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Effects of (−)-nicotine and (−)-cotinine on 6-hydroxydopamine-induced oxidative stress and neurotoxicity: relevance for Parkinson's disease

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Abstract

In view of the apparent controversial properties of (−)-nicotine (NIC) in relation to both oxidative stress and neuroprotection, we studied the effects of NIC on hydroxyl radical (•OH) formation, oxidative stress production by 6-hydroxydopamine (6-OHDA) autoxidation in the presence and absence of ascorbate, and 6-OHDA neurotoxicity. Both NIC and (−)-cotinine (COT) exhibited increased •OH production during 6-OHDA autoxidation. Although the same effect was observed in •OH generation by the Fenton reaction ($H_2O_2 + Fe^{2+}$), this reaction was completely prevented with the previous incubation of Fe^{2+} with NIC or COT. Furthermore, both NIC and COT demonstrated a capacity to be able to reduce the TBARS formation provoked in rat brain mitochondrial preparations by 6-OHDA autoxidation. This effect is assumed as a consequence of the action of NIC and COT on lipid peroxidation propagation. We treated with NIC (1 mg/kg, i.p.) two 6-OHDA-induced rat models of Parkinson's disease. However, only in one of these models did we obtain clear evidence of a neuroprotective effect of NIC on nigrostriatal terminals, as revealed by immunohistochemistry against tyrosine hydroxylase. Thus, the antioxidant properties of both NIC and COT in relation to the lipid peroxidation induced by 6-OHDA autoxidation, together with their reported capacity to prevent the Fenton reaction, probably by sequestration of Fe^{2+}, may contribute to an understanding of its neuroprotective properties. In addition, the reported capacity of both NIC and COT to increase the production of •OH by 6-OHDA autoxidation might help explain the controversial observation found under different experimental conditions.

Keywords: (−)-Nicotine; (−)-Cotinine; 6-Hydroxydopamine; Oxidative stress; Dopaminergic neurotoxicity; Parkinson's disease

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder which primary neurological features include resting tremor, rigidity, and bradykinesia. The disease is characterized by a loss of dopaminergic neurons located in the pars compacta of the substantia nigra [1], which causes a profound dopamine depletion in the striatum [2]. This fact is responsible for most of the motor symptomatology of the disease. Although the cause of the cellular destruction in PD remains unclear, it is now often attributed to a variety of causes: the action of endogenous neurotoxins or xenobiotics which act on the mitochondrial respiratory chain by depleting the production of ATP; to the oxidative stress caused by an excessive production of free radicals or to an alteration in protective antioxidant systems. With regard to oxidative stress, several studies have evidenced certain biochemical changes in the brain of PD patients which appear to be a consequence of this phenomenon [3–5]. However, it is not yet clear whether oxidative stress is actually an etiological factor in PD or a secondary phenomenon which occurs subsequent to an alternate process.

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Abbreviations: PD, Parkinson's disease; 6-OHDA, 6-hydroxydopamine; *p*Q, *p*-quinone of 6-OHDA; •sQ, semiquinone radical of 6-OHDA; •OH, hydroxyl radical; NIC, (−)-nicotine; COT, (−)-cotinine; Asc, ascorbate; DHAsc, dehydroascorbate; CAT, catalase; THA, terephthalic acid; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; KPBS-BSA, potassium phosphate-buffered saline containing bovine serum albumin; TH, tyrosine hydroxylase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

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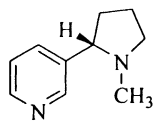
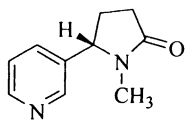
Several epidemiological studies have shown a reduction in the incidence of PD in smokers compared with non-smokers [6–10]. This repeated finding has stimulated investigation into the mechanisms responsible for such a protective effect. (−)-Nicotine (NIC) is the major alkaloid present in *Nicotiana tabacum* and consequently an important constituent of cigarette smoke. The major route of NIC metabolism in humans involves C-oxidation by the isoenzyme P4502A6 to form (−)-cotinine (COT) [11], which is the primary metabolite of NIC into the brain [12]. *In vivo* and *in vitro* studies on the neuroprotective effects of NIC remain controversial: several *in vivo* experiments have reported antioxidant properties of NIC [13,14], which might explain the protective effects of smoking against the development of PD, while other studies have reported that NIC induces *in vivo* oxidative stress in different tissues [15,16]. Similarly, administration of NIC has been reported to reduce, or to enhance, or to have no influence upon nigrostriatal degeneration in animal models of PD [13,17–19]. Thus, it could be possible that only NIC-independent mechanisms were responsible for the protective effects of smoking against PD [20–23].



(-)-nicotine



(-)-cotinine

6-Hydroxydopamine (6-OHDA) is a neurotoxin widely used in experimental studies on the pathogenesis of PD. Furthermore, the reported presence of 6-OHDA in both human brain [24] and urine of parkinsonian patients [25] has led to consider this substance as a putative neurotoxic factor in relation to PD [26,27]. As is well-known, under physiological conditions 6-OHDA is rapidly oxidized by molecular oxygen to give hydrogen peroxide (H_2O_2) and the corresponding *p*-quinone. The *p*-quinone then undergoes a cascade of oxidative reactions which finally results in the formation of an insoluble polymeric pigment related to neuromelanin [28,29]. Evidently, the H_2O_2 resulting from the autoxidation of 6-OHDA may generate hydroxyl radicals ($^{\bullet}OH$) through a metal-catalyzed Fenton-type reaction. Although the precise molecular mechanism of neurotoxicity of 6-OHDA remains uncertain, its neurotoxicity has been often related to the oxidative stress caused by the production of $^{\bullet}OH$ during its autoxidation [30–34]. However, its has also been reported that 6-OHDA can act directly by inhibiting the mitochondrial respiratory chain at the level of complex I [35,36]. The stereotaxic injection of 6-OHDA into the medial forebrain bundle or substantia nigra has been long used to produce animal models of PD [37]. However, this standard model gives rise to a complete or almost complete destruction of the nigral dopaminergic neurons and their terminals in the striatum, and has limited utility for studies on neuroprotection in the nigrostriatal system. More recently, it has been demonstrated that ''partial lesion models'' are more useful for research on neuroprotection and neurotoxicity in PD. These models are 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 the 6-OHDA-derived lesion is easily detectable [38,39], or on small intraventricular injections of 6-OHDA that produce partial and bilateral degeneration of nigrostriatal terminals [40].

Thus, the aim of this study was double. Firstly, we carried out an *in vitro* study in which we investigated the effects of NIC and COT on the generation of $^{\bullet}OH$ during 6-OHDA autoxidation and their actions on the stress oxidative caused by this neurotoxin using mitochondrial preparations obtained from rat brain. In addition, and in view of the suggested ability of NIC to bind iron [41–43], the effect of both compounds on the Fenton reaction was also investigated. Secondly, we investigated *in vivo* the possible neuroprotective effect against 6-OHDA-induced damage of the nigrostriatal system using the two above-mentioned animal models of PD.

2. Materials and methods

2.1. Chemicals and reagents

(−)-Nicotine hydrogen tartrate salt, (−)-cotinine, hydrogen peroxide 30% (w/w), 6-hydroxydopamine hydrobromide, ascorbate (Asc), catalase (CAT), butylated hydroxytoluene crystalline, deferoxamine mesylate, thiobarbituric acid, 1,1,3,3-tetraethoxypropane, sodium dodecylsulfate, Triton X-100, 3,3′-diaminobenzidine, and bovine serum albumin were obtained from Sigma Chemical Co. Ferrous chloride tetrahydrate were purchased from Fluka Chemie AG. Terephthalic acid, disodium salt, was purchased from Aldrich Chemical Co. (Steinheim, Germany). Biotinylated secondary antibody and avidin–biotin–peroxidase complex were obtained from Vector. Normal swine serum was from DAKO (Glostrup, Denmark) and rabbit polyclonal antiserum against tyrosine hydroxylase was from Pel-Freez. The water used for the preparation of solutions was of Milli-RiOs/Q-A10 grade (Millipore Corp.). All remaining chemicals utilized were of analytical grade and were from Fluka Chemie AG. Stock solutions of 6-OHDA were prepared in 1 mM KCl (pH 2) to prevent its autoxidation [44]. Fresh stock solutions of iron were prepared in water immediately before each experiment.

2.2. Preparation of brain mitochondria

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 mitochondrias were then obtained by differential centrifugation with minor modifications to a previously published method [21]. Briefly, after removing blood vessels and pial membranes, the brains were manually homogenized with 4 vol (w/v) of the isolation medium. Then, the homogenate was centrifuged at 900 *g* for 5 min at 4°. The supernatant was centrifuged at 12,500 *g* for 15 min. The mitochondria pellet was then washed once with isolation medium and recentrifuged under the same conditions. Finally, the mitochondrial pellet was reconstituted in a buffer solution (Na_2PO_4/KH_2PO_4 isotonized with KCl, pH 7.4) and stored in aliquots at −40°.

The protein concentration of mitochondrial suspension was determined according to the method of Markwell *et al.* [45], using bovine serum albumin as the standard.

2.3. Determination of •OH production

The generation of •OH was fluorimetrically monitored using a modification to the method of Barreto *et al.* [46] in which THA is used as a chemical dosimeter of •OH. A luminiscence spectrometer Model LS50B (Perkin-Elmer) was used. The cuvette holder was thermostatically maintained at 37° and a magnetic stirrer was used for a continuous mixing of the sample. For •OH production during 6-OHDA autoxidation, 2000 μL of 25 mM phosphate buffer (pH 7.4) containing THA (10 mM) and an aliquot of 25 mM phosphate buffer (pH 7.4) to take the final volume of the incubation to 2.5 mL were incubated in a quartz cuvette for 5 min to reach the temperature. Then, 100 μL of NIC or COT (80 μM) and 20 μL of 6-OHDA (10 μM) were added. In some experiments, 100 μL of Asc (200 μM) were incorporated to the incubation previously to the addition of 6-OHDA. Concentrations of NIC, COT, Asc, and 6-OHDA are final concentrations in the incubation. The monitoring of •OH formation was immediately initiated and maintained for the subsequent 10 min. Wavelengths of 312 and 426 nm were used for excitation and emission, respectively.

For •OH generation from H_2O_2 in the presence of Fe^{2+} by the Fenton reaction, 2000 μL of 25 mM phosphate buffer (pH 7.4) containing THA (10 mM) and an aliquot of 25 mM phosphate buffer (pH 7.4) to take the final volume of the incubation to 2.5 mL were incubated for 5 min to reach the temperature (37°). Prior to this incubation, 100 μL of NIC or COT (320 μM) were also added to investigate the effects of these compounds on the Fenton reaction. Then, 20 μL of H_2O_2 (2 μM) were added and the recording initiated immediately. After 60 s, 20 μL of Fe^{2+} (3.2 μM) were added and the recording maintained for another 120 s. Also several incubations were carried out in which NIC or COT were incubated with Fe^{2+} and H_2O_2 added subsequently. Concentrations of H_2O_2, Fe^{2+}, NIC, and COT are final concentrations in the incubation. The instrumental conditions used were those reported above.

2.4. Determination of O_2 consumption and H_2O_2 accumulation

The O_2 consumption during 6-OHDA autoxidation was monitored polarographically according to a previously published method [34]. A Clark-type O_2-electrode (Digital Oxygen System Model 10, Rank Brothers) equipped with an amplifier, an A/D converter and the appropriate software for data acquisition and control [47] were used. The electrode was assembled in a 5-mL chamber surrounded by a thermostatic water jacket set at 37°. A polarizing voltage of +0.60 V was used. For each assay, 2380 μL of 25 mM phosphate buffer (pH 7.4) and 100 μL of NIC or COT (3.2 mM) were incubated in the electrode chamber for 5 min in order to reach the temperature and the saturating O_2 concentration. Then, 20 μL of 6-OHDA (400 μM) was injected using a Hamilton syringe (Model 702SNR). In some experiments, 2380 μL of 25 mM phosphate buffer (pH 7.4) containing Asc (8 mM) were used. Concentrations of NIC, COT, Asc, and 6-OHDA 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 25 μL of a CAT solution (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 [48].

For assess on the potential O_2-consumption during the Fenton reaction, 2460 μL of 25 mM phosphate buffer (pH 7.4) were incubated with 20 μL H_2O_2 (200 μM) during 5 min to reach equilibrium. Then, 20 μL of Fe^{2+} (320 μM) were injected using a Hamilton syringe. Two thousand units of CAT were then added to estimate the presence of H_2O_2. In order to investigate the potential effects of NIC and COT on the Fenton reaction, 100 μL of each of this compounds (3.2 mM) were incubated with 2360 μL of phosphate buffer and 20 μL H_2O_2 prior to the addition of Fe^{2+}. They have been also made incubations of NIC-(COT) with Fe^{2+} previous to the addition of H_2O_2. The instrumental conditions used were the above reported.

2.5. Determination of TBARS

Lipid peroxidation was assessed by spectrophotometrical determination of TBARS. A modification to a previous published method [49] was used as TBARS assay. To 200 μL of mitochondria preparation (1 mg protein/mL), 25 μL of NIC or COT at a concentration 320 μM or phosphate buffer (pH 7.4) were added followed by 25 μL of 6-OHDA (10 μM). In some experiments, 50 μL

of Asc (100 μM) were incorporated previously to the addition of 6-OHDA. The resulting mixture was incubated at 37° for exactly 10 min. Immediately, 25 μL of butylated hydroxytoluene (1 mM) and 25 μL desferrioxamine (1 mM) were added to prevent the amplification of the lipid peroxidation during the assay. A volume of 750 μL of acetic acid (20%) followed by 200 μL of sodium dodecylsulfate (8%, w/v) were added and the mixture vortexed for 1 min. Then, 750 μL of TBA (0.8%) were added and the resulting mixture incubated at 95° for 60 min. After cooling to room temperature, 3 mL of *n*-butanol were added and the mixture shaken vigorously. After centrifugation at 2.600 *g* for 5 min, the absorbence of the supernatant (organic layer) was measured at 532 nm using an Ultrospec III spectrophotometer (Pharmacia Biotech). For calibration, a standard curve (5–150 nM) was generated using the malondialdehyde derived by the acid hydrolysis (SO_4H_2; 1.5%, v/v) of 1,1,3,3-tetramethoypropane and the TBARS results expressed as nmol MDA/mg protein.

2.6. Animal treatment

A total of 36 adult female Sprague–Dawley rats (weighing about 200 g) were used. All experiments were carried out in accordance with the ''Principles of laboratory animal care'' (NIH Publication no. 86-23, revised 1985). The rats were divided into five groups (A–E). Rats in group A (N = 4) were used as normal (i.e. non-lesioned) controls. Rats in groups B (N = 9) and C (N = 10) were injected in the right striatum with 6 μg of 6-OHDA in 2 μL of sterile saline containing 0.2% of ascorbic acid. Rats in groups D (N = 6) and E (N = 7) were injected in the third ventricle with 300 μg of 6-OHDA in 10 μL of sterile saline containing 0.2% of ascorbic acid. However, rats in groups C and E were injected intraperitoneally with NIC (1 mg/kg) 48, 24 and 4 hr before, and 4, 24, 48 and 72 hr after 6-OHDA injection. Stereotaxic coordinates were 1.0 mm anterior to bregma, 2.7 mm right of midline, 5.5 mm ventral to the dura, and tooth bar at −3.3 for intrastriatal injections, and 0.8 mm posterior to bregma, midline, 6.5 mm ventral to the dura, and tooth bar at 0 for injections in the third ventricle. The solution was injected with a 5 μL (intrastriatal injections) or 10 μL (intraventricular) Hamilton syringe coupled to a motorized injector (Stoelting), at 0.1 μL/min (intrastriatal injections) or 0.5 μL/min (intraventricular injections) 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

One week post-lesion (groups B–E), the animals were deeply anaesthetized 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 TH-immunohistochemistry (as follows). After incubation for 1 hr 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 tyrosine hydroxylase (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% H_2O_2 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 videocamera CCD-72 (Maryland Telecommunications) and a constant illumination light table (Northern Light). In groups B and C (i.e. intrastriatal injections), the TH-negative area (i.e. complete loss of dopaminergic terminals) surrounding the injection site was measured in sections containing the needle track (i.e. the center of the lesion). In groups D and E (i.e. intraventricular injections) and controls (group A), the density of striatal dopaminergic terminals was estimated as optical density of the striatal TH-immunoreactivity. At least four sections through the central striatum of each rat were measured (both the right and left striatum), and for each section optical densities were corrected by subtraction of background as observed in the corpus callosum.

2.8. Statistical analysis

Data are expressed as means ± SEM. Differences between means were statistically evaluated using the one-way ANOVA followed by the Dunnett's test. Statistical differences in immunohistochemical data were tested using ANOVA followed by post-hoc Tukey's 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. In vitro experiments

The production of $^{\bullet}OH$ during 6-OHDA autoxidation was followed fluorimetrically using THA as a chemical dosimeter. The maximal relative fluorescence (ΔF_{max}) after 10 min of reaction was used as a parameter indicative of $^{\bullet}OH$ production. As shown in Fig. 1A, the result of this study showed that $^{\bullet}OH$ production during 6-OHDA (10 μM) autoxidation only take place within the first 2 min of reaction and according to an hyperbolic kinetics.

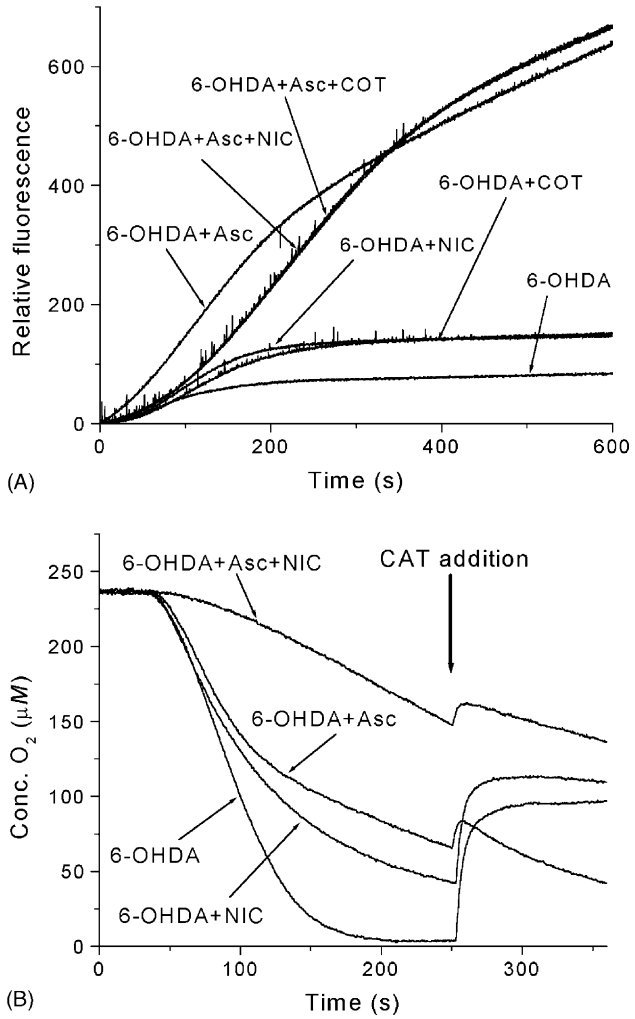


Fig. 1. Representative recordings of: (A) the effects of NIC and COT on •OH generation during 6-OHDA autoxidation in the absence and presence of Asc. Incubations were carried out in 25 mM phosphate buffer (pH 7.4) at 37°, in the following order of addition: 80 μM NIC or COT, 200 μM Asc, and 10 μM 6-OHDA. The •OH formation is indicated by the fluorescence detected using THA as a chemical dosimeter; (B) the effects of NIC on O_2 consumption and H_2O_2 accumulation during 6-OHDA autoxidation in the absence and presence of Asc. The H_2O_2 accumulated after 216 s of reaction was assessed by the addition of 2000 units of CAT. Incubations were carried out in 25 mM phosphate buffer (pH 7.4) at 25°, using the following order of addition: 3.2 mM NIC or COT, 8 mM Asc, and 400 μM 6-OHDA.

The presence of NIC and COT at a concentration 80 μM induced an augmentation in the production of •OH during 6-OHDA autoxidation of 82 and 74%, respectively (Table 1). As illustrated in Fig. 1B, the presence of NIC (3.2 mM) during 6-OHDA (400 μM) caused a significant reduction in both O_2 consumption and H_2O_2 accumulation after 216 s of reaction. Similar effects were observed with COT at the same concentration (Table 1). It is necessary to note that different concentrations of reagents were used in fluorimetric and polarographic assays for an optimal sensitivity of each method. However, the same relationship between concentrations was maintained for statistical comparison of the results obtained with both techniques.

Table 1
Effects of the presence of NIC and COT on the production of •OH and in the accumulation of H_2O_2 during 6-OHDA autoxidation in the presence and absence of ascorbate

Incubation	ΔF_{max} [a]	H_2O_2 (nmol) [b]
6-OHDA	83.8 ± 5.5	459 ± 8.8
6-OHDA + NIC	152 ± 8.3[c]	349 ± 7.4[c]
6-OHDA + COT	147 ± 6.3[c]	379 ± 10.5[c]
6-OHDA + Asc	640 ± 13.5	36.0 ± 1.8
6-OHDA + Asc + NIC	668 ± 8.9[d]	68.5 ± 3.5[d]
6-OHDA + Asc + COT	665 ± 4.4[d]	64.1 ± 4.3[d]

Values are means ± SEM from four independent experiments.

[a] The value of the relative fluorescence after 10 min of reaction was used to assess the production of •OH. Incubations were carried out in 25 mM phosphate buffer (pH 7.4) at 37° and the concentration of reagents was as follows: NIC and COT, 80 μM; Asc, 200 μM; 6-OHDA, 10 μM.

[b] Incubations were carried out in 25 mM phosphate buffer (pH 7.5) at 25°, 2000 units of CAT were added after 216 s of reaction, and the concentration of reagents was as follows: NIC and COT, 3.2 mM; Asc, 8 mM; 6-OHDA, 400 μM.

[c] Statistical significance at $P < 0.05$ (one-way ANOVA and Dunnett's test) in comparison with 6-OHDA group (control).

[d] Statistical significance at $P < 0.05$ (one-way ANOVA and Dunnett's test) in comparison with 6-OHDA + Asc group (control).

As shown in Fig. 1A, the presence of Asc (200 μM) during 6-OHDA autoxidation maintained the production •OH during all the recording time. Furthermore, a continuous production of •OH was observed for at least 30 min (data not shown). In this case, the presence of NIC or COT caused a diminution in •OH production within the first minutes of reaction. However, this lessening was then followed by a slight increase (4%) in •OH production (Table 1). The presence of Asc (8 mM) induced a slight reduction in O_2 consumption during 6-OHDA autoxidation (Fig. 1B), which also was followed by a reduction in the H_2O_2 accumulated after 216 s of reaction (Table 1).

In order to estimate the potential effect of NIC and COT on the Fenton reaction, we registered fluorimetrically the production of •OH after the addition of Fe^{2+} (3.2 μM) to H_2O_2 (2 μM). As illustrated in Fig. 2A, this experiment corroborated a very fast rate for •OH production by the Fenton reaction. The incubation of NIC and COT at a concentration 320 μM with H_2O_2 previous to the addition of Fe^{2+} caused a rise in the production of •OH equivalent to 57 and 53%, respectively (Table 1). As shown in Fig. 2B, this effect was accompanied by a significant consumption of O_2 from the incubation medium without exhibiting accumulation of H_2O_2. However, the incubation of NIC and COT with Fe^{2+} previous to the incorporation of H_2O_2 to the incubation displayed a negligible production of •OH (Fig. 2A). In this case, neither O_2 consumption nor H_2O_2 accumulation was observed (Fig. 2B).

As shown in Fig. 3, the analysis of the presence of TBARS in mitochondria preparations after 10 min of 6-OHDA (10 μM) autoxidation revealed that lipid peroxidation occurred in a significant proportion. Under these experimental conditions, the presence of NIC and COT caused a significant diminution in TBARS production. As

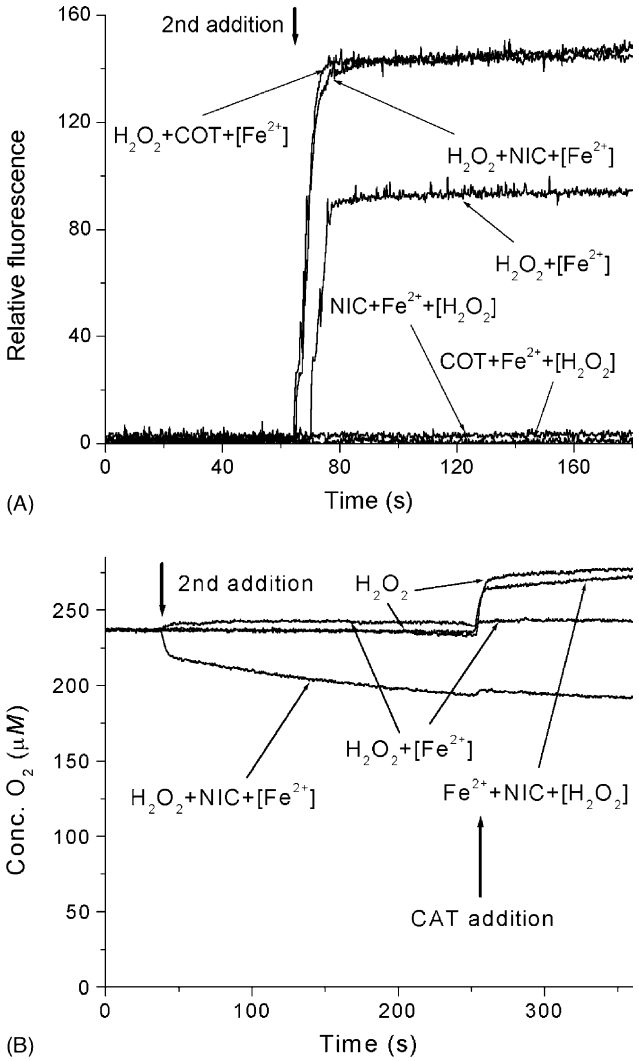


Fig. 2. Representative recordings of: (A) the effects of NIC and COT on •OH generation by Fenton reaction. Incubations were carried out in 25 mM phosphate buffer (pH 7.4) at 37°, using 320 μM NIC or COT, 3.2 mM Fe^{2+}, and 2 μM H_2O_2. The •OH formation is indicated by the fluorescence detected using THA as a chemical dosimeter; (B) the effects of NIC on O_2 consumption and H_2O_2 accumulation in the Fenton reaction. The H_2O_2 accumulated after 216 s of reaction was assessed by the addition of 2000 units of CAT. Incubations were carried out in 25 mM phosphate buffer (pH 7.4) at 25°, using 3.2 mM NIC or COT, 32 μM Fe^{2+}, and 200 μM H_2O_2. Reagents were added in order of their appearance in the figure and those represented between brackets were added at the time indicated as 2nd addition.

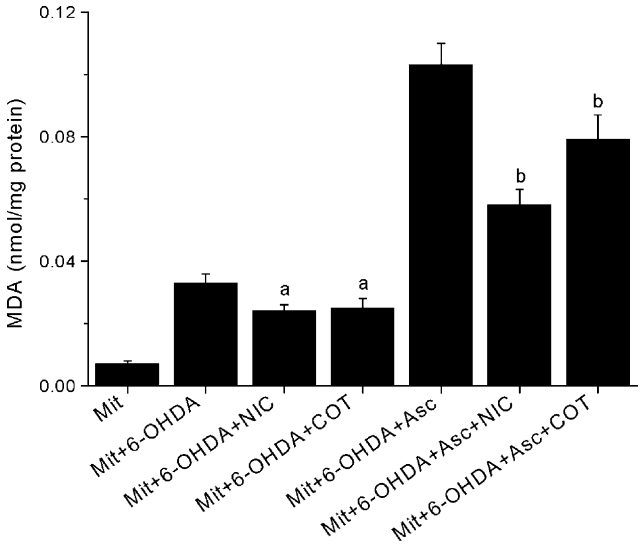


Fig. 3. Effects of NIC and COT in the absence and presence of Asc on the lipid peroxidation induced by 6-OHDA autoxidation on mitochondria preparations from rat brain. The lipid peroxidation was assessed by the formation of TBARS. Mitochondria incubations (1 mg protein/mL) were performed at 37° for 10 min, using the following concentration of reagents and order of addition: NIC and COT, 80 μM; Asc, 200 μM, and 6-OHDA. Data are means ± SEM from four independent experiments. Statistical significance at $P < 0.05$ (one-way ANOVA and Dunnett's test): a, in comparison with Mit + 6-OHDA group (control); b, in comparison with Mit + 6-OHDA + Asc group (control).

expected, the presence of Asc (100 μM) markedly stressed the production of TBARS during 6-OHDA autoxidation (Fig. 3). In this case, the presence of NIC or COT in combination with Asc caused a greater reduction in TBARS formation (48% with NIC and 23% with COT) during 6-OHDA autoxidation when compared to the results obtained with incubations containing Asc + 6-OHDA.

3.2. *In vivo experiments*

As shown in Fig. 4, control rats (i.e. not injected with 6-OHDA; group A) showed a dense and evenly distributed TH-immunoreactivity through the striatum, which indicated the presence of a dense network of nigrostriatal dopaminergic terminals. Rats subjected to intrastriatal injection of 6-OHDA (groups B and C) showed a circumscribed TH-negative area surrounding the injection site that indicated a complete loss of dopaminergic terminals. As illustrated in Fig. 5, the TH-negative area of rats treated with NIC (group C) appeared slightly less (2.65± 0.18 mm^2) than the TH-negative area of non-treated rats (group B; 3.16 ± 0.25 mm^2). However, the difference was not statistically significant.

Rats subjected to intraventricular injection of 6-OHDA (groups D and E) showed an extensive and even decrease of TH-immunoreactivity through both striata (Fig. 4). However, striatal TH-immunoreactivity of rats treated with NIC (group E) was clearly higher (around 61% of the control group) than that of non-treated rats (group D; around 24% of the control group), and this difference was statistically significant (Fig. 5).

4. Discussion

In the first stage, this study has examined the potential mechanisms by which NIC and COT may affect the oxidative stress caused by the autoxidation of 6-OHDA. Thus, it has been observed that the autoxidation of 6-OHDA generates a limited production of •OH. However, this production is enhanced by the presence of Asc, which

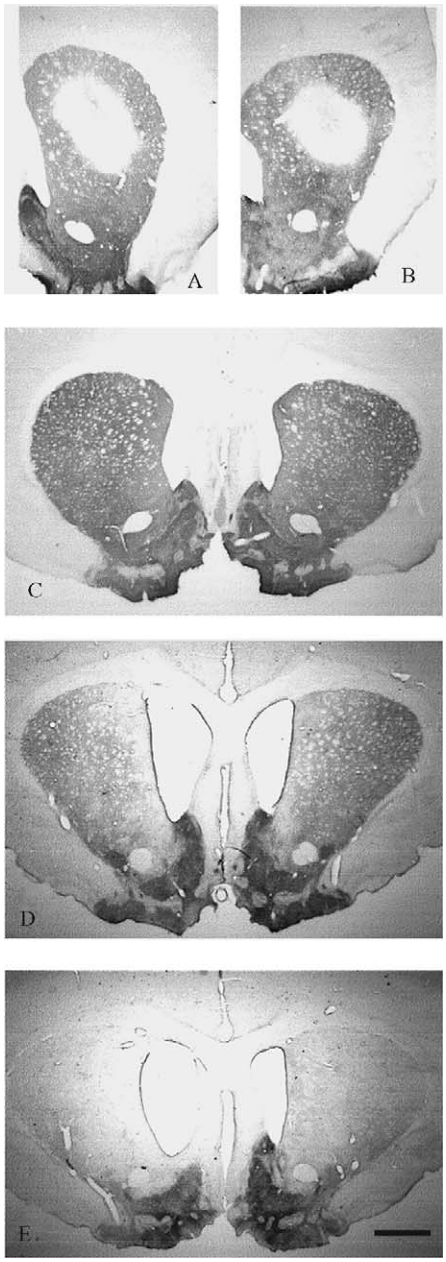


Fig. 4. Microphotographs showing the TH-immunoreactivity in the striata of rats from the different experimental groups. (A and B): sections from the right (i.e. injected) striatum of rats that received an intrastriatal injection of 6-OHDA and were not treated (A) or treated (B) with NIC. Note a clear loss (white) of TH (i.e. dopaminergic terminals, gray) in the striatal area surrounding the injection site. (C–E): sections showing both striata of normal rats (C, controls), and rats injected with 6-OHDA in the third ventricle and treated (D) or non-treated (E) with nicotine. TH-immunoreactivity (i.e. spared dopaminergic fibers) is clearly higher in the treated group (D) than in rats that did not receive NIC (E). Scale bar = 1 mm.

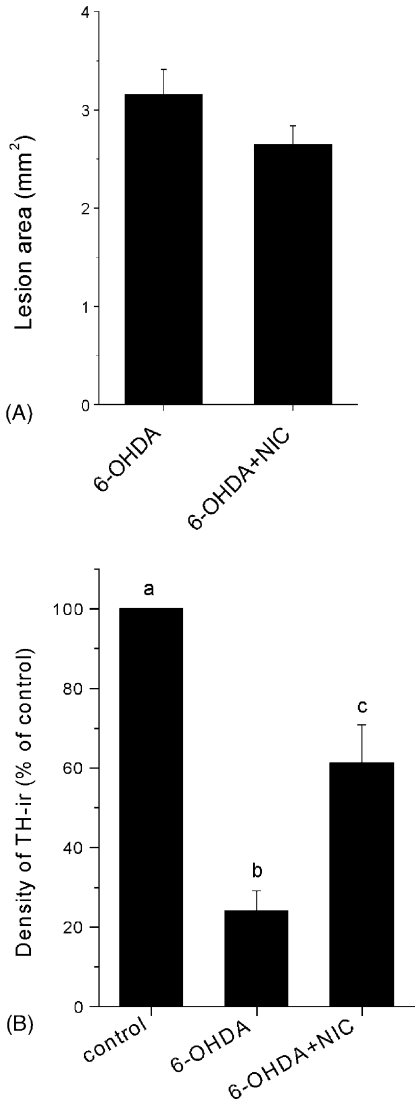


Fig. 5. (A) TH-negative area (i.e. complete loss of dopaminergic terminals; mm^2) surrounding the injection site in rats from groups B and C. The values obtained from rats treated with NIC (group C) were slightly lower than those from untreated rats, but it did not reach statistical significance. (B) Density of TH-immunoreactive (TH-ir) fibers (i.e. striatal dopaminergic terminals) in normal rats (control, group A) and in rats subjected to injection of 6-OHDA in the third ventricle and non-treated (group D) or treated (group E) with NIC. The density of TH-positive fibers (i.e. spared dopaminergic fibers) was significantly higher in the treated group. Data are mean ± SEM. Means that differ significantly are indicated by a different letter (one-way ANOVA followed by post-hoc Tukey's test; $P < 0.05$).

maintained the production of $^{\bullet}$OH due to a redox cycling, as it has been previously postulated [34]. Evidently, this fact helps explain the reported neurotoxicity of 6-OHDA in the presence of Asc because, as is well-known, 6-OHDA solutions for *in vivo* experiments are always prepared in Asc (2%) in order to confer stability to 6-OHDA. Under the here reported experimental conditions, the addition of NIC or COT increased the production of $^{\bullet}$OH during 6-OHDA autoxidation. Furthermore, when the autoxidation of 6-OHDA takes place in the presence of Asc, both NIC and COT also caused an augmentation in $^{\bullet}$OH production. Evidently, these results seem to rule out the direct participation of NIC or COT as scavengers of $^{\bullet}$OH during the autoxidation of 6-OHDA. Taking into account that both O_2-consumption and H_2O_2-accumulation are reduced in the presence of NIC and COT, we hypothesized the participation of NIC and COT as a catalyst in a process involving H_2O_2 and a compound which only is present

at the beginning of the autoxidation. The major candidate appears to be the reaction proposed by Gee and Davison [29] for 6-OHDA autoxidation:

$$QH_2 + H_2O_2 \overset{NIC(COT)}{\rightarrow} {}^{\bullet}sQ + {}^{\bullet}OH + OH^-$$

in which 6-OHDA (QH_2) reacts with the H_2O_2 previously formed in the autoxidation to yield the semiquinone radical ($^{\bullet}sQ$) and $^{\bullet}OH$. Evidently, the acceleration of the above-mentioned reaction might reduce the O_2 consumption main initial reaction of 6-OHDA autoxidation:

$$QH_2 + O_2 \rightarrow pQ + H_2O_2$$

Furthermore, the fast transformation of QH_2 into pQ explain why the generation of $^{\bullet}OH$ only takes place within the first minutes of reaction. This behavior is different in the presence of Asc due to the redox cycling induced by this compound.

We were also able to confirm that the incubation of NIC or COT with H_2O_2 followed by the addition of Fe^{2+} enhances greatly the formation of $^{\bullet}OH$. In view that all the H_2O_2 is consumed by the Fenton reaction (Fig. 2B), we postulated the increase in $^{\bullet}OH$ production is a consequence of its generation from O_2 by the following reactions:

$$O_2 + Fe^{2+} \overset{NIC(COT)}{\rightarrow} O_2{}^{\bullet -} + Fe^{3+}$$

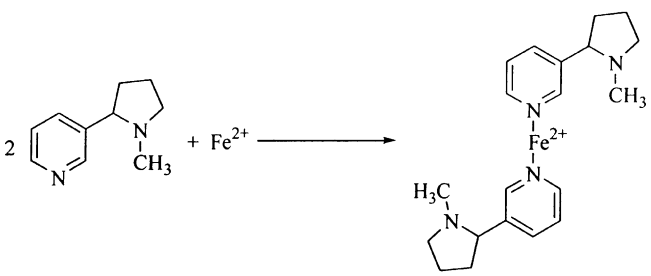
$$2O_2{}^{\bullet -} + 4H^+ \rightarrow 2H_2O_2$$

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

This hypothesis is corroborated by the consumption of O_2 without accumulation of H_2O_2 observed under the reported conditions (Fig. 2B). Evidently, further studies are necessary to understand the molecular mechanism of this catalysis. The ferric iron formed in this process may be regenerated by the reaction:

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2{}^{\bullet -} + 2H^+$$

In contrast, the simultaneous incubation of Fe^{2+} and NIC or COT previously to the addition of H_2O_2 caused a negligible formation of $^{\bullet}OH$. In this case, we were able to prove a negligible consumption of H_2O_2, a fact which demonstrates that under these circumstances both NIC and COT act by blocking the Fenton reaction. In our opinion, these findings are a consequence of the suggested capacity of NIC to chelate Fe^{2+} [11]:



This hypothesis is also corroborated by the activity shown by other iron-chelators to block the Fenton reaction [50,51]. Furthermore, our results are also supported by the antioxidant properties reported for NIC in relation to the formation of 6-OHDA from dopamine with the mediation of the Fenton reaction [14].

Taking into account that the here reported experimental protocol involved the incubation of both NIC and COT previous to the addition of 6-OHDA, the enhancement observed in the production of $^{\bullet}OH$ during the autoxidation of 6-OHDA appears to discard the suggested involvement of traces of iron as a catalyst in the generation of $^{\bullet}OH$ by 6-OHDA autoxidation [52].

Both NIC and COT protect against the lipid peroxidation induced by 6-OHDA on mitochondrial preparations obtained from rat brain as assessed by the reduction observed in TBARS formation. Furthermore, these protective effects are also present to a greater extent when the autoxidation of 6-OHDA takes place in the presence of Asc. Evidently, these effects are not attributable to the antioxidant properties shown by NIC and COT on the Fenton reaction because the production of $^{\bullet}OH$ during 6-OHDA autoxidation is not related to the presence of iron.

Assuming that lipid peroxidation is initiated by removing of a hydrogen from a polyunsaturated fatty acid side chain in a membrane phospholipid (LH) to give a lipid radical ($L^{\bullet}$) which propagate the reaction as follows [53]:

$$LH + {}^{\bullet}OH \rightarrow L^{\bullet} + H_2O$$

$$L^{\bullet} + O_2 \rightarrow L\text{-}O_2{}^{\bullet}$$

$$L\text{-}O_2{}^{\bullet} + LH \rightarrow L\text{-}O_2H + L^{\bullet}$$

our results appear to show that NIC and COT act as chain-breaking antioxidant inhibitors of lipid peroxidation:

$$NIC(COT) + L\text{-}O_2{}^{\bullet} \rightarrow NIC^{\bullet}(COT^{\bullet}) + L\text{-}O_2H$$

which stops the amplification of lipid peroxidation. Furthermore, the greater protection observed with NIC and COT in the presence of Asc are probably a consequence of the antioxidant properties of Asc [54]:

$$Asc\text{-}H + NIC^{\bullet}(COT^{\bullet}) \rightarrow Asc^{\bullet} + NIC(COT)$$

$$2Asc^{\bullet} \rightarrow Asc\text{-}H + DHAsc$$

Evidently, these results contribute to explain the previously reported neuroprotective effects of NIC against the lesion induces by intranigral injection of 6-OHDA to rats [19].

Taking into account the suggested involvement of oxidative stress in the pathogenesis of PD [55], the here reported results clearly confers neuroprotective properties to both NIC and COT. Thus, assuming the suggested role of 6-OHDA as an etiologic factor in PD [27] and that both NIC and COT are able to reduce the lipid peroxidation

provoked by 6-OHDA autoxidation, probably due to their action as a chain-breaking antioxidant inhibitor of lipid peroxidation, this effect represents an important neuroprotective mechanism in relation to this disorder. In addition, it has been demonstrated that both NIC and COT are able to prevent the production of •OH by the Fenton reaction, a fact which is very probably a consequence of their capacity to chelate Fe^{2+}. Clearly, the importance of this effect is enhanced by the fact that dopaminergic neurons are rich in iron and the concentration of this metal is increased in PD patients [56–58]. It is necessary to note that the effect observed with NIC on the lipid peroxidation induced by 6-OHDA autoxidation is greater than that caused by COT, while the effect provoked on the Fenton reaction is similar with both NIC and COT. This fact confirms that the mechanism of these two actions is different, being the difference with regard to lipid peroxidation very probably related with a different reactivity for both compounds.

Recent *in vivo* studies which addressed the protective effect of NIC against 6-OHDA or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have reported controversial results [13,17–19]. These discrepancies have been usually related to differences in doses and administration schedules, with low and intermittent doses (i.e. those used in the present study) being more effective than high and chronic doses. In addition, possible mechanical damage after direct administration of 6-OHDA into the substantia nigra or the medial forebrain bundle, or administration of too high doses of neurotoxin could prevent the observation of the protective effects of NIC in some of the above-mentioned studies. In the present study, we have used the same NIC doses and administration schedule in two recent and different animal models of PD that have been shown to be particularly appropriate for studies on neurotoxicity and neuroprotection [38–40]. However, only with one model (i.e. injection of 6-OHDA into the third ventricle) were we able to obtain clear morphological evidence of the neuroprotective effects of NIC against 6-OHDA *in vivo*. This neuroprotective effect is clearly supported by our *in vitro* results, showing a protective effect of both NIC and COT on lipid peroxidation and on •OH production by the Fenton reaction. Of course, the difficulty in demonstrating the neuroprotective effects of NIC could also be related to the pro-oxidant properties observed for both NIC and COT in relation to the production of •OH during 6-OHDA autoxidation. Finally, regarding the reported protective effects of smoking against PD [6–10], the here reported protection of NIC and COT should be combined with the previously reported publications linking cigarette smoking to preventing the formation of certain dopaminergic neurotoxins [20–23], together with the possible effects of NIC on nicotinic-acetylcholine receptors [19,59] and neurotrophic factors [60,61], all of which contribute to a better understanding of this repeated finding of neuroprotection of smoking against PD.

Acknowledgments

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