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Chronic high dose L-DOPA alone or in combination with the COMT inhibitor entacapone does not increase oxidative damage or impair the function of the nigro-striatal pathway in normal cynomologus monkeys

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Summary. Parkinson's disease (PD) is characterised by a loss of pigmented dopaminergic neurones in the zona compacta of substantia nigra. The mechanisms underlying nigral cell death remain unknown but may involve oxidative damage. There has been concern that L-DOPA treatment may accelerate nigral pathology in PD through chemical and enzymatic oxidation to reactive oxygen species. In the present study, we examined tissues from normal macaque monkeys treated for 13 weeks with high doses of L-DOPA (in combination with the peripheral decarboxylase inhibitor, carbidopa) and/or the COMT inhibitor, entacapone. Plasma was analysed for changes in protein carbonyls as a marker of oxidative damage to protein. Cortical tissue was examined for changes in levels of protein carbonyls, lipid peroxidation and oxidative damage to DNA. The integrity of the nigro-striatal pathway was assessed by nigral tyrosine hydroxylase mRNA levels and specific [^{3}H]mazindol binding to dopaminergic terminals in caudate-putamen.

No alterations in plasma protein carbonyls were observed in any treatment group. An increase was found in the levels of protein carbonyls, lipid peroxidation and 5-OH uracil, but not other products of oxidative DNA damage, in cerebral cortex of monkeys treated with L-DOPA plus carbidopa or with L-DOPA plus carbidopa and entacapone but this was only statistically significant in the latter group. There was no change in nigral tyrosine hydroxylase mRNA levels or specific striatal [^{3}H]mazindol binding in brain tissue from monkeys treated with either L-DOPA plus carbidopa or L-DOPA plus carbidopa and entacapone. The results show that in the normal monkeys L-DOPA does not provoke marked oxidative damage even at high doses, and that there is little or no potentiation of its effects by entacapone. Neither L-

DOPA plus carbidopa nor L-DOPA plus carbidopa and entacapone led to obvious damage to the nigro-striatal pathway.

Keywords: Parkinson's disease, oxidative damage, L-DOPA, substantia nigra.

Introduction

Parkinson's disease (PD) is characterised by a loss of pigmented dopaminergic neurones in the zona compacta of the substania nigra. The cause of this nigral cell loss remains unknown but the progression of the pathological process may involve oxidative damage (Jenner et al., 1992; Jenner, 1997; Jenner and Olanow, 1998). The occurrence of oxidative stress in PD is suggested by studies on nigral tissue showing increased iron accumulation (Sofic et al., 1988; Dexter et al., 1989; Riederer et al., 1989) decreased nigral levels of reduced glutathione (GSH) (Perry and Young, 1986; Sofic et al., 1992; Sian et al., 1994), increased superoxide dismutase levels (Saggu et al., 1989), and decreased activity of complex I of the mitochondrial respiratory chain (Schapira et al., 1990). These phenomena appear not to occur in other areas of the brain. Increased oxidative damage in substantia nigra in PD has been demonstrated by increased lipid peroxidation (Dexter et al., 1989, 1994), protein oxidation (Alam et al., 1997a) and DNA oxidation, (Sanchez-Ramos et al., 1994; Alam et al., 1997b). Alterations in protein oxidation as measured by the formation of protein carbonyls, (a "general" marker of oxidative protein damage, Stadtman and Berlett, 1998), occurred in a range of brain areas suggesting that either oxidative stress in PD is not restricted to the substantia nigra or that chronic treatment with L-DOPA may itself induce widespread damage (Alam et al., 1997a). Indeed, there has been concern that L-DOPA might hasten the progression of nigral pathology in Parkinson's disease through its chemical and enzymatic oxidation to reactive oxygen specie. Dopamine is metabolised by monoamine oxidase to generate H_2O_2, which might then react with transition metal ions such as iron to generate highly reactive and toxic hydroxyl radicals, $OH^{\cdot}$. Dopamine autoxidation results in the generation of not only $O_2^{\cdot -}$ and H_2O_2 but also potential neurotoxins, such as semiquinones and products derived from them (Dexter et al., 1989, 1994; Olanow, 1990; Fahn and Cohen, 1992). In addition, L-DOPA, dopamine and 3-O-methyl-DOPA can cause extensive oxidative damage to DNA in the presence of hydrogen peroxide and traces of divalent metal ions (Spencer et al., 1994, 1996). Indeed, *in vitro* L-DOPA (and dopamine) are toxic to dopaminergic neurones in culture through the formation of reactive oxygen species, implying that chronic treatment of PD patients with L-DOPA may exacerbate neuronal damage and thus accelerate the progression of the disease (Michel et al., 1990; Mena et al., 1992, 1993, 1996; Mytilineou et al., 1993; Basma et al., 1995; Pardo et al., 1995; Ziv et al., 1997). However, *in vivo* investigations in animals and clinical studies failed to show that L-DOPA had a detrimental effect on dopaminergic neurones (Hefti et al., 1981; Reches and Fahn, 1982; Perry et al., 1984; Quinn et al., 1986; Rajput et al., 1997; Murer et al., 1999).

To further investigate the possibility that L-DOPA treatment provokes oxidative damage *in vivo* and impairs the function of the nigro-striatal path-

way, we have examined tissues from normal macaque monkeys chronically treated with a high dose of L-DOPA (80 mg/kg/day, plus the peripheral DOPA decarboxylase inhibitor carbidopa). As part of this study, some monkeys were also treated with entacapone, a specific peripheral catechol O-methyltransferase inhibitor. Entacapone, which does not readily penetrate the brain, acts to prevent L-DOPA metabolism in peripheral tissues. It increases the delivery of L-DOPA to the brain and so theoretically might potentiate any oxidative stress induced by L-DOPA. Plasma was analysed for alterations in protein oxidation products measured as protein carbonyls and brain tissue was examined for changes in levels of products of oxidative damage to lipids, proteins and DNA, and for the integrity of the nigro-striatal pathway as judged by nigral tyrosine hydroxylase mRNA levels and [^{3}H]mazindol binding to dopaminergic terminals in the caudate-putamen.

Materials and methods

Materials

Acetonitrile (silylation grade), N,O-bis-(trimethylsilyl) trifluoroacetamide plus 1% trimethylchlorosilane (BSTFA plus 1% TMS) and the regenerated cellulose tubular membrane (dialysis membrane, Cellu Sep T_1 with a relative molecular mass cut off 3,500) were purchased from Pierce Chemical Co (Chester, UK). 6-Azathymine, 6-azaadenine, 5-hydroxyuracil (isobarbituric acid), 4,6-diamino-5-formamidopyrimidine (Fapy-adenine), 2,5,6-triamino-4-hydroxypyrimidine, 5-(hydroxymethyl)uracil, xanthine, hypoxanthine, proteinase K and phenol/chloroform/isoamyl alcohol (25:24:1), were purchased from Sigma Chemical Co (Dorset, UK). 8-Hydroxyguanine was purchased from Aldrich Chemical Co (Dorset, UK). 8-Hydroxyadenine was synthesized by treatment of 8-bromoadenine with concentrated formic acid (95%) at 150°C for 45 min and purified by crystallization from water (Dizdaroglu, 1994). Fapy-guanine was synthesized by treatment of 2,5,6-triamino-4-hydroxypyrimidine with concentrated formic acid and purified by crystallization from water (Dizdaroglu, 1994). The purity of synthesized standards was assessed by mass spectrometry and all were found to be >99% pure.

Animals

Adult cynomologus monkeys (Macaca fascicularis, n = 32, Huntingdon Research Centre, UK) aged from 1.5 to 3 years old, 16 males and 16 females, weighing 3 to 4 kg, were used in this study which formed part of a regulatory toxicology study. The primary objective of the study was to assess the potential toxic effects of the peripheral acting catechol-O-methyltransferase (COMT) inhibitor entacapone alone or in combination with L-DOPA plus carbidopa. The actions of entacapone are to prevent metabolism of L-DOPA by COMT to 3-O-methyldopa and consequently cause elevated and prolonged plasma and brain levels of L-DOPA (Nissinen et al., 1992). Entacapone has no other known actions. The drug treatment of animals has been described in detail elsewhere (Pearce et al., 2001). Briefly, the animals were divided into the following four treatment groups. Group 1: normal monkeys received sucrose solution as a vehicle control (n = 8); Group 2: entacapone alone (n = 8, 80 mg/kg/day, po). Group 3: L-DOPA plus carbidopa (n = 8, 80 mg/kg/day plus 20 mg/kg/day, po); Group 4: L-DOPA plus carbidopa plus entacapone (n = 8, 80 mg/kg/day plus 20 mg/kg/day, po). The animals were treated with the drugs at 11 am once daily for 13 weeks. The behavioural status of each animals was assessed in blinded fashion 3 times during the 13 week treatment periods by a neurologist and the occurrence of dyskinesia was recorded (Pearce et al., 2001). In groups 3 and 4, 8 of the 16 animals developed severe dyskinesias while the remainder had mild or no involuntary movements.

At the end of 13 weeks of drug treatment, 5 hours after last dose of L-DOPA administration, the animals were given a lethal dose of barbiturate. Blood samples were removed for determination of plasma levels of protein carbonyls as detailed below. At the end of the study, each brain was split into the two hemispheres by a mid-saggital incision immediately after death. One half-brain was fixed in 10% formalin and the other half was flash-frozen in isopentane at −70°C for biochemical studies, autoradiography and in situ hybridisation studies after dissection as described below. Dissection was undertaken on ice, using a disposable blade washed in 100% ethanol to avoid RNAase contamination. The cerebellar hemisphere was removed along the long axis of the brainstem and cut into ca. 0.5 cm slices parasaggitally. The brainstem was then removed and cut into ca 0.5 cm slices perpendicular to its long axis. The cerebral hemisphere was then cut into ca 0.5 cm coronal slices. The sections were flash frozen on a 1 cm thick aluminium plate at −70°C. The flash-frozen brain slices were kept in a −70°C freezer for 15–30 minutes and then packed, labelled and stored at −70°C for further use. For biochemical assays, samples of cortical material were removed and assayed. For autoradiography and in situ hybridisation, hemicoronal sections (20 μm) were cut using a Bright's cryostat at −20°C at the level of AP 15.5–14.6 (Szabo and Cowan, 1984), and were thermally mounted on gelatine/chrome-alum double-coated slides and stored at −70°C until assay. Cortical brain tissue was used to assay for oxidative damage because insufficient tissue was available from the nigrostriatal region to allow accurate analysis, since the extensive washings used in the protein carbonyl assay require 100 mg amounts of starting tissue (Lyras et al., 1996). However, L-DOPA is distributed throughout the brain and in Parkinson's disease marked elevations in protein oxidative damage occur in the cortex as well as in basal ganglia areas (Alam et al., 1997a).

Assay of brain and plasma protein carbonyl content

Brain tissue (200 mg) was homogenised in 2 ml of homogenising buffer (100 mM KH_2PO_4-K_2HPO_4, pH 7.4 plus 0.1% (w/v) digitonin) in a 2 ml glass homogeniser. The homogenised tissue was transferred to a plastic tube and a 10% (w/v) streptomycin sulphate solution was added to a final concentration of 1%. The solution was mixed and left to stand at room temperature for 10 minutes. It was then centrifuged for 10 minutes and the supernatant was removed and 800 μl was divided equally into two 12 ml plastic centrifuge tubes. For each 100 μl of supernatant that was present, 0.4 ml of 10 mM dinitrophenylhydrazine (DNPH) (in 2 M HCl) was added to one tube and 0.4 ml of 2 M HCl to the other tube. The tubes were then incubated for 1 hour on a rotator and the protein precipitated by adding an equal volume of 20% (w/v) trichloroacetic acid. The protein was spun down, the supernatant discarded and the pellet washed with 1.5 ml of an ethyl acetate : ethanol mixture (1 : 1) to remove excess DNPH. This was repeated three times. The final protein pellet was dissolved in 1.25 ml of 6 M gunaidine HCl and the absorbance of samples measured at 280 nm and 370 nm. Carbonyl values were calculated as in Lyras et al. (1996). Plasma protein carbonyls were measured on 100 μl aliquots using the same method, but omitting homogenisation and the addition of streptomycin sulphate solution.

Measurement of thiobarbituric acid (TBA)-reactive material in brain by HPLC

Measurement of TBA was performed according to Chirico et al. (1993). Briefly, brain tissue (100 mg) was added to 0.9 ml homogenisation buffer [KH_2PO_4-KOH buffer (pH 7.4) plus 0.1% digitonin]. 100 μl of butylated hydroxytoluene/L (2 g/l in ethanol) was added and the tissue homogenised in a glass homogeniser. Two 0.25 ml samples were taken and 1.5 ml of 0.44 mol H_3PO_4/L was added to each. Samples were left standing for 10 min at room temperature and then 0.5 ml 1% (w/v) TBA was added. Samples were heated at 90°C for 30 min, allowed to cool, centrifuged and 20 μl aliquots of the clear supernatants were injected onto a Spherisorb 5ODS2 (C18) column with a guard

column (Hiber C8). These were eluted with 65% (v/v) 50 mmol KH_2PO_4-KOH buffer pH 7.0 and 35% (v/v) methanol at 1 ml/min. Absorbance of the sharp peak at retention time 4.8 min was read at 532 nm. Results are expressed as malondialdehyde (MDA) equivalents.

Extraction of DNA from brain tissue and measurement of DNA oxidation products

Freeze-clamped tissue (100 mg) was thawed and homogenised in 2 ml of digestion buffer (100 mM NaCl, 10 mM tris-HCl, 25 mM EDTA and 0.5% SDS in 250 ml of water, pH of 8.0) with added proteinase K (final conc. of proteinase K = 0.1 mg/ml). The homogenate was then incubated at 50°C in a water bath for 2 hours after which it was vortexed with a mixture of phenol:chloroform:isoamylalcohol (25:24:1) (1 ml for every 1 ml of tissue extract). This was then centrifuged for 10 min and the upper aqueous layer was collected. The DNA was precipitated using 3 M sodium acetate buffer (pH 6.0) and 100% ethanol by adding 3 M sodium acetate (1/10th of initial volume of aqueous layer) and ethanol (2.5 volumes of final mixture of sodium acetate plus aqueous layer). The precipitated DNA was left in the freezer at −70°C for 1 hour or overnight and then centrifuged for 10 min. The supernatant was discarded and the DNA pellet washed 3 times with 70% ethanol. The washed DNA pellet was finally dissolved in a solution of tris-EDTA buffer (10 mM tris-HCl containing 1 mM EDTA at a final pH of 8.0). The DNA concentration was assessed by spctrophotometry at 260 nm (50 μg/ml of DNA gives an A_{280} of 1.0). A260/A280 values were also measured and indicated that good quality DNA was obtained (ratio ≈ 1.8).

Treatment of DNA

Treatment of DNA samples were carried out according to Lyras et al. (1997). DNA samples were dialysed for approximately 24 hours against ultra pure water (Elga purification system). Dialysis membranes had a relative molecular mass cut off of 3,500. After dialysis the amount of DNA was remeasured at 260 nm. Aliquots of 100 μg of DNA were taken and 1 nmol of 6-azathymine and 2 nmol of 8-azaadenine added as internal standards, followed by lyophilization. The samples were then hydrolysed in evacuated and sealed tubes for 45 minutes at 150°C using 0.5 ml of formic acid (60% v/v). After cooling, samples were transferred to poly(tetrafluoroethylene) hypovials and lyophilized to remove the acid. The samples were then trimethylsilylated by the addition of 0.1 ml BSTFA (Bis(trimethylisilyl)trifluoracetamide) containing 1% TMCS (trimethylchlorosilane) and acetonitrile (4:1 v/v) mixture into capped hypovials, and heated in a sand bath at 90°C, for 1 hour. Derivatized samples were then transferred to auto-injector vials and analysed on a GC-MC (Hewlett-Packard 5890II gas chromatograph interfaced with a Hewlett-Packard 5917A mass selective detector). The injection port and the GC-MS interface were kept at 220°C and 280°C respectively. Separations were carried out on a fused silica column (12 m long, 0.2 mm i.d.) coated with cross-linked 5% phenylmethylsiloxane (film thickness 0.33 mm) (Hewlett-Packard). Helium was the carrier gas with a flow rate of 0.91 ml/min. Derivatized sample (4 μl) was injected onto the column using a split ratio of 8:1, resulting in approximately 0.5 μg of sample being loaded onto the column. Column temperature was increased from 145°C to 190°C at 10°C/min after 2 minutes at 145°C, then from 190°C to 270°C at 30°C/min and finally held at 270°C for 2 minutes. Selected ion monitoring was performed using the electron-ionization mode at 70 eV with the ion source maintained at 180°C.

Relative molar response factors (K values) were obtained, using linear regression analysis, from the slopes of calibration curves. Product yields were calculated according to the K value, internal standard concentration and the peak area ratio (compound:internal standard) appropriate for each compound. Comparison of mass spectra and retention time with the authentic compound enabled unequivocal identification.

[^{3}H]Mazindol autoradiography

[^{3}H]Mazindol (DuPont NEN, UK; specific activity 17 Ci/mmol) binding was performed according to Javitch et al. (1985). Briefly, sections were thawed at room temperature, and binding performed at 4°C. Non-specific binding was determined by the addition of 10 μM mazindol. Noradrenaline uptake sites were inhibited by the inclusion of 0.3 μM desmethylimipramine (DMI). Sections for non-specific binding was incubated for 45 min in buffer (Tris-HCl 50 mM containing 300 mM NaCl and 5 mM KCl, pH 7.9 at 4°C) containing 4 nM [^{3}H]mazindol, 0.3 μM DMI, and 10 μM mazindol. Sections for total binding were incubated for 45 min in buffer containing DMI and [^{3}H]mazindol. This was followed by two consecutive 1 min washes in Tris buffer, then slides were dipped in distilled water and dried under a stream of cold air. Autoradiograms were generated by apposing slide-mounted sections to tritium Hyperfilm (Amersham) for 6 weeks at 4°C, together with 10 Amersham tritium microscale reference standards (range of tissue-equivalent tritium concentration 0.06–32.0 nCi/mg tissue wet weight, using 30 μm brain sections). Films were developed in Kodak D19.

Using computerised densitometry (Imaging Research Inc., Canada), the levels of density of binding sites on the autoradiograms was determined. Both caudate nucleus and putamen, at the level of AP 15.5–14.6 (Szabo and Cowan, 1984) were examined for [^{3}H]mazindol binding in both hemispheres. The Amersham tritium microscales were used to create a standard curve relating absorbance to known molar quantities of bound ligand (nCi/mg). The absorbance was converted into nCi/mg from the standard curve, and then to fmol/mg wet weight of tissue from the specific activity of the ligand. Specific binding in fmol/mg was obtained by subtraction of non-specific from total binding values.

Tyrosine hydroxylase (TH) gene expression by in situ hybridisation

Oligonucleotide probes complementary to bases 578–606 of human TH cDNA (O'Malley et al., 1987) were used. The probes were labelled using terminal deoxynucleotidyl transferase. The labelling reaction mixture consisted of 40 U terminal deoxynucleotidyl transferase (Promega), 30 pmol [^{35}S] dATP (1,300 Ci/mmol), 3.2 pmol of the probe, 8 μl of trailing buffer and distilled water to 40 μl final volume. Following 30 min incubation at 37°C, the reaction was stopped by the addition of 400 μl 10 mM Tris-HCl, 1 mM EDTA buffer (pH 7,6) and 1 μl tRNA (50 g/l). The oligonucleotide probes was separated by Nick column (Pharmacia Biotech, Sweden). Specific activity of the probes obtained was in the range of 8–11 $\times$ 10^3 Ci/mmol.

The specificity of probes was verified using brain sections pretreated with RNAase (50 μg/ml) at 37°C for 30 min prior to hybridisation to destroy meassage. In addition, sections were hybridised in the presence of a 50-fold excess of unlabelled probe to displace specific labelling. In both cases only evenly distributed background signal was detected. In situ hybridisation procedures were carried out as described previously (Zeng et al., 1995). Briefly, sections were warmed to room temperature and rinsed in 0.1 M phosphate buffer/solution (PBS) and then acetylated in freshly made 0.25% acetic anhydride in 0.1 M triethanolamine pH 8.0 for 10 min, dehydrated through ascending concentrations of ethanol (70, 80, 95 and 100%) and delipidated in chloroform 2 $\times$ 5 min. Partially rehydrated (95% ethanol) sections were air dried before hybridisation. Labelled probes were diluted in hybridisation buffer to a concentration 5–7 $\times$ 10^6 dpm/ml (0.64 pmol/ml). The buffer contained 50% deionized formanide, 4 $\times$ saline-sodium-citrate buffer (SSC, 1 $\times$ SSC contains 0.15 M NaCl and 0.15 M sodium citrate), 10% dextran sulphate, 1 $\times$ Denhardt's solution (0.02% ficol, 0.02% polyvinylpyrrolidone, 10 mg/ml bovine serum albumin, Sigma), 0.5 mg/l boiled salmon sperm ssDNA, 0.25 g/l tRNA, 12 nM dATP and 1 mM DTT. A Pap pen (Agar Scientific, UK) was used to make a boundary around the sections on the slides and hybridisation buffer (50 μl) was applied to each section. Sections were then allowed to incubate overnight in a humidified box at 37°C. Following hybridisation, slides were rinsed in 1 $\times$ SSC and sequentially washed in

1 × SSC at 55°C 4 × 15 min and then rinsed in 1 × SSC for 2 × 20 min at room temperature. Finally, slides were dipped in distilled water and blow dried.

To generate autoradiograms, the slides were apposed to X-ray film (TM-Bmax, Amersham) together with brain-paste standards impregnated with ^{35}S. After 2–3 weeks exposure time, films were developed and autoradiograms were analysed by computerised densitometry (MCID, Imaging Research Inc). For each section, the areas of interest were outlined with a mouse-controlled cursor and the absorbancies of the outlined areas were converted to nCi/μg wet weight of tissue from the standard curve and the values obtained were taken for statistical analysis. Non-specific signal, as assessed from RNAse treated sections, was subtracted from these values.

Statistical analysis

All values are expressed as the mean ± S.E.M. Differences between groups were analyzed using a two-way analysis of variance (ANOVA). When ANOVA showed differences between groups, these were determined using a Bonferroni/Dunn's test.

Results

Protein carbonyl levels in plasma

There was a tend for plasma protein carbonyl levels to be decreased (−11%, $p = 0.056$) in animals treated with L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg) and entacapone (80 mg/kg; Group 4) compared with vehicle treated animals (Group 1). This was not observed in animals treated with entacapone (80 mg/kg) alone (Group 2) or in those treated with L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg; Group 3). None of the differences were statistically significant.

Protein carbonyl levels in cortical tissue

Protein carbonyl levels in cortical tissue from animals treated with L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg) (Group 3) were not different from vehicle treated animals (Group 1; Fig. 2). There was a small but significant increase (+16%, $p = 0.015$) in protein carbonyl levels in animals receiving L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg) and entacapone (80 mg/kg; Group 4) compared to vehicle controls (Group 1; Fig. 1).

Lipid peroxidation in cortical tissue

Lipid peroxidation was measured as the formation of thiobarbituric acid-reactive material (TBARM), using HPLC to remove interfering chromogens (Chirico et al., 1993). Levels in cortical tissue from animals treated with L-DOPA (80 mg/kg) and carbidopa (20 mg/kg; Group 3) were not different from those receiving vehicle (Group 1). There was a small but significant increase (+17%, $p = 0.022$) in TBARM adducts levels in animals receiving L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg) and entacapone (80 mg/kg; Group 4) compared to vehicle treated animals but there was no difference in lipid peroxidation levels compared to animals receiving L-DOPA plus carbidopa (Group 3; Fig. 2).

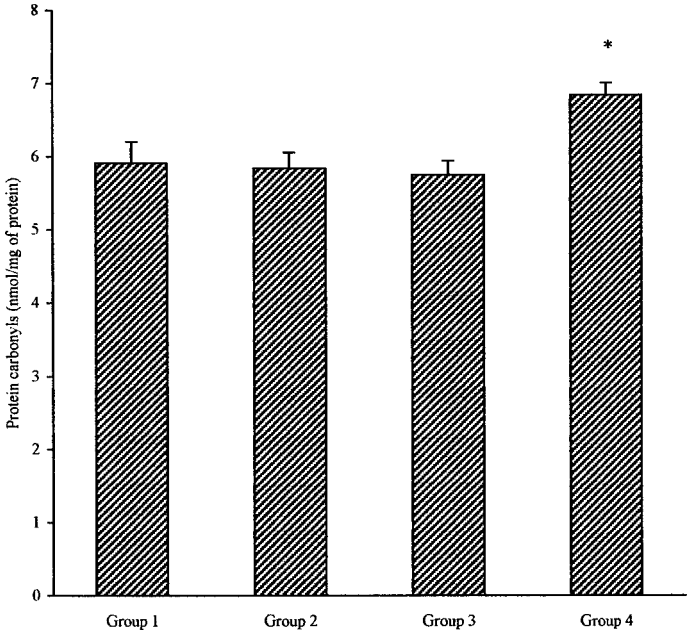

Fig. 1. Protein carbonyl levels of cortical tissue from Macacque monkeys treated with L-DOPA and/or entacapone. Animals were treated fro 13 weeks with L-DOPA plus carbidopa and/or entacapone by oral gastric infusion. Group 1: normal controls; Group 3: L-DOPA plus carbidopa (80 mg/kg + 20 mg/kg); Group 4: L-DOPA plus carbidopa and entacapone (80 mg/kg + 20 mg/kg + 80 mg/kg). The values shown represent the means ± SEM for eight animals per group. *$P < 0.013$ vs Group 1

Products of oxidative damage to DNA bases in cortical tissues

There were no significant differences in any products of DNA oxidation between animals in Groups 1, 3 and 4, except for 5-hydroxyuracil. Levels of 5-hydroxyuracil were significantly increased ($p = 0.013$) in animals treated with L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg, Group 3, +33%) and L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg) and entacapone (80 mg/kg; Group 4, +34%) compared to vehicle treated animals (Group 1; Table 1). However, there was no significant difference in the levels of 5-hydroxyuracil between animals treated with L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg) and entacapone (80 mg/kg; Group 4) compared to animals treated with L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg; Group 3; Table 1).

Tyrosine hydroxylase (TH) mRNA

TH mRNA labelling was strongly expressed in cell clusters throughout the substantia nigra pars compacta. Overall there was no significant difference in the levels of TH mRNA labelling between normal monkeys receiving L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg; Group 3) and animals treated with entacapone alone (80 mg/kg; Group 2) and animals treated with

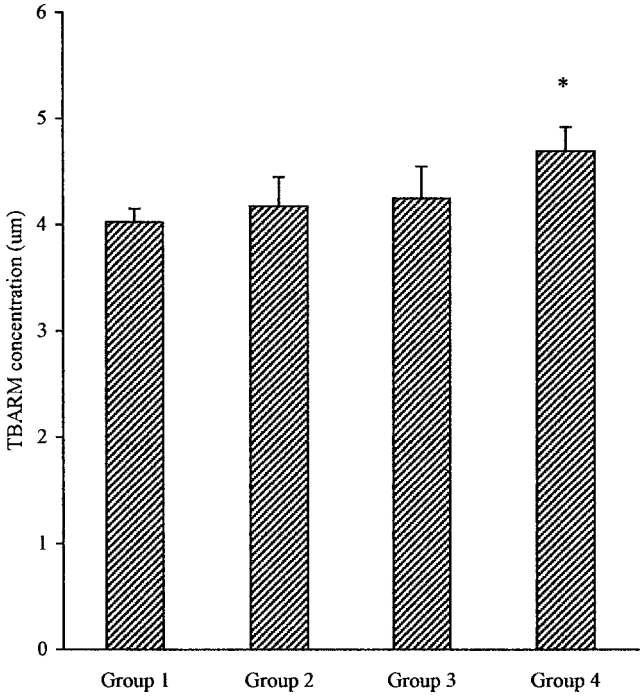

Fig. 2. Lipid peroxidation levels in cortical tissue from Macacque monkeys treated with L-DOPA and/or entacapone. Animals were treated fro 13 weeks with L-DOPA plus carbidopa and/or entacapone by oral gastric infusion. Group 1: normal controls; Group 3: L-DOPA plus carbidopa (80 mg/kg + 20 mg/kg); Group 4: L-DOPA plus carbidopa and entacapone (80 mg/kg + 20 mg/kg + 80 mg/kg). The values shown represent the means ± SEM for eight animals per group. *P < 0.022 vs Group 1. Peroxidation was expressed as the amouont of TBA-reactive material (TBARM) calculated as μm level of malondialdehyde (MDA)

Table 1. Levels of DNA oxidation products in cortical tissue from normal Macacque monkeys treated with L-DOPA and/or entacapone

Base oxidation products (nmol/mg)	Group 1	Group 2	Group 3	Group 4
5-OH Uracil	0.032 ± 0.003	0.038 ± 0.002	0.048 ± 0.007*	0.049 ± 0.005*
5-OH Me Uracil	0.011 ± 0.003	0.011 ± 0.003	0.011 ± 0.003	0.002 ± 0.005
8-OH Adenine	0.125 ± 0.014	0.135 ± 0.013	0.145 ± 0.013	0.146 ± 0.028
Fapy guanine	0.709 ± 0.115	0.908 ± 0.123	1.102 ± 0.164	0.986 ± 0.148
8-OH Guanine	1.376 ± 0.165	1.392 ± 0.191	1.497 ± 0.224	1.391 ± 0.175

Animals were treated fro 13 weeks with L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg) and/or entacapone (80 mg/kg) by oral gastric infusion, see Materials and methods for more details. The values shown represent the means ± SEM for eight animals per group. *P < 0.013 vs Group 1

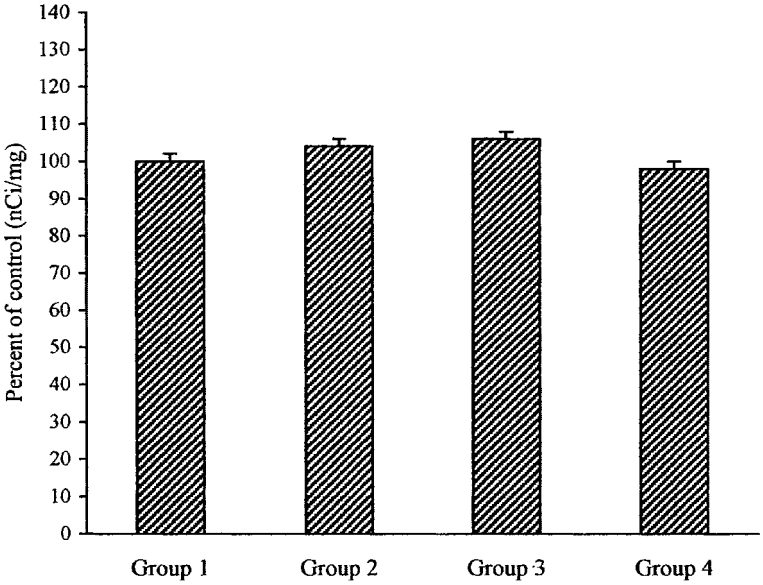

Fig. 3. Nigral tyrosine hydroxylase gene expression from Macacque monkeys treated with L-DOPA and/or entacapone. Animals were treated fro 13 weeks with L-DOPA plus carbidopa and/or entacapone by oral gastric infusion. Group 1: normal controls; Group 2: entacapone alone (80 mg/kg); Group 3: L-DOPA plus carbidopa (80 mg/kg + 20 mg/kg); Group 4: L-DOPA plus carbidopa and entacapone (80 mg/kg + 20 mg/kg + 80 mg/kg). The values shown represent the means ± SEM for eight animals per group

L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg) and entacapone (80 mg/kg; Group 4) when compared to the normal controls (Group 1; Fig. 3).

[^{3}H]Mazindol binding

There was no overall significant difference in the specific [^{3}H]mazindol binding in the caudate nucleus and putamen of monkeys treated with vehicle (Group 1) or entacapone (80 mg/kg; Group 2) compared to those receiving L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg; Group 3) or L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg) and entacapone (80 mg/kg; Group 4; data not shown). Subregional analyse showed a non-significant decrease in the specific [^{3}H]mazindol binding in the lateral region of the putamen of Group 2 (entacapone alone, 80 mg/kg, −13%, Table 2) compared to the Group 1 (vehicle control; Table 2).

Discussion

The occurrence of oxidative stress and subsequent oxidative damage to lipid, proteins and DNA is established as contributory to the pathology of PD damage (Jenner et al., 1992; Jenner, 1997; Jenner and Olanow, 1998). The ability of L-DOPA to form reactive oxygen species and to induce oxidative stress has led to the suggestion that its use in PD might increase the rate of progression of nigral cell degeneration (Michel et al., 1990; Tanaka et al.,

Table 2. Specific [^{3}H]mazindol binding density (fmol/mg) in the caudate-putamen of normal Macacque monkeys treated with L-DOPA and/or entacapone

	Caudate nucleus		Putamen			
	lateral	medial	dorsolateral	dorsomedial	ventrolateral	ventromedial
Group 1 (n = 8)	294.0 ± 32.5	321.3 ± 42.3	242.2 ± 23.0	228.7 ± 23.3	286.5 ± 39.0	266.6 ± 38.9
Group 2 (n = 8)	302.1 ± 26.2	313.4 ± 30.7	212.2 ± 17.8	214.9 ± 18.9	245.5 ± 22.0	242.7 ± 19.5
Group 3 (n = 8)	315.5 ± 22.7	336.8 ± 21.7	254.8 ± 21.1	239.7 ± 14.8	291.3 ± 21.1	259.3 ± 13.7
Group 4 (n = 8)	264.4 ± 17.0	298.0 ± 23.5	223.7 ± 9.21	221.8 ± 5.1	262.4 ± 15.2	253.2 ± 10.5

Animals were treated fro 13 weeks with L-DOPA (80 mg/kg) plus carbidopa (20 mg/kg) and/or entacapone (80 mg/kg) by oral gastric infusion, see Materials and methods for more details. The values shown represent the means ± SEM for eight animals per group

1991; Mena et al., 1992, 1993, 1996; Mytilineou et al., 1993; Basma et al., 1995; Pardo et al., 1995; Ziv et al., 1997). Despite extensive investigations this question remains unresolved. The availability of brain tissue from cynomologus monkeys receiving high dose L-DOPA treatment for 13 weeks has provided an opportunity to determine in vivo whether oxidative damage or nigro-striatal degenration occurs. The doses of L-DOPA plus carbidopa used were well above those used to treat patients with PD (by a factor of 5–10) and so could be expected to induce oxidative stress and nigro-striatal damage, if these were feature of the drug's action.

Measurement of indices of altered plasma and brain protein oxidation, lipid peroxidation and oxidative DNA damage showed that these indices were not markedly altered by chronic high dose treatment with L-DOPA plus carbidopa. This contrasts with the in vitro data showing that L-DOPA can generate radical species inducing oxidative damage to biomolecules. (Michel et al., 1990; Basama et al., 1995). However, L-DOPA can have both pro-oxidant and anti-oxidant effects depending on the circumstance under which it is examined (Li et al., 1995; Spencer et al., 1996). The COMT inhibitor entacapone alone also had no effects on the same indices of oxidative damage but when administered in conjunction with the high dose of L-DOPA/carbidopa, a small, but significant, increase in protein cabonyl formation and lipid peroxidation in cortical tissue was observed. This may reflect the potentiation of L-DOPA exposure of brain produced by the COMT inhibitor, but both changes were minor compared to the expected alteration in brain L-DOPA levels. Levels of 5-hydroxyuracil were significantly increased by the L-DOPA/carbidopa/entacapone combinations compared to vehicle treated animals. However, this difference was not significant compared to animals treated with L-DOPA plus carbidopa alone and no other markers of oxidative DNA damage were altered by any of the drug treatments, especially, not 8-hydroxyguanine which is shown to be elevated in PD (Alam et al., 1997b). There has been considerable debate on methods to measure oxidative DNA damage and the method used in this paper may not have been optional in view of recent methodological developments (Halliwell, 2000). Nevertheless, the

methods used in this study have readily detected increased oxidative damage in brain samples from patients with PD, Alzheimer's disease and SDAT (Alam et al., 1997b; Lyras et al., 1997), so that they would have been capable of detecting significant oxidative damage if it had occurred in the monkey tissues. Overall, there was little indication of any real oxidative stress produced by exposure to large amount of L-DOPA even with long periods of time.

The current study was undertaken using cortical tissue rather than tissue from basal ganglia which was not available in the quantity required for the assay procedures. This should not have any major influence on the results obtained since L-DOPA is widely distributed in brain and there is also no indication of elevated oxidative protein damage in plasma. It might be argued that the substantia nigra could be exceptional as it has a high degree of basal oxidative metabolism and contains high concentration of iron that might catalyse L-DOPA induced oxidative damage accumulation (Sofic et al., 1988; Dexter et al., 1989; Riederer et al., 1989; Spencer et al., 1996). In fact, L-DOPA probably accumulates more in the striatum which also contains a large amount of iron but where there is no evidence of damage induced by L-DOPA in these animals or in PD. Studies in PD have indicated elevated carbonyls in all brain regions (Alam et al., 1997a), but the data in this paper suggests that L-DOPA administration is not responsible for this.

Our data suggesting that a high doses of L-DOPA do not induce significant oxidative damage in brain are strengthened by the histochemical findings. There was no effect of L-DOPA plus carbidopa, entacapone alone or entacapone plus L-DOPA and carbidopa on the number of dopaminergic cells present in the substantia nigra or the innervation of the striatum by dopaminergic fibres as assessed by specific [^{3}H]mazindol binding. We conclude that L-DOPA is not highly neurotoxic to the nigro-striatal pathway even in doses well above those used in clinical patients with PD (Hefti et al., 1981; Reches and Fahn, 1982; Perry et al., 1984; Quinn et al., 1986; Rajput et al., 1997). This is in agreement with a recent studies showing that long-term L-DOPA administration had no detectable detrimental effect on dopaminergic neurones in rats with moderate nigro-striatal lesion (Murer et al., 1999; Datla et al., 2001). These findings appear to contrast with a variety of evidence from in vitro studies of L-DOPA toxicity and also from some in vivo studies in rodents (Mena et al., 1993, 1996; Mytilineou et al., 1993; Pardo et al., 1995; Han et al., 1996; Carvey et al., 1997). However, not all studies using L-DOPA have shown it to be neurotoxic, there are reports that it can be neuroprotective, that elevations in GSH can occur and that neurotrophic actions of L-DOPA can be detected (Mytilineou et al., 1993). However, this is first study of chronic treated with L-DOPA in primates and this in vivo data appears consistent with the belief that L-DOPA is not toxic in man based on an inability of L-DOPA to induce dyskinesia in normal individuals and the lack of evidence for any augmentation of the progression of PD. It could still be argued that L-DOPA might enhance the oxidative stress which occurs in damaged substantia nigra in PD and so increase the rate of nigral cell death. This remains a possibility which can only be solved by studies in patient

populations since no progressive animal models of PD has been established. However, it does seems that inherently L-DOPA is not neurotoxic and does not cause oxidative stress is contrast to previously held views, although the dose of L-DOPA used was sufficient to induce serve dyskinesia in these animals (Pearce et al., 2001).

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