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Effects of aluminum and zinc on the oxidative stress caused by 6-hydroxydopamine autoxidation: relevance for the pathogenesis of Parkinson's disease

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Abstract

Aluminum and zinc have been related to the pathogenesis of Parkinson's disease (PD), the former for its neurotoxicity and the latter for its apparent antioxidant properties. 6-Hydroxydopamine (6-OHDA) is an important neurotoxin putatively involved in the pathogenesis of PD, its neurotoxicity often being related to oxidative stress. The potential effect of these metals on the oxidative stress induced by 6-OHDA autoxidation and the potential of ascorbic acid (AA), cysteine, and glutathione to modify this effect were investigated. Both metals, particularly Al^{3+}, induced a significant reduction in $^{\bullet}OH$ production by 6-OHDA autoxidation. The combined action of AA and a metal caused a significant and sustained increase in $^{\bullet}OH$ generation, particularly with Al^{3+}, while the effect of sulfhydryl reductants was limited to only the first few minutes of the reaction. However, both Al^{3+} and Zn^{2+} provoked a decrease in the lipid peroxidation induced by 6-OHDA autoxidation using mitochondrial preparations from rat brain, assessed by TBARS formation. In the presence of AA, only Al^{3+} induced a significant reduction in lipid peroxidation. After intrastriatal injections of 6-OHDA in rats, tyrosine hydroxylase immunohistochemistry revealed that Al^{3+} reduces 6-OHDA-induced dopaminergic lesion in the striatum, which corroborates the involvement of lipid peroxidation in 6-OHDA neurotoxicity and appears to discard the participation of this mechanism on PD by Al^{3+} accumulation. The previously reported antioxidant properties of Zn^{2+} appear to be related to the induction of Zn^{2+}-containing proteins and not to the metal per se.

Keywords: 6-Hydroxydopamine; Aluminum; Zinc; Antioxidant; Oxidative stress; Parkinson's disease

1. Introduction

Parkinson's disease (PD) is a late-onset, progressive neurodegenerative disease, which is considered the most frequent disorder of the basal ganglia. The clinical characteristics are resting tremor, rigidity, bradykinesia, and postural disturbance. These motor abnormalities are associated with a specific loss of dopaminergic neurons in the substantia nigra pars compacta and the subsequent depletion of dopamine levels in the striatum. The clinical manifestations of PD only appear when 70–80% of the men-

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tioned dopaminergic neurons are lost. Although several molecular mechanisms have been proposed for the pathogenesis of this disorder [1], the etiology of PD remains unclear. However, there are increasing data suggesting that oxidative stress may be involved in the neuronal death which occurs in PD [2–4].

6-Hydroxydopamine (6-OHDA) is a neurotoxin which, in the form of stereotaxic injections into the medial forebrain bundle or substantia nigra, has been used for years to produce a rodent model of PD [5]. More recently, a 'partial lesion model' has been developed which is particularly useful for research on neuroprotection and neurotoxicity [6,7]. This model is based on small intrastriatal injections of 6-OHDA that produce the degeneration of nigrostriatal terminals in a reduced striatal area, in which a drug-induced increase or decrease of 6-OHDA derived lesion is easily detectable. Under physiological conditions 6-OHDA suffers autoxidation by molecular oxygen to give a cascade of oxidative reactions resulting in the formation of hydrogen peroxide (H_2O_2) and an insoluble polymeric pigment related to neuromelanin [8]. Although it has been reported that 6-OHDA may act by inhibiting complex I of the mitochondrial respiratory chain [9,10], its neurotoxicity has often been linked to the production of free radicals [11–13]. Evidently, it is possible that the H_2O_2 resulting from the autoxidation of 6-OHDA is easily reduced by the metal-catalyzed Fenton reaction to generate the hydroxyl radical ($^{\bullet}OH$), which is considered the most damaging free radical for living cells.

Assuming the great ability of 6-OHDA to suffer autoxidation and produce $^{\bullet}OH$ by a metal-catalyzed reaction, the effects of metals on this process would seem particularly useful for a better understanding of both the mechanism underlying the neurotoxicity of 6-OHDA and the suggested involvement of this neurotoxin in the pathogenesis of PD [14,15]. Aluminum (Al^{3+}) and zinc (Zn^{2+}) are two non-transition metal ions which have often been related to the pathogenesis of PD [16–19], and several studies have shown that Al^{3+} is able to increase oxidative stress in both rat brain [20,21] and cultured neurons [22]. In addition, it has also been suggested that the parkinsonism–dementia found on Guam Island is related to the accumulation of Al^{3+} in certain brain areas of the patients suffering this disorder [23,24]. However, taking into account that Al^{3+} is not a transition metal, and hence cannot participate in redox reactions, the molecular mechanism underlying this effect remains unclear [25]. Regarding Zn^{2+}, antioxidant properties in relation to brain oxidative stress have been reported [26–28]. Although it has been suggested that these antioxidant properties may be related to the capacity shown by Zn^{2+} to induce the synthesis of metallothionein [26], the precise mechanism of this effect is still unclear [28]. Furthermore, the complexity of the physiological effects of Zn^{2+} is to be expected, given its proven ability to inhibit Ca^{2+} transport [29], induce neuronal apoptosis [30,31], inhibit (Na,K)-ATPase [32] and provoke seizures [33].

Although it is well established that reductant substances may act as scavengers of free radicals, it is also known that under certain conditions these substances may also display pro-oxidant properties. Thus, although it has been reported that $^{\bullet}OH$ production by H_2O_2+Fe^{2+} is enhanced by the presence of ascorbic acid (AA), cysteine (CySH), and glutathione (GSH) [34], it has also been found that GSH is able to protect DNA from the oxidation caused by H_2O_2+Fe^{2+} [35]. Furthermore, it has recently been reported that AA may enhance the neurotoxicity of 6-OHDA by inducing a redox cycle during 6-OHDA autoxidation, while CySH and GSH protect against the neurotoxicity of 6-OHDA [36]. For this reason, it might be expected that the presence of these antioxidants could also affect the potential effects induced by the assayed metal ions on the production of $^{\bullet}OH$ by 6-OHDA.

Thus, this study was undertaken to determine the potential effects of the presence of Al^{3+} and Zn^{2+} on the generation of $^{\bullet}OH$ during 6-OHDA autoxidation, together with the role which certain antioxidant substances such as AA, CySH, and GSH might play. We have also studied the effect of the presence of these metal ions on the lipid peroxidation induced by the autoxidation of 6-OHDA in mitochondria preparations obtained from rat brain. Finally, the capacity of Al^{3+} to lesion, either by itself or in combination with 6-OHDA, the nigrostriatal dopaminergic system, was also investigated using a 'partial lesion model' of PD in rats. Evidently, knowledge of the molecular mechanisms underlying the action of potential risk factors or neuroprotectors for PD may lead to a better understanding of the pathogenesis

of this disorder and might also help to develop new strategies for its prevention.

2. Materials and methods

2.1. Chemicals

6-OHDA hydrobromide, AA, CySH, GSH, H_2O_2, catalase (CAT), butylated hydroxytoluene crystalline, deferoxamine mesylate, thiobarbituric acid, 1,1,3,3-tetraethoxypropane, sodium dodecyl sulfate, Triton X-100, 3,3′-diaminobenzidine, and bovine serum albumin were obtained from Sigma (St. Louis, MO, USA). Zinc chloride and ferrous chloride tetrahydrate were purchased from Fluka (Buchs, Switzerland). Aluminum chloride hexahydrate was obtained from Riedel-de Haën (Seelze, Germany). Terephthalic acid (THA) disodium salt was purchased from Aldrich (Steinheim, Germany). Biotinylated secondary antibody and avidin–biotin–peroxidase complex were purchased from Vector (Burlingame, CA, USA). Normal swine serum was obtained from Dako (Glostrup, Denmark), and rabbit polyclonal antiserum to tyrosine hydroxylase (TH) from Pel-Freez (Rogers, AK, USA). The water used for the preparation of solutions was of Milli-RiOs/Q-A10 grade (Millipore, Bedford, MA, USA). All remaining chemicals used were of analytical grade and were from Fluka. Stock solutions of 6-OHDA were prepared in 1.0 mM KCl (pH 2.0) to prevent its autoxidation [37]. Fresh stock solutions of antioxidants and metal ions were used, and prepared in phosphate buffer (25 mM, pH 7.4) and water, respectively. In order to prevent contamination by metal ions, the contact of the different solutions used with glassware was avoided as much as possible.

2.2. Mitochondria preparation

Male Sprague–Dawley rats weighing 200–250 g were used. The rats were received from the breeder at least 4 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 (Na_2PO_4/KH_2PO_4 isotonized with sucrose, pH 7.4). Brain mitochondria were then obtained by differential centrifugation with minor modifications to a previously published method [38]. Briefly, after removing blood vessels and pial membranes, the brains were manually homogenized with 4 vols. (w/v) of the isolation medium. The homogenate was then centrifuged at $900\times g$ for 5 min at 4°C, and the supernatant centrifuged at $12\,500\times g$ for 15 min. The mitochondria pellet was washed once with isolation medium and recentrifuged under the same conditions. Finally, the mitochondria pellet was reconstituted in a buffer solution (Na_2PO_4/KH_2PO_4 isotonized with KCl, pH 7.4) and stored in aliquots at −40°C.

The protein concentration of mitochondria preparations was determined according to the method of Markwell et al. [39], using bovine serum albumin as the standard.

2.3. Production of $^{\bullet}OH$

The generation of $^{\bullet}OH$ was fluorimetrically monitored according to a modification made to a previously published method [40], in which THA is used as a chemical dosimeter of $^{\bullet}OH$. A luminescence 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 continuous mixing of the sample. For each assay, 2000 μl of 25 mM phosphate buffer (pH 7.4) containing THA (10 mM) was incubated in a quartz cuvette (optical path length 10 mm) for 5 min to reach the temperature. An aliquot of 25 mM phosphate buffer (pH 7.4) was added to take the final volume of the incubation to 2.5 ml. Then, 20 μl of metal (800 nM) and/or 100 μl of antioxidant (100 μM) and/or 20 μl of a stock solution of 6-OHDA (5 μM) were added. All concentrations are final concentrations in the incubation. The monitoring of $^{\bullet}OH$ formation was immediately initiated and maintained for the subsequent 4 min, using 312 and 426 nm as excitation and emission wavelengths, respectively. All fluorescence measurements were expressed relative to the initial reading.

2.4. Consumption of O_2 and accumulation of H_2O_2

The O_2 consumption during 6-OHDA autoxida-

tion was monitored polarographically using a Clark-type O_2 electrode (Digital Oxygen System Model 10, Rank Brothers, Cambridge, UK) equipped with a device consisting of an amplifier, an A/D converter (Model AD12, AM-Systems, Jaén, Spain) and the corresponding software (LipSoft v. 2.0, AM-Systems) for data acquisition and control [41]. 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, 2460 μl 25 mM phosphate buffer (pH 7.4) or 2460 μl of this phosphate buffer containing antioxidant (8 mM) was incubated in the electrode chamber for 5 min in order to reach the temperature and the saturating O_2 concentration. Then, 20 μl of metal ion (64 μM) and/or 20 μl of 6-OHDA (400 μM) were injected using a Hamilton syringe (Model 702SNR). All concentrations are final concentrations in the incubation. A magnetic agitator maintained the solution in the electrode chamber continuously under vigorous stirring. The potential accumulation of H_2O_2 was estimated from the production of O_2 after the injection of 2000 μmol/min of CAT. 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 [42].

2.5. Evaluation of lipid peroxidation

Lipid peroxidation in mitochondria preparations was assessed by spectrophotometric determination of TBARS according to a modification made to a previously published method [43] as follows. To 200 μl of mitochondria preparation (1 mg protein/ml), 25 μl of Al^{3+} or Zn^{2+} (1.6 μM) or phosphate buffer (pH 7.4) were added, followed by 25 μl of 6-OHDA (10 μM). In some experiments, 50 μl of AA (100 μM) were incorporated prior to the addition of 6-OHDA. The resulting mixture was incubated at 37°C for exactly 10 min. Lipid peroxidation was stopped by the addition of 25 μl of butylated hydroxytoluene (1 mM) and 25 μl desferrioxamine (1 mM). A volume of 750 μl of acetic acid (20%) followed by 200 μl of sodium dodecyl sulfate (8%, w/v) was added and the mixture vortexed for 1 min. Then, 750 μl of TBA (0.8%) were 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 4000 rpm 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 derived by acid hydrolysis (SO_4H_2; 1.5%, v/v) of 1,1,3,3-tetramethoxypropane and the TBARS results expressed as nmol MDA/mg protein.

2.6. Animal treatment

A total of nine male Sprague–Dawley rats (weighing about 200 g at the beginning of the experiments) were used. All experiments were carried out in accordance with the 'Principles of laboratory animal care' (NIH publication No. 86-23, revised 1985). All rats received bilateral intrastriatal injections, and were divided into two groups. Rats in group A ($n=4$) were injected with 2 μl of sterile saline containing 0.48 μg of Al^{3+} into the right striatum, and 2 μl of sterile saline into the left striatum. Rats in group B ($n=4$) were injected into the right striatum with 2 μl of sterile saline containing 0.48 μg of Al^{3+}, and 10 min later with 6 μg of 6-OHDA in 2 μl sterile saline containing 0.2% AA. Group B rats were injected into the left striatum with 2 μl of sterile saline, and 10 min later with 6 μg of 6-OHDA in 2 μl sterile saline containing 0.2% AA. Stereotaxic coordinates were 1.0 mm anterior to bregma, 2.7 mm left or right of midline, and 5.5 mm ventral to the dura; tooth bar at −3.3. The solution was injected with a 5 μl Hamilton syringe at 0.2 μl/min and the cannula was left in situ for 5 min after injection. All surgery was performed under equithesin anesthesia (3 ml/kg i.p.).

2.7. Immunohistochemistry

A week post lesion, the animals were deeply anesthetized with chloral hydrate and perfused first with 0.9% saline and then with cold 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were removed and subsequently washed and cryoprotected in the same buffer containing 20% sucrose, and finally cut on a freezing microtome. Sections were processed by TH immunohistochemistry (as follows). After incubation for 1 h in 10% normal swine serum

with 0.25% Triton X-100 in 0.02 M potassium phosphate-buffered saline containing 1% bovine serum albumin (KPBS-BSA), sections were incubated overnight at room temperature with rabbit polyclonal antiserum to TH (1/500 in KPBS-BSA containing 2% normal swine serum and 0.25% Triton X-100). The sections were subsequently incubated first for 90 min with the corresponding biotinylated secondary antibody (diluted 1/200) and then for 90 min with an avidin–biotin–peroxidase complex. Finally, the labeling was visualized with 0.04% hydrogen peroxide and 0.05% 3,3′-diaminobenzidine. TH immunohistochemistry was quantified with the aid of NIH-Image 1.55 image analysis software (Wayne Rasband, MIMH) on a personal computer coupled to a video camera (CCD-72, MTI). The lesioned area surrounding the needle track on the left and right striatum was evaluated using a constant illumination light table (Northern Light, St. Catharines, Canada) coupled to the video camera.

2.8. Statistical analysis

Data are expressed as the mean ± S.E.M. Differences between means were statistically evaluated using a two-sided Student's *t*-test. The statistical significance of differences in the rates of $^{\bullet}OH$ production in the presence of AA was assessed by testing the slopes of the regression lines for parallelism using the analysis of covariance (ANCOVA) [44]. Statistical differences in dopaminergic lesions were tested using one-way ANOVA followed by a post-hoc Tukey's test. Normality of populations and homogeneity of variances were tested before each ANOVA. The accepted level of significance in all cases was $P < 0.05$.

3. Results

The formation of $^{\bullet}OH$ during 6-OHDA autoxidation was followed fluorimetrically and the maximum value of the relative fluorescence (ΔF_{max}) after 4 min of reaction was used to quantify the production of $^{\bullet}OH$. As shown in Fig. 1, the autoxidation of 6-OHDA (5 μM) caused an immediate generation of $^{\bullet}OH$ according to a hyperbolic kinetics. The O_2 consumption during the autoxidation of 6-OHDA was followed polarographically and the H_2O_2 accumulation was calculated from the polarographic recording obtained with the addition of CAT (2000 units) after 216 s of reaction and taking into account the stoichiometry of the reaction catalyzed by CAT. As il-

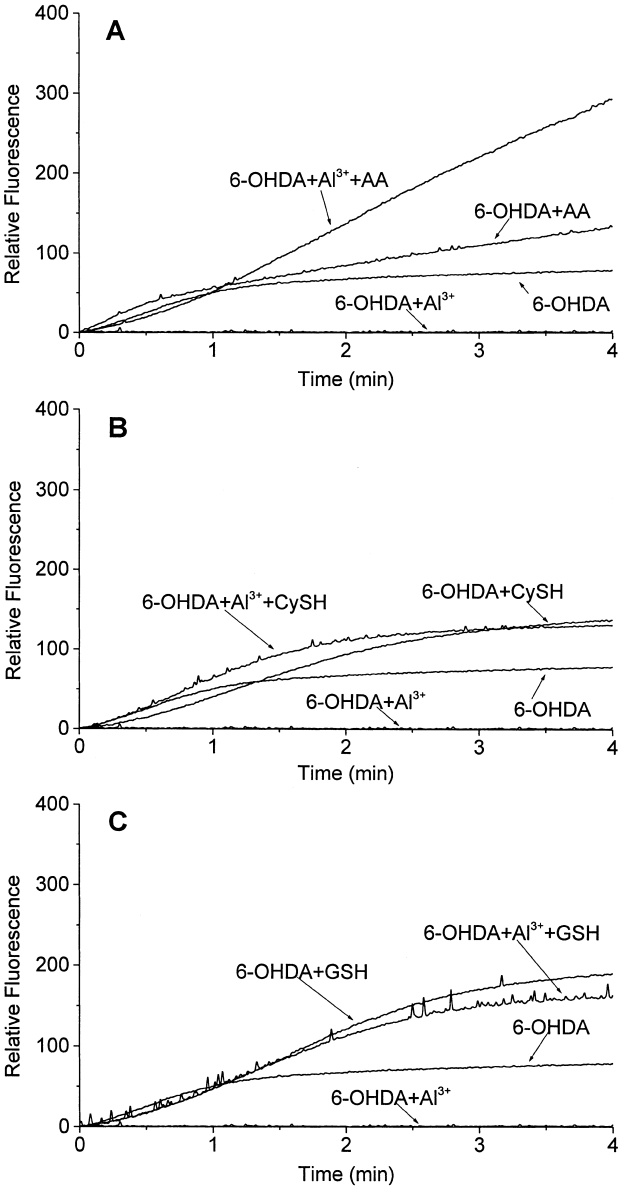


Fig. 1. Effects of the presence of Al^{3+} on $^{\bullet}OH$ formation during 6-OHDA autoxidation in the absence and presence of: (A) ascorbic acid (AA), (B) L-cysteine (CySH), and (C) glutathione (GSH). Incubations were carried out in 25 mM phosphate buffer (pH 7.5) at 37°C. Concentration of reagents was as follows: 6-OHDA, 5 μM; Al^{3+}, 800 nM; antioxidant, 100 μM. $^{\bullet}OH$ formation is indicated by the detection of fluorescence using THA as a chemical dosimeter.

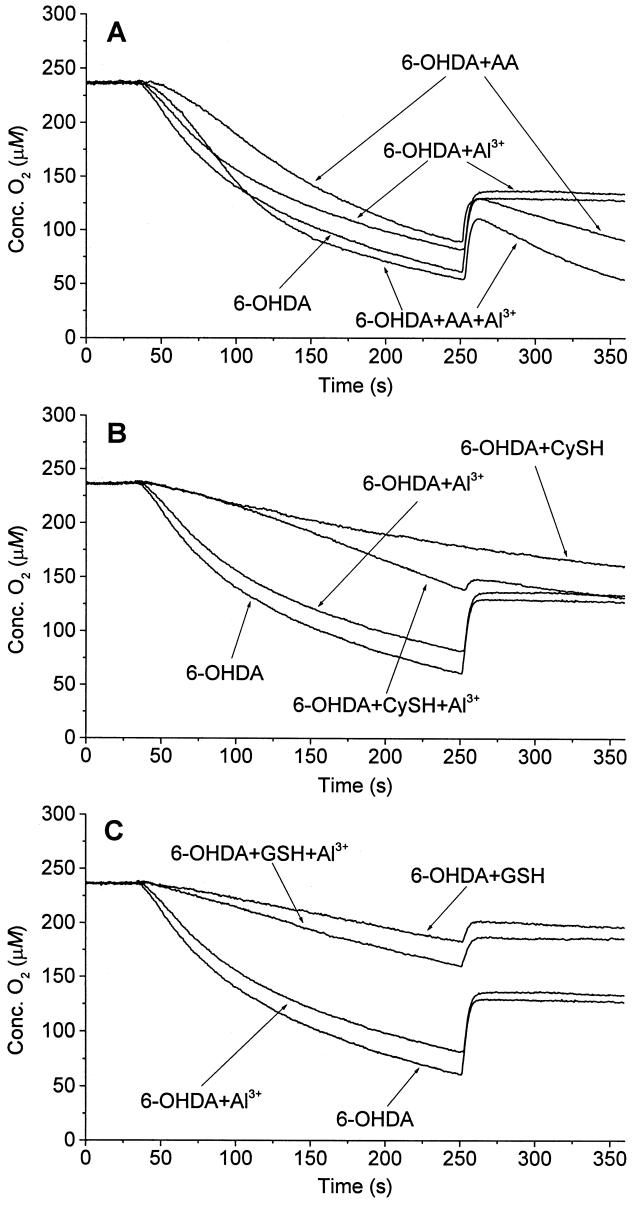


Fig. 2. Polarographic monitoring of the effects of the presence of Al^{3+} on oxygen consumption during 6-OHDA autoxidation in 25 mM phosphate buffer (pH 7.5) in the absence and presence of: (A) ascorbic acid (AA), (B) L-cysteine (CySH), and (C) glutathione (GSH). 2000 μmol/min of CAT were added after 216 s of reaction. Concentrations in the reaction mixture were as follows: 6-OHDA, 400 μM; Al^{3+}, 64 μM; antioxidant, 8 mM.

lustrated in Fig. 2, the autoxidation of 6-OHDA (400 μM) was accompanied by a significant consumption of O_2 and a considerable production of H_2O_2 (Fig. 2). Table 1 shows the corresponding values obtained during the autoxidation of 6-OHDA (control values).

In order to achieve the best analytical response, different concentrations of reagents were used in fluorimetric, polarographic, and spectrophotometric assays. However, the same relationship between concentrations was used in each analytical method for statistical comparison of the results.

For a better understanding of the effect of each metal ion on $^{\bullet}OH$ production by 6-OHDA autoxidation in the presence of an antioxidant, we previously studied the potential effects of the assayed antioxidants on the mentioned process. As shown in Fig. 1A, the autoxidation of 6-OHDA (5 μM) in the presence of AA (100 μM) caused a continuous production of $^{\bullet}OH$, which continued for at least 30 min (data not shown). In this case, the amount of $^{\bullet}OH$ generated involved an increase of 72% when compared to the corresponding control value (Table 1). A significant visual diminution in O_2 consumption and a significant reduction (−42%) in H_2O_2 accumulation were also observed during the autoxidation of 6-OHDA (400 μM) in the presence of AA (8 mM) (Table 1). Although the presence of a sulfhydryl reductant (CySH and GSH) also caused a significant increase in the amount generated of $^{\bullet}OH$ (+75% for CySH and +143% for GSH), in this case the kinetics of $^{\bullet}OH$ production maintained its hyperbolic shape (Fig. 1B,C). This phenomenon was accompanied by a significant visual reduction in O_2 consumption and a drastic reduction in H_2O_2 accumulation (−100% for CySH and −60% for GSH) (Fig. 3B,C, Table 1).

As shown in Fig. 1, the presence of Al^{3+} (800 nM) drastically reduced (−91%) the production of $^{\bullet}OH$ during 6-OHDA (5 μM) autoxidation (Table 1). This effect was accompanied by both a considerable reduction in O_2 consumption (Fig. 2) and a significant decrease (−22%) in H_2O_2 accumulation during the autoxidation of 6-OHDA (400 μM) in the presence of Al^{3+} (64 μM) (Table 1). As illustrated in Fig. 1A, when the autoxidation of 6-OHDA takes place under the simultaneous presence of AA+Al^{3+}, a marked increase (+257%) in $^{\bullet}OH$ production was observed (Table 1). At this point it is important to remark that the slope of the continuous $^{\bullet}OH$ production observed after the first minute of the reaction with 6-OHDA+Al^{3+}+AA (slope = 78.5 ± 0.37, r = 0.998) was significantly ($P < 0.001$) higher than that obtained with 6-OHDA+AA (slope = 29.4 ± 0.40, r = 0.986), which represents an important increase

in the rate of •OH production. This effect was accompanied by a very slight reduction in O_2 consumption but a significant decrease (−19%) in H_2O_2 accumulation. As shown in Fig. 1B,C, the simultaneous presence of a sulfhydryl reductant and Al^{3+} also caused a significant increase (+66% for CySH and +126% for GSH) in •OH production during 6-OHDA autoxidation, but in both cases the kinetics maintained its hyperbolic shape. With regard to the accumulation of H_2O_2, a drastic reduction (−88% for CySH and −74% for GSH) was observed for both antioxidants (Fig. 2B,C, Table 1). As illustrated in Fig. 2B,C, this effect were also accompanied by a significant reduction in O_2 consumption.

The presence of Zn^{2+} (800 nM) during the autoxidation of 6-OHDA (5 μM) induced a significant reduction in the •OH production (−48%), maintaining the shape of the 6-OHDA kinetics (Fig. 3). In addition, the autoxidation of 6-OHDA (400 μM) in the presence of Zn^{2+} (64 μM) caused a notable diminution in O_2 consumption (Fig. 4) and a significant reduction (−62%) in H_2O_2 accumulation (Table 1). The effect achieved with Zn^{2+} in the presence of AA was a marked increase (+141%) in •OH generation, which maintained the continuity of the production during the autoxidation of 6-OHDA (Fig. 3A). A significant ($P < 0.01$) increase in the rate of •OH

Table 1
Effects of the presence of Al^{3+} and Zn^{2+} and/or an antioxidant on the production of •OH[a] and the formation of H_2O_2[b] during 6-OHDA autoxidation

Incubation system	ΔF_{max}(a.u.)	H_2O_2 (nmol)
6-OHDA (control)	78.7 ± 3.21	347 ± 22.8
6-OHDA+AA	135 ± 14.0***	202 ± 13.4***
6-OHDA+CySH	138 ± 7.25***	N.D.
6-OHDA+GSH	191 ± 5.82***	139 ± 11.1***
6-OHDA+Al^{3+}	6.91 ± 0.33***	270 ± 9.35**
6-OHDA+Zn^{2+}	40.5 ± 2.22***	131 ± 8.09***
6-OHDA+AA+Al^{3+}	281 ± 5.81***	281 ± 15.1***
6-OHDA+AA+Zn^{2+}	190 ± 9.89***	309 ± 17.3*
6-OHDA+CySH+Al^{3+}	131 ± 3.11***	43.4 ± 2.79***
6-OHDA+CySH+Zn^{2+}	142 ± 2.09***	27.0 ± 5.11***
6-OHDA+GSH+Al^{3+}	178 ± 7.23***	91.5 ± 7.42***
6-OHDA+GSH+Zn^{2+}	225 ± 3.71***	34.0 ± 2.78***

Data are means ± S.E.M. from four determinations. N.D. means not detected. Statistical significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (two-sided Student's *t*-test).

[a]Incubations were carried out in 25 mM phosphate buffer (pH 7.5) at 37°C and the concentration of reagents was as follows: 6-OHDA, 5 μM; metal ion, 800 nM; antioxidant, 100 μM.

[b]Incubations were carried out in 25 mM phosphate buffer (pH 7.5) at 25°C, 2000 μmol/min CAT were added after 216 s of reaction, and the concentration of reagents was as follows: 6-OHDA, 400 μM; metal ion, 64 μM; antioxidant, 8 mM.

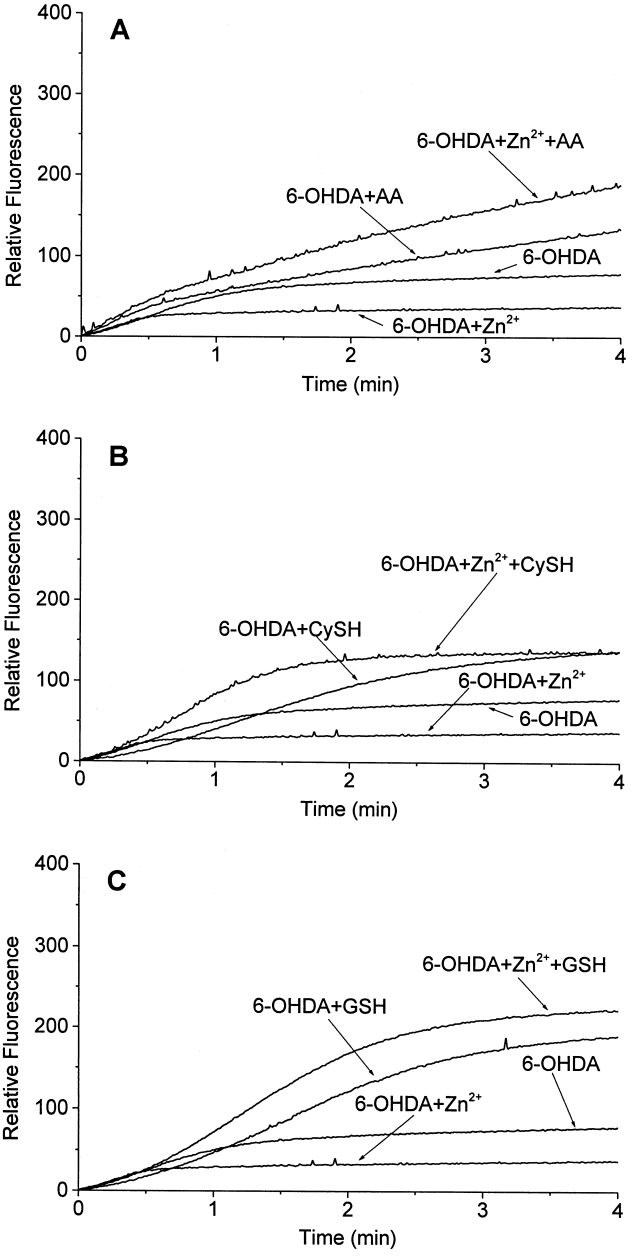


Fig. 3. Effects of the presence of Zn^{2+} on •OH formation during 6-OHDA autoxidation in the absence and presence of: (A) ascorbic acid (AA), (B) L-cysteine (CySH), and (C) glutathione (GSH). Incubations were carried out in 25 mM phosphate buffer (pH 7.5) at 37°C. Concentration of reagents was as follows: 6-OHDA, 5 μM; Zn^{2+}, 800 nM; antioxidant, 100 μM. •OH formation is indicated by the detection of fluorescence using THA as a chemical dosimeter.

production after the first minute of the reaction (slope = 43.6 ± 0.26, $r = 0.990$) was also observed. The reported effect also involved a slight increase in O_2 consumption and a slight but significant reduction (−11%) in H_2O_2 accumulation (Fig. 4A, Table

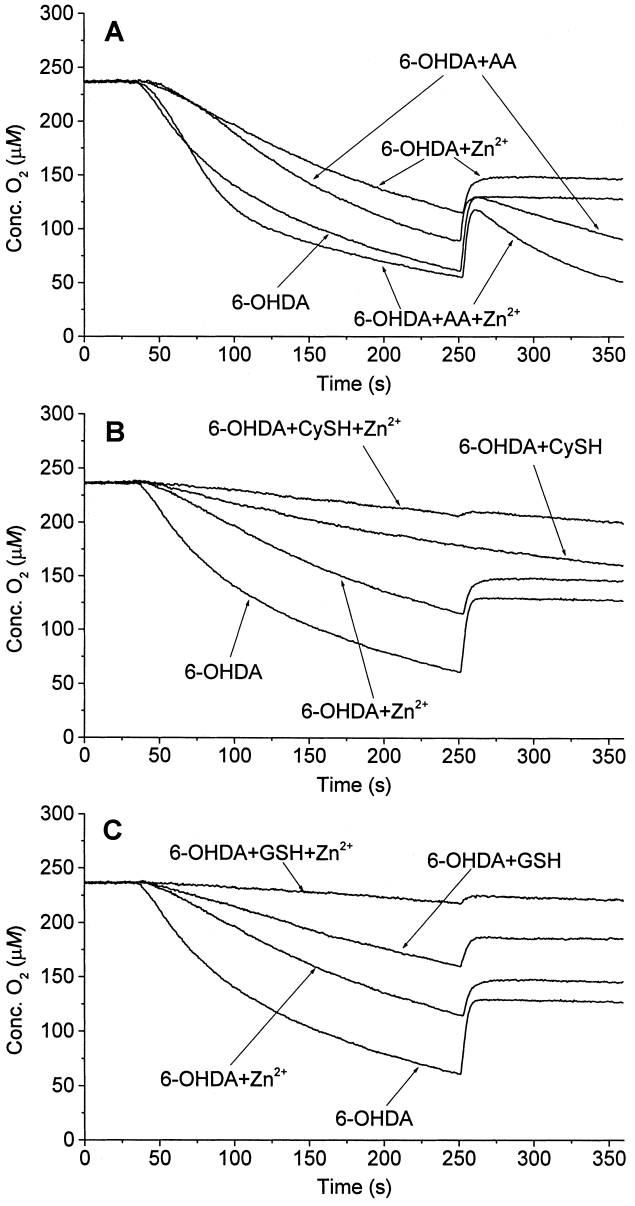


Fig. 4. Polarographic monitoring of the effects of the presence of Zn^{2+} on oxygen consumption during 6-OHDA autoxidation in 25 mM phosphate buffer (pH 7.5) in the absence and presence of: (A) ascorbic acid (AA), (B) L-cysteine (CySH), and (C) glutathione (GSH). 2000 μmol/min of CAT were added after 216 s of reaction. Concentrations in the reaction mixture were as follows: 6-OHDA, 400 μM; Zn^{2+}, 64 μM; antioxidant, 8 mM.

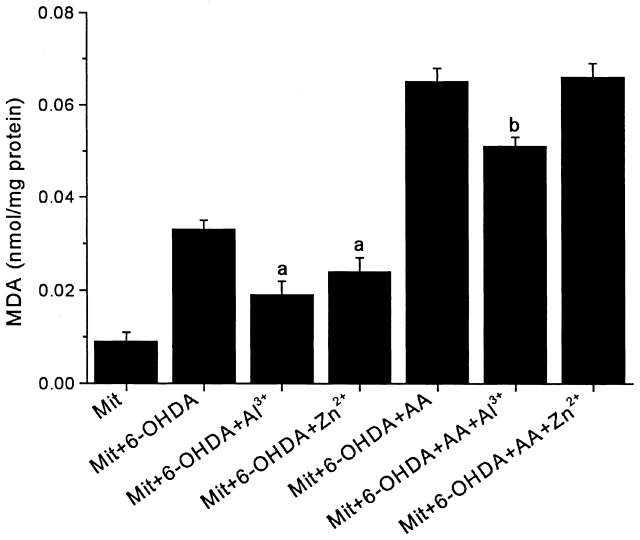


Fig. 5. Influence of Al^{3+} and Zn^{2+} in the presence and absence of ascorbic acid (AA) on TBARS production by 6-OHDA autoxidation on mitochondria preparations from rat brain. Mitochondria (1 mg protein/ml) were incubated in Na_2PO_4/KH_2PO_4 buffer isotonized with KCl (pH 7.4) with the corresponding chemicals for 10 min at 37°C. Reagent concentrations: 6-OHDA, 10 μM; Al^{3+} and Zn^{2+}, 1.6 μM; AA, 200 μM. Malondialdehyde (MDA) was used to quantify TBARS formation. Bars represent means ± S.D. from four independent experiments. Statistical significance (two-sided Student's *t*-test): $^{a}P < 0.05$ when compared with Mit+6-OHDA (control); $^{b}P < 0.05$ when compared with Mit+6-OHDA+AA (control).

1). When the antioxidant used was a sulfhydryl substance, the effect observed with Zn^{2+} was a significant augmentation (+80% for CySH and +186% for GSH) in $^{\bullet}OH$ production (Fig. 3B,C, Table 1) and a marked reduction (−92% for CySH and −90% for GSH) in H_2O_2 accumulation (Fig. 4B,C, Table 1). As can be seen in Fig. 4B,C, this effect was also accompanied by a considerable diminution in O_2 consumption.

The incubation of 6-OHDA (10 μM) with brain mitochondria at 37°C for 10 min induced a significant production of TBARS (Fig. 5), which was markedly increased (+97%) when the autoxidation of 6-OHDA took place in the presence of AA (100 μM). As shown in Fig. 5, a significant reduction (−42%) in TBARS production was observed when the autoxidation of 6-OHDA occurred in the presence of Al^{3+} (1.6 μM). However, the observed reduction was lower (−22%) in the presence of AA when compared with the results obtained with 6-OHDA+AA. The presence of Zn^{2+} (1.6 μM) also caused

a significant diminution (−27%) in TBARS formation during 6-OHDA autoxidation (Fig. 5). However, when the autoxidation of 6-OHDA occurred in the presence of AA, Zn^{2+} did not modify significantly the production of TBARS.

In the in vivo experiments, in group A rats, both right and left striata showed a dense TH immunoreactivity, where only the needle track could be distinguished, with no significant additional lesion. No significant differences were observed between the striatum injected with Al^{3+} alone and the striatum injected with saline (Fig. 6A). In group B rats both the right and left striata showed an extensive loss of TH immunoreactivity (i.e. loss of nigrostriatal dopaminergic terminals) around the injection site (Fig. 6B). However, the striatal area of dopaminergic denervation induced by injection of 6-OHDA+Al^{3+} (i.e. right striatum) was statistically significantly less than that observed after injection of 6-OHDA+saline (i.e. left striatum; Fig. 7). It is interesting to remark that in this study, comparison of lesions on the left and right striatum of the same rats minimizes the risk of influence of possible additional factors on the results.

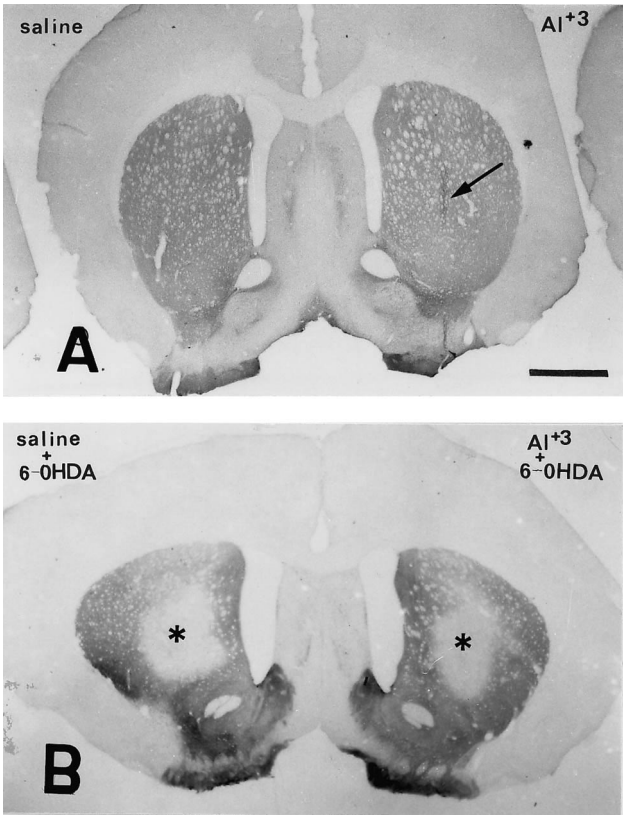


Fig. 6. Microphotographs of tyrosine hydroxylase-immunostained (TH-ir; gray) sections through the striatum of a group A rat (A) and a group B rat (B). Group A rats (A) were injected with saline into the left striatum (control) and with saline+Al^{3+} into the right striatum. No significant lesion was observed surrounding the needle track (arrow). Group B rats (B) were injected with saline+6-OHDA into the left striatum and with Al^{3+}+6-OHDA into the right striatum. Note a clear loss (white, asterisks) of TH-ir (i.e. dopaminergic terminals; gray) in the striatal area surrounding the injection site. Scale bar = 2 mm.

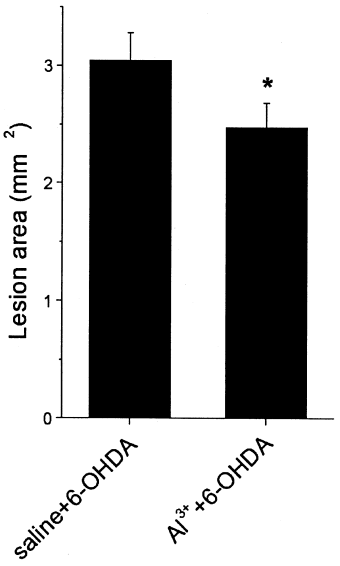


Fig. 7. TH-negative area (i.e. complete loss of dopaminergic terminals; mm^2) surrounding the injection site in group B rats. The values obtained from the left striatum (i.e. the side injected with saline+6-OHDA) were significantly higher than those obtained from the right striatum (i.e. the side injected with Al^{3+}+6-OHDA). Data are mean ± S.E.M. ($n = 5$) and the asterisk indicates significant differences (one-way ANOVA followed by post-hoc Tukey's test; $P < 0.05$).

4. Discussion

The results of this study have shown that, under physiological conditions of pH and temperature, 6-OHDA suffers autoxidation to give H_2O_2 and $^{\bullet}OH$. It is important to point out that the formation of $^{\bullet}OH$ only occurs within the first 2 min of the reaction, which appears to demonstrate that this process is not a consequence of a Fenton-type reaction on the H_2O_2 formed. This suggestion is supported by the fact that the formation of $^{\bullet}OH$ is negligible after the first 2 min of the reaction, when the concentra-

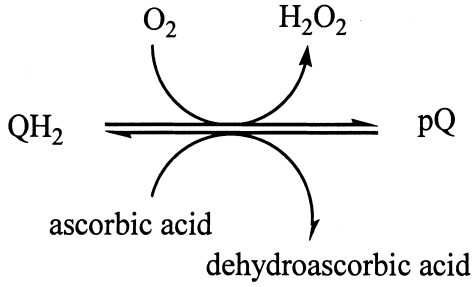
tion of H_2O_2 is still considerable. Furthermore, this fact also seems to demonstrate that the generation of $^{\bullet}OH$ in the absence of a transition metal occurs through the involvement of a compound that is present only at the initiation of 6-OHDA autoxidation. The major candidate to explain this process is the reaction suggested by Gee and Davison [8] in which the reduced form of 6-OHDA (QH_2) is oxidized by the H_2O_2 previously formed to give the semiquinone radical ($^{\bullet}sQ$) and $^{\bullet}OH$:

$$QH_2 + H_2O_2 \rightarrow {}^{\bullet}sQ + {}^{\bullet}OH + OH^-$$

Then, $^{\bullet}sQ$ may be oxidized by molecular oxygen to yield the corresponding *p*-quinone (pQ) and a superoxide radical ($O_2^{\bullet-}$):

$$^{\bullet}sQ + O_2 \rightarrow pQ + O_2^{\bullet-}$$

The presence of AA during 6-OHDA autoxidation induces a redox cycle, which causes a continuous production of $^{\bullet}OH$ to AA exhaustion:



Clearly, this fact contributes to interpreting the suggested role of AA in the neurotoxicity of 6-OHDA [36]. However, the presence of a sulfhydryl reductant (CySH or GSH) only induces an increase in $^{\bullet}OH$ production within the first 3 min of the reaction, which precludes the existence of a redox cycle in the presence of such antioxidants. Evidently, this observation also agrees with previous work showing a protective effect of sulfhydryl reductants against the neurotoxicity of 6-OHDA [36].

The presence of Al^{3+} causes a drastic diminution in $^{\bullet}OH$ production during 6-OHDA autoxidation, which is also accompanied by a significant reduction in both O_2 consumption and H_2O_2 accumulation. Taking into account the fixed oxidation number of Al^{3+} and consequently its inability to participate in redox reactions, the only possible mechanism which could integrate or explain the results of this study would be the binding of Al^{3+} to 6-OHDA, an eventuality which would hinder the interaction between 6-OHDA and H_2O_2:

$$QH_2 + Al^{3+} \rightarrow QH_2{\cdot}Al^{3+}$$

$$QH_2{\cdot}Al^{3+} + H_2O_2 \rightarrow {}^{\bullet}sQ{\cdot}Al^{3+} \text{ (or } {}^{\bullet}sQ + Al^{3+}) +$$

$$^{\bullet}OH + OH^- \text{ (slow)}$$

However, the combined action of Al^{3+} and AA causes a marked increase in $^{\bullet}OH$ production during 6-OHDA autoxidation, which is accompanied by a slight augmentation in O_2 consumption and a significant reduction in H_2O_2 accumulation. These findings show how the presence of AA not only inhibits the effect caused by Al^{3+} on $^{\bullet}OH$ production, but also even significantly activates the production of $^{\bullet}OH$ during 6-OHDA autoxidation. Probably, the molecular mechanism implicated in this process might involve a catalytic effect of Al^{3+} on the interaction between AA and 6-OHDA or the *p*-quinone. Considering that the concentration of AA in brain tissue is in the mM order [45] and that oxidative stress has been shown to facilitate the accumulation of Al^{3+} in rat brain synaptosomes [46], this effect would explain some stressor properties reported for this metal [20,47]. However, the combined action of Al^{3+} and a sulfhydryl reductant (CySH or GSH) only exhibited a slight reduction in $^{\bullet}OH$ production when compared to the effect induced by such reductants in the absence of the metal ion. In addition, once again the formation of $^{\bullet}OH$ only takes place within the first 3 min of the reaction. Clearly, Al^{3+} does not contribute greatly to the oxidative stress induced by 6-OHDA when this process occurs in the presence of a sulfhydryl reductant such as CySH and GSH. Evidently, this fact appears to support a neuroprotective function for sulfhydryl reductants, particularly CySH, which may explain the reported increase in the γ-glutamyltranspeptidase activity found in the substantia nigra of patients suffering from PD [48].

Although the reduction induced by the presence of Al^{3+} on TBARS production by 6-OHDA autoxidation in mitochondria preparations appears to agree with the diminution observed in $^{\bullet}OH$ formation, the reduction on TBARS production in the presence of AA contrasts with the increase observed under these conditions in $^{\bullet}OH$ formation. Evidently, this fact appears to agree with the lack of correlation observed

by other authors [49]. However, assuming that (a) Al^{3+} can associate to phosphate groups and accumulate in membranes due to its low exchange rate [50] and (b) lipid peroxidation occurs in negatively charged micelles and not in positively charged micelles [51], the here reported reduction of lipid peroxidation in the presence of Al^{3+} appears to be a consequence of the expected diminution of negative charge density provoked by the association of this metal ion to membranes. Evidently, our findings contrast with the reported increase in Fe^{2+}-induced lipid peroxidation by Al^{3+} [52,53], a fact that might be related to a potential interaction between Fe^{2+} and Al^{3+} and not to an increase in the rate of propagation of lipid peroxidation caused by alterations of lipid packing in membrane. Although Al^{3+} is an acidic metal that at physiological pH seems to be in the form of poly-hydrated hydroxides [54]:

$$Al^{3+}(aq) + H_2O \rightleftharpoons [Al(OH)^{2+}(aq) + H^+$$

$$K_a = 1.12 \times 10^{-5}$$

the presence of the negatively charged phospholipids of the membrane together with the high capacity shown by the phosphate group to bind Al^{3+} [55] will shift this hydrolysis equilibrium to the left, as it has been previously suggested by Verstraeten et al. [56].

Stereotaxic injection of 6-OHDA into the medial forebrain bundle or substantia nigra has been used for years to produce a rodent model of PD. Dopaminergic axons and neuron bodies are densely packed within these structures, and this injection originates a complete, or almost complete, destruction of the nigral dopaminergic neurons and their terminals in the striatum, producing a model for the end stage of PD [5]. However, this model has limited utility for research on neuroprotection or neurotoxicity in the nigrostriatal system. More recently, a partial lesion model has been developed, which is particularly useful for research on neuroprotection and neurotoxicity in PD [6,7]. In this model a small volume of 6-OHDA is injected into the striatum, which is a much larger structure, and selectively eliminates the dopaminergic axons and terminals within a circumscribed area surrounding the injection site, followed by a degeneration of the parent nigral neurons. Intrastriatal injection of 6-OHDA causes rapid degeneration of nigrostriatal terminals within as early as 24 h, and the loss of TH immunoreactivity increases for up to 5 days following the lesion [57]. In the present in vivo study, the quantification of the lesion a week after 6-OHDA injections reflects, therefore, the long-term damage of the dopaminergic terminals [8]. In the present work we have made an immunohistochemical study of the lesion induced in the striatal dopaminergic terminals after injection of 6-OHDA, 6-OHDA+Al^{3+}, Al^{3+} alone, or saline alone. The results obtained in this study showed reduction in the nigrostriatal 6-OHDA-induced lesion in the presence of Al^{3+}, as well as a lack of significant striatal lesion after injection of Al^{3+} alone, which agrees with the reported protection of Al^{3+} against the lipid peroxidation induced by 6-OHDA on mitochondria preparations from rat brain.

The presence of Zn^{2+} causes a significant reduction in the production of $^{\bullet}OH$ during 6-OHDA autoxidation, which was also accompanied by an important reduction in both O_2 consumption and H_2O_2 accumulation. Although the effects caused by this metal ion are less important in relation to those achieved with Al^{3+}, the observed behavior was very similar, which agrees with the fact that Zn^{2+} is another non-redox cation. At this point, it is important to note that a light inhibition of dopamine autoxidation in the presence of Zn^{2+} was previously reported by other authors [58]. Once again, the combined action of AA and Zn^{2+} also induced a significant increase in $^{\bullet}OH$ production, which was greater than that induced by AA in the absence of zinc. Thus, although Zn^{2+} might act as an antioxidant preventing the formation of $^{\bullet}OH$ during 6-OHDA autoxidation, the guaranteed presence of a relatively high concentration of AA in the brain [45] may contribute to enhance the oxidative stress caused by 6-OHDA. Furthermore, this phenomenon is reinforced by the reported mobilization of Zn^{2+} from metallothionein under conditions of oxidative stress [59]. Obviously, the reported ability of Zn^{2+} to increase the production $^{\bullet}OH$ by 6-OHDA in the presence of AA is particularly negative in relation to the development of PD because an increase in the brain levels of this metal has been reported in patients suffering this disorder [60].

Once again, although the reported reduction exhibited by the presence of Zn^{2+} on TBARS formation by 6-OHDA autoxidation on mitochondria preparations agrees with the diminution observed in $^{\bullet}OH$ production, it contrasts with the nonsignificant effect observed in TBARS production in the presence of AA which was accompanied by an increase in $^{\bullet}OH$ production. The explanation of these findings may be the same as those given for Al^{3+}, but in this case the protection of Zn^{2+} against lipid peroxidation is not so important as that observed with the presence of Al^{3+}, which perhaps may be related to the fact that Zn^{2+} is a divalent ion with a charge density ($z/r = 2.74$) lower than that of Al^{3+} ($z/r = 5.56$).

In summary, it is important to note that the data reported here show for the first time how Al^{3+} in the presence of AA contributes to enhance the production of $^{\bullet}OH$ by the autoxidation of 6-OHDA, but also reduces the lipid peroxidation caused by 6-OHDA autoxidation in a mitochondria preparation from rat brain. Furthermore, the presence of Al^{3+} is able to reduce the extension of the lesions induced by 6-OHDA in the nigrostriatal system of the rat. Thus, taking into account our results and the suggested contribution of Al^{3+} accumulation in the brain to the pathogenesis of PD [16,17,23,24], this latter fact could be a long-term consequence of the here reported augmentation of $^{\bullet}OH$ production by Al^{3+} during 6-OHDA autoxidation, but not related to the enhancement of lipid peroxidation, at least in the absence of high concentrations of free Fe^{2+}. Evidently, further investigations will be performed in order to corroborate this hypothesis. The effects induced by the presence of Zn^{2+} are similar to those reported by Al^{3+} but much less significant, which appears to show that the reported antioxidant properties of Zn^{2+} might be related to the induction of Zn^{2+}-containing proteins involved in the defence against oxidative stress. Finally, our results have also shown that the presence of sulfhydryl reductants, particularly CySH, might contribute to the reduction in the potential oxidative stress caused by the combined action of 6-OHDA and AA.

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