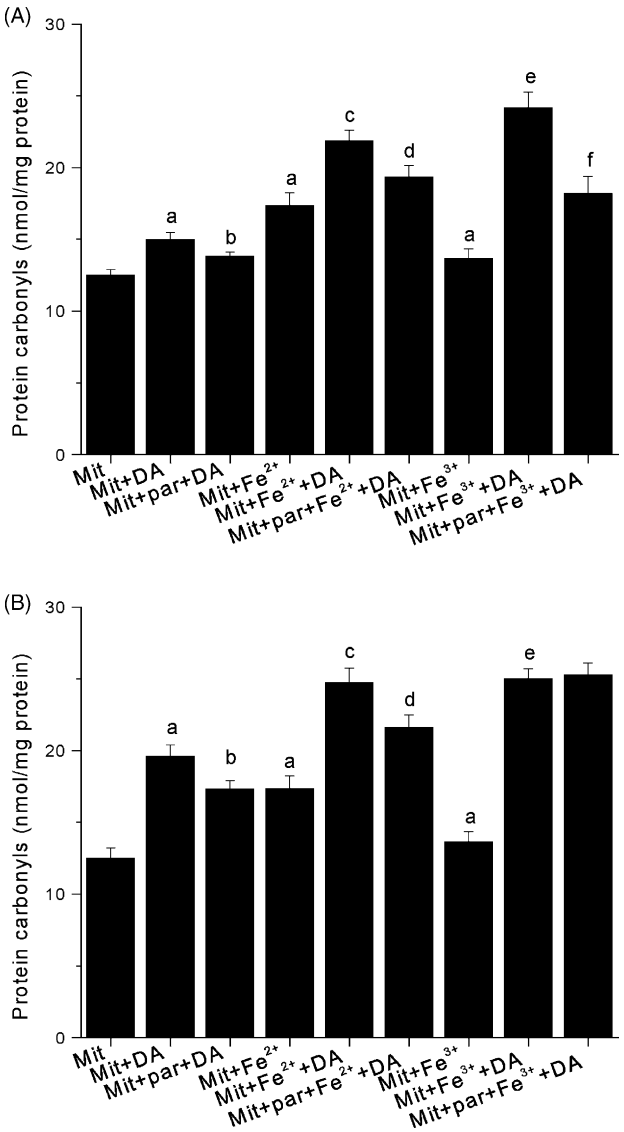


Fig. 4. DA effects on the protein carbonyl content detected after the incubation of mitochondrial preparations (1 mg protein/ml) from rat brain at 37 °C for 20 min: effects caused by the presence of Fe^{2+} and Fe^{3+}. DA concentration in the incubation was (A) 400 μM and (B) 1 mM. Pargyline (10 μM) was used to inhibit DA metabolism by MAO. The concentration used of Fe^{2+} and Fe^{3+} was 100 μM. Data are mean ± S.E.M. from four independent experiments. Statistical significance at $P < 0.05$ (one-way ANOVA and Bonferroni test) was established in comparison with the following controls: a, Mit; b, (Mit + DA); c, (Mit + Fe^{2+}); d, (Mit + DA + Fe^{2+}); e, (Mit + Fe^{3+}); f, (Mit + DA + Fe^{3+}).

3.5. Effects of DA metabolism and autoxidation on protein thiol content

Fig. 5 shows how the incubation of mitochondrial preparations (1 mg protein/ml) with DA at 37 °C for 20 min caused a significant reduction in thiol content of mitochondrial proteins, which was dependent of the concentration of DA used (−14% for 400 μM DA and −23% for 1 mM DA). When mitochondrial preparations were pre-incubated with pargyline (10 μM), a slight increase in thiol content (+5% for 400 μM DA and +17% for 1 mM DA) was observed in relation to the values found with DA alone. The addition of Fe^{2+} (100 μM) to mitochondrial incubations caused a significant reduction (−26%) in thiol content. However, no significant changes in thiol content were observed with the combined action of DA (400 μM and 1 mM) and Fe^{2+} (100 μM) when compared with the values obtained in the presence of Fe^{2+} alone. As illustrated in Fig. 5, the inhibition of MAO activity with the pre-incubation of mitochondrial preparation with pargyline previous to the addition both Fe^{2+} and DA caused no significant change in thiol content. Although, the presence of Fe^{3+} (100 μM) did not caused any significant change in the thiol content of mitochondrial proteins, the simultaneous presence of Fe^{3+} and DA led to a slight reduction (−4% for 400 μM DA and −14% for 1 mM DA) in its corresponding value in relation to that observed in the presence of Fe^{3+} alone (Fig. 5). However, the pre-incubation of mitochondrial preparations with pargyline (10 μM) provoked a slight augmentation (+6% for 400 μM DA and +7% for 1 mM DA) in the protein thiol content when compared with the value obtained in the absence of pargyline.

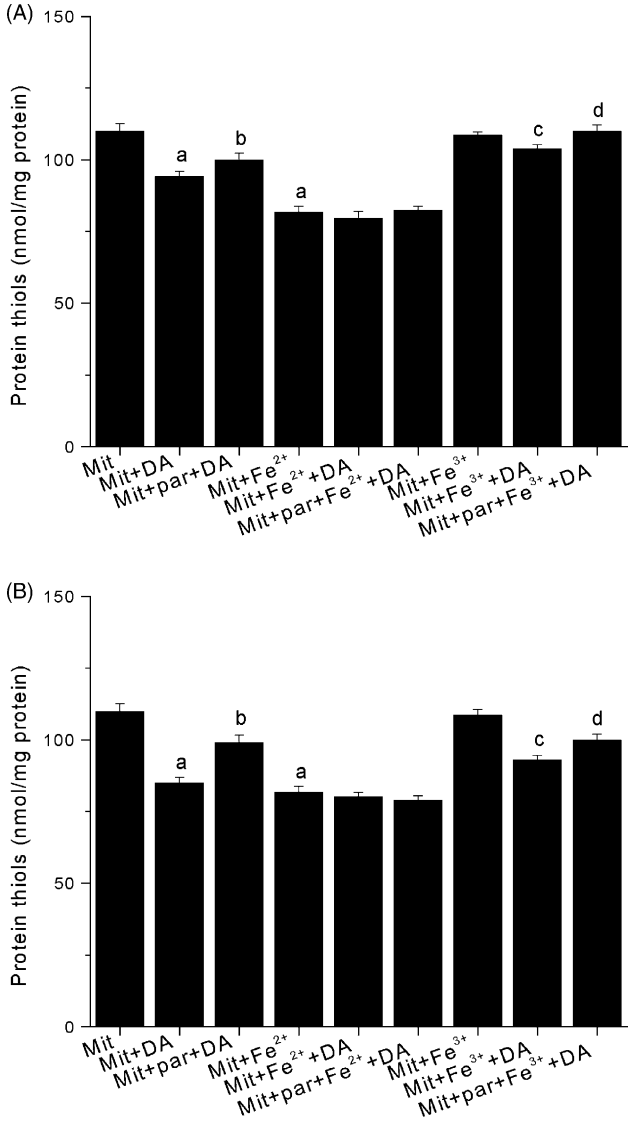


Fig. 5. DA effects on the protein thiol content detected after the incubation of mitochondrial preparations (1 mg protein/ml) from rat brain at 37 °C for 20 min: effects caused by the presence of Fe^{2+} and Fe^{3+}. DA concentration in the incubation was (A) 400 μM and (B) 1 mM. Pargyline (10 μM) was used to inhibit DA metabolism by MAO. The concentration used of Fe^{2+} and Fe^{3+} was 100 μM. Data are mean ± S.E.M. from four independent experiments. Statistical significance at $P < 0.05$ (one-way ANOVA and Bonferroni test) was established in comparison with the following controls: a, Mit; b, (Mit + DA); c, (Mit + Fe^{2+}); d, (Mit + DA + Fe^{2+}); e, (Mit + Fe^{3+}); f, (Mit + DA + Fe^{3+}).

3.6. Effects of DA metabolism and autoxidation on MAO activity

In order to examine the potential capacity of the metabolism and autoxidation of DA to modify MAO activity, mitochondrial preparations (1 mg protein/ml) were incubated with DA or without DA (control) at 37 °C for 20 min, previously to the determination of MAO activity. As shown in Table 3, the pre-incubation of mitochondrial preparations with 400 μM DA caused no significant change in either MAO-A or MAO-B activities. However, the use of a DA concentration of 1 mM caused a significant increase (+29%) in MAO-B activity. When DA pre-incubations was performed in the presence of Fe^{2+} (100 μM), a significant reduction in both MAO-A and MAO-B activities was observed, which was dependent on DA concentration. Thus, the reduction caused by 400 μM DA was of −22% for MAO-A and −33% for MAO-B, while with 1 mM DA the diminution was of −48% for MAO-A and of −53% for MAO-B. The results obtained with the pre-incubation of DA in the presence of Fe^{3+} (100 μM) were qualitatively similar to those reported for Fe^{2+}. However, the reduction observed in MAO activity in this case only was significant for MAO-B. Thus, the decrease in MAO-B activity was of −18% with the use of a concentration of DA of 400 μM and of −26% with DA concentration of 1 mM. The pre-incubation of mitochondrial preparations with a metal ion (Fe^{2+} or Fe^{3+}) at 37 °C for 20 min caused no significant change in either MAO-A or MAO-B activities (data not shown).

Table 3
Effects caused by the metabolism and autoxidation of DA on both MAO-A and MAO-B activity: contribution of Fe^{2+} and Fe^{3+}

Pre-incubation[a]	MAO activity[b]	
	MAO-A	MAO-B
Control	1.01 ± 0.07	2.45 ± 0.11
DA (400 μM)	0.95 ± 0.10	2.14 ± 0.15
DA (400 μM) + Fe^{2+}	0.79 ± 0.05 a	1.65 ± 0.17 b
DA (400 μM) + Fe^{3+}	0.87 ± 0.04	2.01 ± 0.12 a
DA (1 mM)	1.09 ± 0.05	3.15 ± 0.09 b
DA (1 mM) + Fe^{2+}	0.53 ± 0.03 c	1.16 ± 0.05 c
DA (1 mM) + Fe^{3+}	0.82 ± 0.07	1.80 ± 0.10 b

Values are mean±S.E.M. from four determinations. Statistical significance of differences between means (one-way ANOVA and Bonferroni test) is: a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$.

[a] Mitochondrial preparations (1 mg protein/ml) were pre-incubated at 37 °C for 20 min. The concentrations used of Fe^{2+} and Fe^{3+} were of 100 μM.

[b] MAO activity was calculated using kynuramine as substrate and expressed as nmol 4-hydroxyquinoline/(mg protein min).

4. Discussion

To gain additional insight into the nature of the molecular mechanisms involved in the neurotoxicity of DA, the present study investigated the potential of the metabolism and autoxidation of DA to generate oxidative stress in rat brain mitochondria. Indeed, the here reported data show for the first time a continuous production of •OH during the incubation of DA with mitochondrial preparations obtained from rat brain, maintained under physiological conditions of pH and temperature. This production was reduced when MAO activity was inhibited by a pre-incubation of mitochondrial preparations with pargyline. Evidently, these data appear to

demonstrate that $^{\bullet}OH$ production is preferentially caused by DA autoxidation, as previously suggested by other authors (Graham et al., 1978; Chiueh et al., 1993; Linert et al., 1996; Nappi and Vass, 1997). The molecular mechanism generally accepted for $^{\bullet}OH$ production during DA autoxidation is the following:

$$QH_2 + O_2 \rightarrow sQH + O_2^{\bullet -} + H^+$$

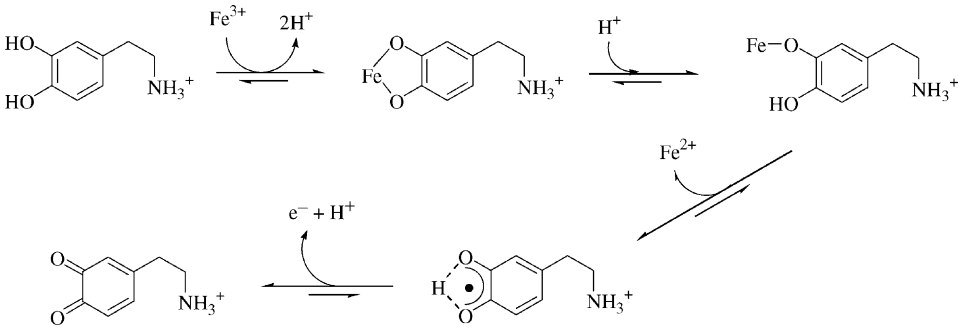
$$^{\bullet}sQH + O_2 \rightarrow oQ + O_2^{\bullet -} + H^+$$

$$O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + {}^{\bullet}OH + Fe^{3+}$$

which involves an initial production of the superoxide anion ($O_2^{\bullet -}$), from which H_2O_2 is then formed, to finally give the $^{\bullet}OH$ through the participation of the Fenton reaction. As can be seen, this process also involves the formation of *o*Q from the corresponding semiquinone radical ($^{\bullet}sQH$).

Furthermore, our study reports a significant increase in $^{\bullet}OH$ production when DA incubations are performed in the presence of iron (Fe^{2+} or Fe^{3+}). Although, these data appear to reflect the involvement of Fe^{2+} in DA autoxidation, the polarographic study of this process in the presence of Fe^{2+} has revealed increases in both O_2 consumption and H_2O_2 accumulation during DA autoxidation, which precludes the involvement of Fe^{2+} in the production of $^{\bullet}OH$ by the Fenton reaction. At this point it is interesting to note that similar results were previously obtained with 6-hydroxydopamine (Méndez-Álvarez et al., 2001). Thus, the observed effect are probably a consequence of the reported capacity of Fe^{3+} to catalyze the autoxidation of DA (Linert and Jameson, 2000), according to the following mechanism:



As can be seen, dioxygen (O_2) is involved in this process as a single-electron acceptor to yield $O_2^{\bullet -}$ which then forms H_2O_2. This hypothesis is supported by the fact that the here reported increase in $^{\bullet}OH$ production was greater with Fe^{3+} than with Fe^{2+}. Evidently, in order to understand the effect caused by Fe^{2+} it appears necessary to consider than this metal ion, after being chelated with DA, is oxidized by $^{\bullet}OH$ to Fe^{3+}, according to the following reaction (Halliwell and Gutteridge, 1999):

$$Fe(II)\text{-}DA + {}^{\bullet}OH \rightarrow Fe(III)\text{-}DA + OH^-$$

Very probably, the suggested chelation of Fe^{2+} by DA is the reason for which this metal ion is not involved in the Fenton reaction. Furthermore, the formation of a complex with a higher number of coordination is possible (Raymond et al., 1976; Avdeef et al., 1978), a fact which may easily contribute to impede the accessibility of this metal ion to H_2O_2.

The use of pargyline as an inhibitor of the metabolism of DA by MAO caused a significant decrease in the rate of $^{\bullet}OH$ production during DA autoxidation, both in the absence and in the presence of iron (Fe^{2+} and Fe^{3+}). Presumably, this finding might be, at least in part, a consequence of the inhibition of DA metabolism because we were able to demonstrate that the rate of $^{\bullet}OH$ production is higher during DOPAC autoxidation than during DA autoxidation. Evidently, this fact allows us to assign an important physiological role to the COMT in the metabolism of DA. Furthermore, it helps explain the reported increase in $^{\bullet}OH$ production observed with the use of COMT inhibitors in L-DOPA/carbidopa treated rats (Gerlach et al., 2001). Nevertheless, a slight contribution of the capacity shown by pargyline to trap $^{\bullet}OH$ is a possibility that may be attributed to the presence of an acetylenic group on the molecular structure of pargyline. This hypothesis is supported by a similar behavior previously reported for other acetylenic MAO inhibitors such as (−)-deprenyl (Wu et al., 1993; Thomas et al., 1997).

The incubation of mitochondrial preparations under physiological conditions of pH and temperature is accompanied by a significant increase in the levels of TBARS, a fact which is interpreted as a consequence of the lipid peroxidation which takes place in the membranes of these organelles during the incubation. This process could be induced by the potential presence of iron in mitochondrial preparations due to the high content of mitochondria in both iron–sulfur proteins and cytochromes. However, the addition of DA to mitochondrial incubations provoked a marked reduction in the levels of TBARS. This result corroborate previous studies reporting an inhibitory effect of DA on lipid peroxidation induced under different experimental conditions in brain homogenates (Zaleska and Floyd, 1985; Dostert et al., 1991; Yen and Hsieh, 1997), liposomes (Spencer et al., 1996), and linoleic acid (Liu and Mori, 1993). Although, some of these authors have suggested that this effect is due to the potential of DA to trap $^{\bullet}OH$, the here reported capacity of DA to generate this free radical during the metabolism and autox-

idation of DA appears to contradict this hypothesis. In our opinion, the most probable interpretation is that the $^{\bullet}$sQH formed during DA autoxidation may act as a chain-breaking antioxidant by reacting with lipid radicals ($L^{\bullet}$), thus preventing the propagation of lipid peroxidation:

$$L^{\bullet} + {}^{\bullet}\text{sQH} \rightarrow \text{LH} + o\text{Q}$$

$$LO^{\bullet} + {}^{\bullet}\text{sQH} \rightarrow \text{LOH} + o\text{Q}$$

$$LOO^{\bullet} + {}^{\bullet}\text{sQH} \rightarrow \text{LOOH} + o\text{Q}$$

The addition of iron to mitochondrial preparations previous to its incubation causes an increase in the concentration of TBARS, which is dramatically greater with Fe^{2+} than with Fe^{3+}. Assuming that the hydroperoxyl radical ($HO_2^{\bullet}$) is considered the free radical involved in the initiation of lipid peroxidation (Aikens and Dix, 1991; Gebicki and Gebicki, 1993), the following two reactions are probably responsible for the observed effect:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -}$$

$$O_2^{\bullet -} + H^{+} \rightleftharpoons HO_2^{\bullet}$$

Nevertheless, we cannot discard the potential contribution of some lipid hydroperoxides (LOOH) (Svingen et al., 1979), whose probable presence in mitochondrial membranes would lead to the propagation of the lipid peroxidation, according to the following reaction:

$$Fe^{2+} + \text{LOOH} \rightarrow Fe^{3+} + LO^{\bullet} + OH^{-}$$

In the presence of both Fe^{2+} and Fe^{3+}, the addition of DA to the incubation provokes a reduction in the concentration of TBARS when this value is compared with that obtained with the corresponding metal ion (control). Once again, the inhibition of the lipid peroxidation by DA appears to be a consequence of the suggested capacity of $^{\bullet}$sQH to block the propagation of this process. Evidently, this hypothesis allows us to explain the greater ability shown by DA in the presence of Fe^{3+} (the actual catalyst of DA autoxidation) to inhibit lipid peroxidation in comparison to that found for DA in the presence of Fe^{2+}.

The pre-addition of pargyline to DA incubation with mitochondria provokes a significant increase in the levels of TBARS. This finding is in accordance with a previous report (Thomas et al., 1997) in which the addition of (−)-deprenyl caused an increase in the concentration of TBARS after the incubation of liposomes with a mixture of H_2O_2, Fe^{2+}-EDTA, and ascorbate. We observed a similar effect on the presence of both Fe^{2+} and Fe^{3+}. In our opinion, this phenomenon is viewed as a consequence of the suggested capacity of $^{\bullet}$OH to abstract hydrogen ($H^{\bullet}$) from the acetylenic group (Pryor, 1976) to form a pargylinyl radical ($Par^{\bullet}$), a fact which also contributes to explain the reduction of $^{\bullet}$OH formation by DA autoxidation in the presence of pargyline. This fact may be eventually followed by the abstraction of hydrogen from a membrane phospholipid (LH) to form a lipid radical, which may act as initiator of lipid peroxidation, and regenerate the pargyline:

$$\text{ParH} + {}^{\bullet}\text{OH} \rightarrow Par^{\bullet} + H_2O$$

$$\text{LH} + Par^{\bullet} \rightarrow L^{\bullet} + \text{ParH}$$

It is interesting to note that in this way the acetylenic group of pargyline remains intact, and thus guarantees the participation of this group in the inhibition of MAO activity (Kalir et al., 1981; Fowler et al., 1982). Evidently, the here suggested capacity of an acetylenic MAO inhibitor to induce lipid peroxidation could help to explain both the increase in DA neurotoxicity after the administration of pargyline to rats (Ben-Shachar et al., 1995) and the observed morbidity of the coadministration of levodopa and selegiline in some patients suffering from Parkinson's disease (Lees, 1995).

With regard to the effect of the metabolism and autoxidation of DA on the oxidant status of mitochondrial proteins, it has been shown that the incubation of DA with mitochondria causes an increase in the protein carbonyl content. The addition of iron to mitochondrial incubations in the absence of DA provokes a significant increase in carbonyl content, which is greater with Fe^{2+} than with Fe^{3+}. However, the increase observed with the combined action of DA and iron was greater with Fe^{3+} than with Fe^{2+}. Taking into account the effects observed with Fe^{2+} and Fe^{3+} on both $^{\bullet}$OH formation and TBARS accumulation, our findings appear to corroborate the previously suggested involvement of $HO_2^{\bullet}$ in the generation of carbonyl groups on proteins (Neuzil and Stocker, 1993). In addition to the here reported data, it appears reasonable that an uncharged free radical with a relatively non short half-life will be involved in this process because this fact facilitates both the approach of the radical to the hydrophobic environment conducive to the formation of carbonyl groups (Gebicki and Gebicki, 1993; Fu et al., 1995; Ayala and Cutler, 1996) and the access of the corresponding free radical to proteins located in the mitochondrial matrix. This latter fact could explain the remarkable increase in carbonyl content observed in some of the experimental conditions in this study. The mechanism suggested for the formation of carbonyl groups (see Fig. 6) involves a hydrogen abstraction from a β-carbon followed by dioxygen addition to generate a peroxyl radical ($ROO^{\bullet}$). This free radical may react with another $R'OO^{\bullet}$ (generated either by protein oxidation or lipid peroxidation) to form a tetraoxide (Dean et al., 1997) which subsequently breaks down to give an alkoxyl radical ($R'O^{\bullet}$) and the corresponding side-chain radical, which in turn cleaves to finally generate an α-carbonyl group. Evidently, the drastic increase in the carbonyl content exhibited by the combined action of (DA + Fe^{3+}) is viewed as a consequence of the catalytic effect of Fe^{3+} on DA autoxidation which might facilitate the generation of $HO_2^{\bullet}$ by promoting $O_2^{\bullet -}$ production. However, in the absence of DA, Fe^{2+} is the ion directly involved in $HO_2^{\bullet}$ production. In general, the addition of pargyline

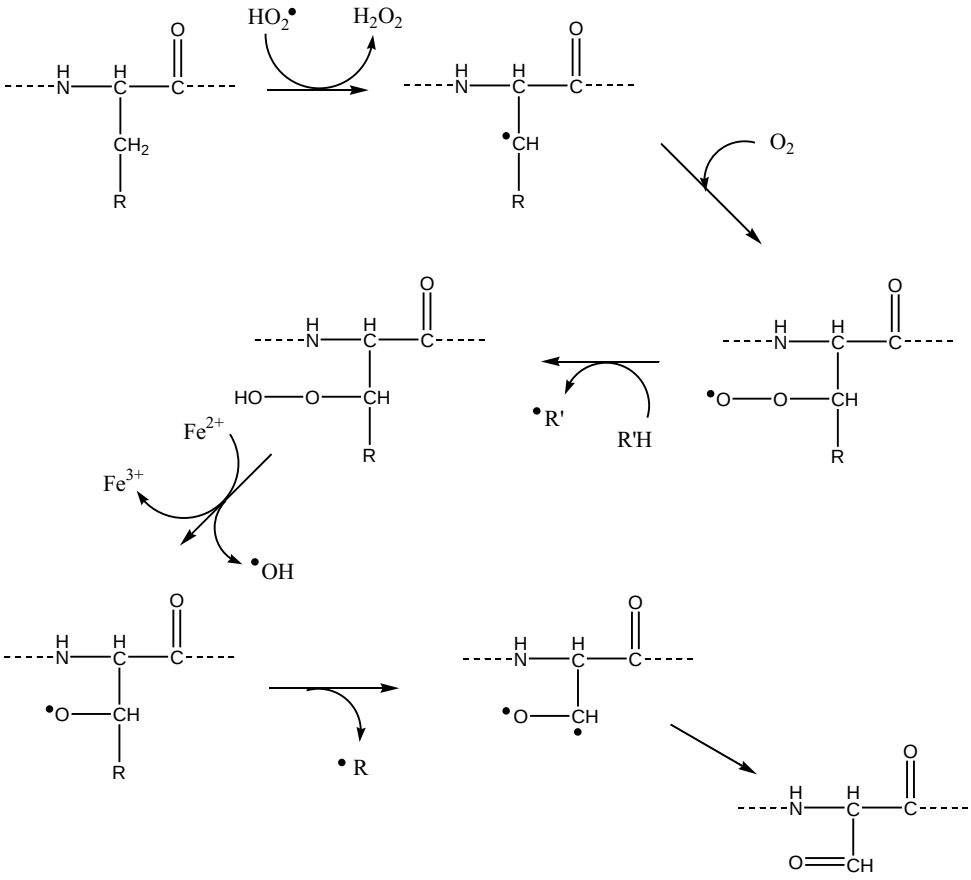
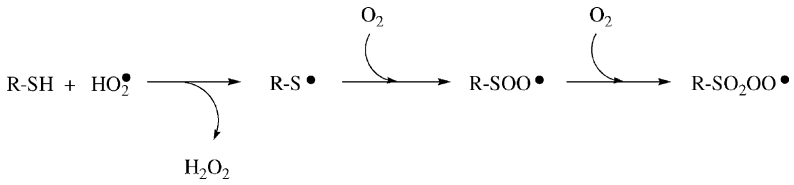


Fig. 6. Molecular mechanism proposed for the formation of carbonyl groups in proteins, which initiation involves the abstraction of one hydrogen from the side chain of the amino acid by hydroperoxyl radical.

caused a reduction in carbonyl content in proteins under each of the different experimental condition used in this study which agrees with the here reported ability of pargyline to inhibit the formation of $^{\bullet}OH$ and consequently $HO_2^{\bullet}$ production.

Another interesting aspect of protein damage by oxidative stress is the loss of free thiol groups in proteins. Our research have shown that the incubation of mitochondria with DA provokes a significant decrease in the thiol content of mitochondrial proteins, which is dependent of the DA concentration used. In addition, the presence of Fe^{2+} also caused a drastic fall in protein thiol content. Although the effect observed with the combined action of (Fe^{2+} + DA) does not differ from that found with Fe^{2+} alone, the cause could be a limit to the number of thiol groups able to undergo oxidation. Thus, in the case of Fe^{3+} in which the presence of this metal ion alone does not affect the protein thiol content, the combined action of (Fe^{3+} + DA) caused a reduction in protein thiol content which is attributed to the autoxidation of DA. In our opinion, the here reported effects could be related to the generation of $HO_2^{\bullet}$, which, as previously mentioned, is facilitated by the presence of Fe^{2+} and the autoxidation of DA. Furthermore, this hypothesis is supported by the reported inability of catalase (Chakrabarti et al., 1990) and certain $^{\bullet}OH$ scavengers (Chakraborty et al., 2001) to inhibit the reduction of protein thiol content under different experimental conditions. The mechanism suggested for the oxidation of thiol groups under aerobic conditions is as follows:after



hydrogen abstraction of $HO_2^{\bullet}$ from the thiol group to form the thiyl radical (R–$S^{\bullet}$), it reacts with oxygen to give sequentially the sulfonyl (R–$SOO^{\bullet}$) and the sulfonyl peroxyl radicals (R–$SO_2OO^{\bullet}$), which then undergo reduction to finally give sulfenic (R–SOH) and sulfonic (R–SO_3H) acids, respectively. Evidently, the capacity shown by pargyline to

inhibit the reduction of protein thiol content by DA autoxidation may be related with the here reported capacity of this MAO inhibitor to reduce the production of $^{\bullet}OH$ and consequently of the radical directly involved in the oxidation of thiol groups ($HO_2^{\bullet}$).

Finally, we have investigated the potential of the metabolism and autoxidation of DA to modify MAO activity, a parameter which has often been used to assess the capacity of oxidative stress to affect mitochondrial functions (Dean et al., 1986, 1991; Thomas et al., 1989). Under the experimental conditions used, only a high DA concentration causes significant MAO-B activation. This effect is attributed to the accumulation of H_2O_2, a compound which has been shown to enhance particularly MAO-B activity (Konradi et al., 1986). In contrast, the combined action of (DA + Fe^{2+}) causes an inhibitory effect on MAO activity, dependent on DA concentration, and which is more relevant for MAO-B than for MAO-A. However, the combined action of (DA + Fe^{3+}) only was able to inhibit MAO-B activity. Taking into account the effects observed by the combined action of DA with Fe^{2+} and Fe^{3+} on both $^{\bullet}OH$ production and TBARS accumulation, the inhibition of MAO activity is viewed as a consequence of the lipid peroxidation induced on mitochondrial membranes, which shows the important role played by the lipid environment of the mitochondrial membrane on MAO activity (Fowler et al., 1980).

Thus, from our results we conclude that DA is a double-edged sword which, on the one hand protects against both the hazardous Fenton reaction and the propagation of lipid peroxidation, but on the other hand generates $^{\bullet}OH$ and promotes protein oxidation. Furthermore, these properties are enhanced in different manners by the presence of Fe^{2+} and Fe^{3+}. In our opinion, the pro-oxidant properties of this neurotransmitter may be well-controlled by the defense systems which dopaminergic neurons have against oxidative stress. However, problems arise with high concentrations of DA, because of the possibility that the resulting oxidative stress cannot be controlled by the corresponding defense systems, a fact that may be exacerbated by the reported inhibition of MAO activity. Evidently, our findings contribute to explain the progression of Parkinson's disease due to the reported increase in DA turnover and also the long-term side effects derived from the treatment of Parkinson's disease with L-DOPA which maintains brain levels of DA over those found in non-parkinsonian brains. Finally, the capacity shown by DOPAC to generate $^{\bullet}OH$ could also explain some of the side effects arising from the use of COMT inhibitors in the treatment of Parkinson's disease.

Acknowledgements

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References

Aikens, J., Dix, J.A., 1991. Perhydroxyl radical (HOO$^{\bullet}$) initiated lipid peroxidation. The role of fatty acid hydroperoxides. J. Biol. Chem. 266, 15091–15098.

Avdeef, A., Sofen, S.R., Bregante, T.L., Raymond, K.N., 1978. Coordination chemistry of microbial iron transport compounds. Part 9. Stability constants for catechol models of enterobactin. J. Am. Chem. Soc. 100, 5362–5370.

Ayala, A., Cutler, R.G., 1996. The utilization of 5-hydroxyl-2-amino valeric acid as a specific marker of oxidized arginine and proline residues in proteins. Free Radic. Biol. Med. 21, 65–80.

Ben-Shachar, D., Zuk, R., Glinka, Y., 1995. Dopamine neurotoxicity: inhibition of mitochondrial respiration. J. Neurochem. 64, 718–723.

Bernheimer, H., Birkmayer, W., Hornykiewicz, O., Jellinger, K., Seitelberger, F., 1973. Brain dopamine and the syndromes of Parkinson and Huntington—clinical, morphological and neurochemical correlations. J. Neurol. Sci. 29, 415–455.

Chakrabarti, S., Naik, A.A., Reddy, G.R., 1990. Phenylhydrazine mediated degradation of bovine serum albumin and membrane proteins of human erythrocytes. Biochim. Biophys. Acta 1028, 89–94.

Chakraborty, H., Ray, S.N., Chakrabarti, S., 2001. Lipid peroxidation associated protein damage in rat brain crude synaptosomal fraction mediated by iron and ascorbate. Neurochem. Int. 39, 311–317.

Chiueh, C.C., Miyake, H., Peng, M.T., 1993. Role of dopamine autoxidation, hydroxyl radical generation and calcium overload in underlying mechanisms involved in MTP-induced parkinsonism. Adv. Neurol. 60, 251–258.

Clark, J.B., 1993. Electrochemical assay. In: Eisenthal, R., Danson, M.J. (Eds.), Enzyme Assay: A Practical Approach. IRL Press, Oxford, pp. 181–190.

Cohen, G., 1990. Monoamine oxidase and oxidative stress at dopaminergic synapse. J. Neural Transm. 32, 229–238.

Dean, R.T., Fu, S., Stocker, R., Davies, M.J., 1997. Biochemistry and pathology of radical-mediated protein oxidation. Biochem. J. 324, 1–18.

Dean, R.T., Hunt, J.V., Grant, A.J., Yamamoto, Y., Niki, E., 1991. Free radical damage to proteins: the influence of the relative localization of radical generation, antioxidants, and target proteins. Free Radic. Biol. Med. 11, 161–168.

Dean, R.T., Thomas, S.M., Garner, A., 1986. Free radical-mediated fragmentation of monoamine oxidase in the mitochondrial membrane. Roles for lipid radicals. Biochem. J. 240, 489–494.

Dostert, P., Strolin Benedetti, M., Frigerio, E., 1991. Effect of L-DOPA, oxyferriscorbone, and ferrous iron on in vivo lipid peroxidation. J. Neural Transm. 84, 119–128.

Enoch, W.S., Sarna, T., Zecca, L., Riley, P.A., Swartz, H.M., 1994. The roles of neuromelanin, binding of metal ions, and the oxidative cytotoxicity in the pathogenesis of Parkinson's disease: a hypothesis. J. Neural Transm. 7, 83–100.

Filloux, F., Townsend, J.J., 1993. Pre- and postsynaptic neurotoxic effects of dopamine demonstrated by intrastriatal injection. Exp. Neurol. 119, 79–88.

Fornstedt, B., 1990. Role of catechol autoxidation in the degeneration of dopamine neurons. Acta Neurol. Scand. 129, 12–14.

Fornstedt, B., Pileblad, E., Carlsson, A., 1990. In vivo autoxidation of dopamine in guinea pig striatum increases with age. J. Neurochem. 55, 655–659.

Fowler, C.J., Callingham, B.A., Mantel, T.J., Tipton, K.F., 1980. The effect of lipophilic compounds upon the activity of rat liver mitochondrial mono-oxidase-A and -B. Biochem. Pharmacol. 29, 1177–1183.

Fowler, C.J., Mantel, T.J., Tipton, K.F., 1982. The nature of the inhibition of rat liver monoamine oxidase types A and B by the acetylenic inhibitors clorgyline, L-deprenyl and pargyline. Biochem. Pharmacol. 31, 3555–3561.

Fu, S., Gebicki, S., Jessup, W., Gebicki, J.M., Dean, R.T., 1995. Biological fate of amino acid, peptide and protein hydroperoxides. Biochem. J. 311, 821–827.

Gebicki, S., Gebicki, J.M., 1993. Formation of peroxides in amino acids and proteins exposed to oxygen free radicals. Biochem. J. 289, 743–749.

Gerlach, M., Xiao, A.Y., Kuhn, W., Lehnfeld, R., Waldmeier, P., Sontag, K.H., Riederer, P., 2001. The central catechol-*O*-methyltransferase inhibitor tolcapone increases striatal hydroxyl radical production in L-DOPA/carbidopa treated rats. J. Neural Transm. 108, 189–204.

Graham, D.G., 1978. Oxidative pathway for catecholamines in the genesis of neuromelanin and cytotoxic quinones. Mol. Pharmacol. 14, 633–643.

Graham, D.G., Tiffany, S.M., Bell, W.R., Gutknecht, W.F., 1978. Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells in vitro. Mol. Pharmacol. 14, 644–653.

Habeeb, A.F.S.A., 1972. Reaction of protein sulfhydryl groups with Ellman's reagent. Methods Enzymol. 25, 457–464.

Halliwell, B., Gutteridge, J.M.C. (Eds.), 1999. Free Radicals in Biology and Medicine, third ed. Oxford University Press, Oxford.

Hastings, T.G., Lewis, D.A., Zigmond, M.J., 1996. Role of oxidation in the neurotoxic effects of intrastriatal dopamine injections. Proc. Natl. Acad. Sci. U.S.A. 93, 1956–1961.

Kalir, A., Sabbagh, A., Youdim, M.B., 1981. Selective acetylenic "suicide" and inhibitors of monoamine oxidase types A and B. Br. J. Pharmacol. 73, 55–64.

Konradi, C., Riederer, P., Youdim, M.B.H., 1986. Hydrogen peroxide enhances the activity of monoamine oxidase type-B but not type-A: a pilot study. J. Neural Transm. 22, 61–73.

Langston, J.W., 1990. Predicting Parkinson's disease. Neurology 40 (Suppl.), 70–74.

Lees, A.J.,. On the behalf of the Parkinson's Disease Research Group of the United Kingdom, 1995 1995. Comparison of the therapeutic effects and mortality data of levodopa and levodopa combined with selegiline in patients with early, mild Parkinson's disease. Br. Med. J. 311, 1602–1607.

Li, C.-L., Werner, P., Cohen, G., 1995. Lipid peroxidation in brain: interactions of L-DOPA/dopamine with ascorbate and iron. Neurodegeneration 4, 147–153.

Linert, W., Herlinger, E., Jameson, R.F., Kienzl, E., Jellinger, K., Youdim, M.B.H., 1996. Dopamine, 6-hydroxydopamine, iron, and dioxygen—their mutual interactions and possible implications in the development of Parkinson's disease. Biochim. Biophys. Acta 1316, 160–168.

Linert, W., Jameson, G.N.L., 2000. Redox reactions of neurotransmitters possibly involved in the progression of Parkinson's disease. J. Inorg. Biochem. 79, 319–326.

Liu, J., Mori, A., 1993. Monoamine metabolism provides an antioxidant defense in the brain against oxidant- and free radical-induced damage. Arch. Biochem. Biophys. 302, 118–127.

Mann, D.M.A., Yates, P.O., 1983. Possible role of neuromelanin in the pathogenesis of Parkinson's disease. Mech. Age Dev. 21, 193–203.

Markwell, M.A.K., Haas, S.M., Bieber, L.L., Tolbert, N.E., 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87, 206–210.

McGeer, P.L., McGeer, E.G., Suzuki, J.S., 1977. Aging and extrapyramidal function. Arch. Neurol. 34, 33–35.

Méndez-Álvarez, E., Soto-Otero, R., Hermida-Ameijeiras, A., López-Martín, M.E., Labandeira-García, J.L., 2001. Effect of iron and manganese on hydroxyl radical production by 6-hydroxydopamine: mediation of antioxidants. Free Radic. Biol. Med. 31, 986–998.

Méndez-Álvarez, E., Soto-Otero, R., Sánchez-Sellero, I., López-Rivadulla Lamas, M., 1997. Inhibition of brain monoamine oxidase by adducts of 1,2,3,4-tetrahydroisoquinoline with components of cigarette smoke. Life Sci. 60, 1719–1727.

Michel, P.P., Hefti, F., 1990. Toxicity of 6-hydroxydopamine and dopamine for dopaminergic neurons in culture. J. Neurosci. Res. 26, 428–435.

Nappi, A.J., Vass, E., 1997. Comparative studies of enhanced iron-mediated production of hydroxyl radical by glutathione, cysteine, ascorbic acid, and selected catechols. Biochim. Biophys. Acta 1336, 295–301.

Neuzil, J., Stocker, R., 1993. Bilirrubin attenuates radical-mediated damage to serum albumin. FEBS Lett. 331, 281–284.

Palkovits, M., Brownstein, M., 1989. Catecholamines in the central nervous system. In: Trendelenberg, U., Weiner, N. (Eds.), Catecholamines II. Springer, Berlin, pp. 1–26.

Pedrosa, R., Soares-da-Silva, P., 2002. Oxidative and non-oxidative mechanism of neuronal cell death and apoptosis by L-3,4-dihydroxyphenylalanine (L-DOPA) and dopamine. Br. J. Pharmacol. 137, 1305–1313.

Pryor, W.A., 1976. The role of free radical reactions in biological systems. In: Pryor, W.A. (Ed.), Free Radicals in Biology, vol. 1. Academic Press, New York, pp. 1–49.

Ravinovic, A.D., Lewis, D.A., Hastings, T.G., 2000. Role of oxidative changes in the degeneration of dopamine terminals after injection of neurotoxic levels of dopamine. Neuroscience 101, 67–76.

Raymond, K.N., Isied, S.S., Brown, L.D., Fronczek, F.R., Nibert, J.H., 1976. Coordination isomers of biological iron transport compounds. Part VI. Models of the enterobactin coordination site. J. Am. Chem. Soc. 98, 1767–1774.

Reznick, A.Z., Packer, L., 1994. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Methods Enzymol. 233, 357–363.

Richter, H.W., Waddell, W.H., 1983. Mechanism of the oxidation of dopamine by the hydroxyl radical in aqueous solution. J. Am. Chem. Soc. 105, 5434–5440.

Schild, L., Reinheckel, T., Wiswedel, I., Augustin, W., 1997. Short-term impairment of energy production in isolated rat liver mitochondria by hypoxia/reoxygenation: involvement of oxidative protein modification. Biochem. J. 328, 205–210.

Severson, J.A., Marcusson, J., Winblad, B., Finch, C.E., 1982. Age-correlated loss of dopaminergic binding sites in human basal ganglia. J. Neurochem. 39, 1623–1631.

Slivka, A., Cohen, G., 1985. Hydroxyl radical attack on dopamine. J. Biol. Chem. 260, 15466–15472.

Soto-Otero, R., Méndez-Álvarez, E., Hermida-Ameijeiras, A., López-Real, A.M., Labandeira-García, J.L., 2002. Effects of (−)-nicotine and (−)-cotinine on 6-hydroxydopamine-induced oxidative stress and neurotoxicity: relevance for Parkinson's disease. Biochem. Pharmacol. 64, 125–135.

Soto-Otero, R., Méndez-Álvarez, E., Hermida-Ameijeiras, A., Muñoz-Patiño, A.M., Labandeira-García, J.L., 2000. Autoxidation and neurotoxicity of 6-hydroxydopamine in the presence of some antioxidants: potential implication in relation to the pathogenesis of Parkinson's disease. J. Neurochem. 74, 1605–1612.

Spencer, J.P.E., Jenner, A., Butler, J., Aruoma, O.I., Dexter, D.T., Jenner, P., Halliwell, B., 1996. Evaluation of the pro-oxidant and antioxidant actions of L-DOPA and dopamine in vitro: implications for Parkinson's disease. Free Radic. Res. 24, 95–105.

Svingen, B.A., Buege, J.A., O'Neal, F.O., Aust, S.D., 1979. The mechanism of NADPH-dependent lipid peroxidation. The propagation of lipid peroxidation. J. Biol. Chem. 254, 5892–5899.

Tanaka, M., Sotomatsu, A., Kanai, H., Hirai, S., 1991. Dopa and dopamine cause cultured neuronal death in the presence of iron. J. Neurol. Sci. 101, 198–203.

Thomas, S.M., Gebicki, J.M., Dean, R.T., 1989. Radical initiated alpha-tocopherol depletion and lipid peroxidation in mitochondrial membranes. Biochim. Biophys. Acta 1002, 189–197.

Thomas, C.E., Huber, E.W., Ohlweiler, D.F., 1997. Hydroxyl and peroxyl radical trapping by monoamine oxidase-B inhibitors deprenyl and MLD 72,974A: implications for protection of biological substrates. Free Radic. Biol. Med. 22, 733–737.

Wu, R.-M., Chiueh, C.C., Pert, A., Murphy, D.L., 1993. Apparent antioxidant effect of L-deprenyl on hydroxyl radical formation and nigral injury elicited by MPP^+ in vivo. Eur. J. Pharmacol. 243, 241–247.

Yen, G.-C., Hsieh, C.-L., 1997. Antioxidant effects of dopamine and related compounds. Biosci. Biotech. Biochem. 61, 1646–1649.

Zaleska, M.M., Floyd, R.A., 1985. Regional lipid peroxidation in rat brain in vitro: possible role of endogenous iron. Neurochem. Res. 10, 397–410.

Zhang, F., Dryhurst, G., 1993. Oxidation chemistry of dopamine: possible insights into the age-dependent loss of dopaminergic nigrostriatal neurons. Bioorg. Chem. 21, 392–410.