Free Radical Research, June 2010; 44(6): 635–644

Specific oxidative stress profile associated with partial striatal dopaminergic depletion by 6-hydroxydopamine as assessed by a novel multifunctional marker molecule

Y. ALUF[1,2], J. VAYA[1], S. KHATIB[1], Y. LOBODA[2], S. KIZHNER[2] & J. P. M. FINBERG[2]

[1]*Laboratory of Natural Medicinal Compounds, Migal-Galilee Technology Center, and Tel Hai College, Kiryat Shmona, Israel, and* [2]*Department of Molecular Pharmacology, Rappaport Medical Faculty, Technion, Haifa, Israel*

(Received date: 8 December 2009; In revised form date: 25 January 2010)

Abstract

Real time oxidative stress in the extracellular compartment of rat striatum was characterized by microdialysis with synthetic non-dialyzable marker molecules composed of linoleic acid, tyrosine and guanosine (N-linoleoyl tyrosine (LT) and N-linoleoyl tyrosine 2´-deoxyguanosyl ester (LTG)). Partial dopaminergic deafferentation was induced by injection of 6-hydroxydopamine (250 μg) to the left lateral ventricle, which depleted ipsilateral striatal dopamine by 46% and dopaminergic cells in left substantia nigra by 44%, 5 weeks after administration. Resting microdialysate dopamine levels in dopamine-depleted striatum were not different from sham-operated rats, although the ratio of oxidized metabolites of dopamine to free dopamine was significantly increased. Hydroperoxide and epoxy products of the linoleoyl portion of LT and LTG were detected in the striatal microdialysate by LC/MS/MS following initial separation by HPLC and were significantly increased in dopamine-depleted compared with control striatum without an increase in guanosine or tyrosine oxidation or nitration. Systemic administration of N-acetyl cysteine (350 mg/kg i.p.) decreased the increment in hydroperoxide and epoxy metabolites to levels not significantly different from control. Oxidation activity towards polyunsaturated fatty acids is present in the extracellular space of partially dopamine-denervated striatum, whereas oxidized glutathione and oxysterol levels in striatal tissue are decreased, possibly indicative of a compensatory response.

Keywords: *Compensatory mechanisms, 6-hydroxydopamine, markers, Parkinson's disease, polyunsaturated fatty acids, striatal microdialysis*

Abbreviations: *DA, Dopamine; 6OHDA, 6-Hydroxy Dopamine; ICV, Intra-cerebroventricular; LT, N-linoleoyl tyrosine; LTG, N-linoleoyl tyrosine 2´-deoxyguanosyl ester; NAC, N-Acetyl Cysteine; PD, Parkinson's disease*

Introduction

Although the pathological phenotype and clinical symptoms of Parkinson's disease (PD) are well described, the aetiology of the disease and the mechanism responsible for the progression of neurodegeneration are still unknown. Clinical and biochemical studies have raised hypotheses that inflammation, excessive nitric oxide generation, mitochondrial dysfunction, proteosomal dysfunction and genetic polymorphisms are involved in the pathogenesis of PD [1,2]. In addition, abundant evidence points to a role of oxidative stress in causing the neuronal death seen in PD [3]. This association is based on: (i) biochemical features of dopaminergic neurons, including increased amounts of iron (which serves as a catalyst for hydroxyl radical formation via the Fenton reaction) and accumulation of neuromelanin, together generating highly reactive free radicals; (ii) evidence of oxidative stress in human post-mortem brain tissue including an increase in superoxide dismutase and a

Correspondence: Professor John P. M. Finberg, Department of Molecular Pharmacology, Rappaport Family Faculty of Medicine, Technion, Haifa, 31096, Israel. Tel: +972-4-8295272. Fax: +972-4-8295271. Email: finberg@tx.technion.ac.il

ISSN 1071-5762 print/ISSN 1029-2470 online © 2010 Informa UK Ltd. (Informa Healthcare, Taylor & Francis AS)
DOI: 10.3109/10715761003692529

decrease in glutathione (GSH) levels in substantia nigra [4,5], (iii) the formation of protein carbonyls and 8-hydroxy-2-deoxyguanosine, reflecting oxidative damage to proteins and DNA, respectively, and (iv) increased levels of the lipid peroxidation products malondialdehyde and lipid hydroperoxides in the substantia nigra pars compacta of deceased PD patients [6]. Oxidative stress and changes in redox balance of tissues may exert collateral damage to major biomolecules and to normal neighbouring cells [7]. Thus, free radical generation may be the initiating pathway, leading to cell death and to the development of pathological conditions such as PD.

In the current work, a new methodology for characterizing real time oxidative stress in biological systems is described using previously described synthetic marker molecules. The sensitive marker molecules were designed, synthesized and constructed from various endogenous sub-units, which were connected covalently together to form a novel probe not present as such in tissues (Figure 1). Each marker molecule contains two or three of the major groups from which the body is composed: linoleic acid (LA) represents the polyunsaturated fatty acids (PUFA), the tyrosine residue represents proteins and the hydrophilic subunit 2′-deoxyguanosine represents DNA. Each part of the synthetic marker is well known to be easily oxidized and to form specific products, depending on the type of ROS/RNS present [8–12]. The markers used in this experiment were N-linoleoyl tyrosine (LT) and N-linoleoyl tyrosine 2′-deoxyguanosyl ester (LTG). Analysis of the oxidized markers provides information about the presence and type of the oxidative stress under study.

The novel marker molecules were perfused by microdialysis in the striatum of rats in which partial dopaminergic (DA-ergic) deafferentation had been produced by intracerebroventricular (i.c.v.) injection of 6-hydroxydopamine (6OHDA) in order to monitor real time oxidative stress. The partial lesion model was chosen to mimic the situation of early Parkinson's disease. The synthetic marker molecules do not pass the microdialysis membrane and so the changes in their composition found *in vivo* are *only* caused by diffusion of free radicals from the extracellular space into the microdialysate. This approach represents an important difference from other dialysable markers (chiefly salicylate) which have been used previously because (i) unlike salicylate, our marker sub-units are naturally present in tissues and (ii) other dialysable markers may diffuse into the tissue, enter surrounding cells and their oxidized products may diffuse back into the microdialysis probe not accurately demonstrating the level of oxidative stress in the extracellular space. An increased generation of free radicals in the extracellular space of striatum partially depleted of DA was detected using the present technique, lending support for the hypothesis that partial dopaminergic denervation increases oxidative stress on the remaining neuronal population in the striatum and laying the foundation for a new method of real time measurement of oxidative stress.

Materials and methods

Animals and lesioning procedure

All procedures with animals were authorized by the Technion Animal Care and Use Committee, whose ethical standards are based on those detailed in the National Institutes of Health (Bethesda, MD) Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (200–250 g; Harlan, Jerusalem, Israel) were anaesthetized with ketamine/xylazine (70/35 mg/kg i.p.) and placed in a stereotaxic frame (Kopf, CA). A heating pad maintained a constant body temperature of 37°C. In an initial study aimed to quantify lesion extent, one of three doses of 6OHDA hydrochloride: 125, 250 or 325 μg (in 10 μL 0.85% saline containing 0.1% ascorbic acid) was injected i.c.v. into the left lateral ventricle using co-ordinates AP –0.8, LR 1.4, DV –3.5 mm with respect to bregma [13]. The noradrenergic neurons were protected by injecting desipramine (10 mg/kg s.c.) 30 min prior to the 6OHDA injection. Sham-lesioned rats were subjected to the same procedures using vehicle. For the microdialysis study, two groups of rats were subjected to the same protocol, sham lesioned and 250 μg 6OHDA.

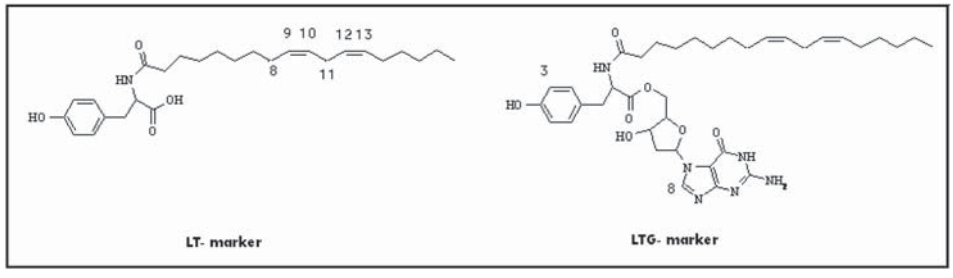

Figure 1. The structure of a custom-designed synthetic marker composed of tyrosine (T) and linoleic acid (L) with and without the addition of guanosine (G).

Immunohistochemistry

For immunohistochemistry, rats were formalin-fixed *in vivo*. Briefly, rats were anaesthetised deeply with pentobarbital and perfused through the heart with phosphate-buffered saline (PBS) followed by 4% formalin in PBS, brains were removed and fixed in formalin for 5 days. Following the fixation, brains were freeze-protected in 25% sucrose and frozen in tissue-freezing medium (Triangle Biomedical Science, Durham, NC, USA). Cryosections (25μm) were made from the substantia nigra area, washed in PBS and treated with 0.5% Triton (X-10, Sigma, St Louis, MO, USA) for 10 min. Endogenous peroxidase activity was blocked by 30 min incubation with 3% hydrogen peroxide in methanol. Non-specific binding was blocked with a mixture of 5% bovine serum albumin and 5% foetal calf serum and the sections were then incubated overnight at 4°C with mouse anti-tyrosine hydroxylase (TH) primary antibody (1:1000; Sigma, T1299). The following day, detection of primary antibody was carried out using anti-mouse IgG and HRP-streptavidin-coupled peroxidase activity with AEC (3-amino-9-ethyl carbazole) chromogen reagent as indicator (Zymed Histostain kit, Zymed, San Francisco, CA, USA). Sections of substantia nigra were counter-stained with cresyl violet acetate (Sigma). Tyrosine hydroxylase-positive cells were manually counted in the left substantia nigra pars compacta by a blinded observer in every fourth brain section between bregma –4.8 to –6.0 (14 sections per brain).

Microdialysis study

Five weeks after the lesion of DA-ergic neurons and 1 day prior to microdialysis, rats were anaesthetized with ketamine/xylazine and a silicon guide cannula (CMA/12, CMA/Microdialysis, Stockholm, Sweden) was implanted with its tip at the dorsal limit of the left striatum (AP +0.02, LR +0.3, DV –0.35 from bregma) [14]. A dummy microdialysis probe was inserted into the guide cannula, so that the necessary brain tissue damage would be done at the time of guide cannula insertion, but not at the time of active microdialysis probe placement. One day later, rats were anaesthetized with isoflurane (Nicholas Piramal, London, UK) while maintaining a constant body temperature of 37°C with a heating pad. The dummy probe was removed and a microdialysis probe (CMA/12, 4 mm membrane length, polyarylethersulphone membrane, cut-off 20 kDa, CMA/Microdialysis, Stockholm, Sweden) was inserted into the guide cannula and perfused with artificial cerebro-spinal fluid (ACSF; NaCl 147 mmol/L, KCl 2.7 mmol/L, $CaCl_2$ 1.2 mmol/L, $MgCl_2$ 0.85 mmol/L) containing a mixture of markers (LT and LTG, 160 μM) and Tween 20 (0.01 %) at a flow rate of 1 μL/min. After a 1 h equilibration period, dialysates were collected every hour into refrigerated tubes containing 20 μL of 0.1 M perchloric acid and frozen on dry ice. After the third dialysate collection, rats were administered either N-acetyl cysteine (NAC, 350 mg/kg, s.c.) or saline. Samples were collected for an additional 3 h and then analysed by HPLC equipped with electrochemical detector for DA and its metabolites and LCMS for markers and metabolites. Immediately at the end of the microdialysis, rats were sacrificed and the striata were removed and homogenized with 10 volumes of 0.1 M perchloric acid in saline (w/v), sonicated for 5 min and kept frozen at –70°C until analysis.

Analysis of catecholamines in the dialysate and in the striatal tissue

For DA determination in tissue, 100 μl of striatal homogenate (diluted with 0.1 M perchloric acid in saline 1:10 w/v) were centrifuged for 10 min at 10, 000 g. The supernatant was injected into the solvent stream of an HPLC apparatus. The dialysate samples were similarly injected directly to the HPLC. The separation of DA and its metabolites was achieved using an Inertsil ODS-2 column 5 μm 4.6 × 150 mm (GL Sciences, Tokyo, Japan) with a mobile phase composed of 100 mM NaH_2PO_4, 1 mM octanesulphonic acid, 2.5 % methanol, 4.5% acetonitrile and 269 μM sodium EDTA dissolved in HPLC grade deionized water and pH adjusted to 2.75. The flow rate through the system was 1 mL/min. Detection of compounds was enabled by a model 5011A analytical cell and model 5200 Coulochem II electrochemical detector (ESA, Chelmsford, MA, USA) operated in redox mode. Column eluates were initially oxidized at a potential of +300 mV using an ESA guard cell placed before the detector, reduced to +100 mV at detector 1 and measured at –400 mV at detector 2. Catecholamines were normalized to protein quantity using the Lowry assay for protein determination.

Analysis of oxidative stress marker molecules and their metabolites

Dialysates were diluted 1:4 in acetonitrile and the detection of oxidized and unoxidized LT marker and oxidized and unoxidized LTG marker was performed by LC/MS/MS. Initial separation and detection of compounds was performed using HPLC (Waters 2790) and a Waters photodiode array detector (model 996). The HPLC column was 3.5 μm C18 ODS XTerra (Waters, MA) and the compounds were eluted using a gradient of solution A (0.1% acetic acid in acetonitrile) and solution B (0.1% acetic acid in double-distilled water, DDW) as follows: start with 40% A; followed by an increase to 60% A for 2 min; and

then to 80% A for 10 min. Finally, the column was washed with a solution of 98% A. In the separation of LTG and oxidized LTG, in solvents A and B, acetic acid was omitted. MS/MS (Micromass Quattro Ultima MS, Manchester, UK) analysis of the oxidized products was performed in scan mode using electrospray negative ions (ES^-). The MS source temperature was set at 150°C, with a cone gas flow of 22 L/h and a desolvation gas flow of 600 L/h. Peak spectra were monitored between 30–900 m/z. Collision-induced dissociation MS was performed with collision energy of 25–30 eV and 3–3.5 kV capillary voltages. Multiple-reaction-monitoring was performed under the same conditions. A calibration curve of LT and LTG was run with each set of analyses.

Reduced and oxidized glutathione derivatization and detection in the striatal tissue

The analysis of GSH and GSSG was performed according to Hammermeister et al. [15], with slight modifications as follows. Striatal homogenate (50 μL) was added to 2.5 μL phosphate buffer (1 M, pH 6.25) and 10 μL of perchloric acid 60% and was centrifuged for 10 min. To 50 μL of aliquot from the homogenate aqueous layer or standard solution (0.1–20 μM of GSH and GSSG in 10% perchloric acid) was added 6 μL of 72.6 μM internal standard (γ-Glu-Glu), 12.5 μL of an indicator buffer solution containing 1.5 mM bathophenanthroline disulphonic acid (BPSA), 0.15 M boric acid, 10 μM cresol red, all in 5% perchloric acid, and 5 μL of 33 mM iodoacetic acid, in an ice bath. The solution was mixed and adjusted to pH 8.6–8.8 (first appearance of purple colour) with 40–48 μL of 2 M NaOH. The solution was stored at room temperature in the dark (0.5 h). Dansyl chloride (94 μL, 15 mM in acetonitrile) was added and the samples were mixed and stored at 4°C. Samples were analysed using HPLC-fluorescence derivatization (FLD) methodology. Twenty microlitres of sample were injected into a model 1050 HPLC system (Hewlett-Packard, Palo Alto, CA) fitted with a Hypersil (Hypersil Inc., Cheshire, UK) aminopropyl AE column (APS-2, 150 × 4.6 mm) and a model 1046A FLD (Hewlett-Packard) optimized at 258 nm excitation and 515 nm emission. The 20 min gradient elution (1.5 mL/min) was carried out at room temperature: solvent A was 55% acetonitrile in double-distilled water and B was 800 mM ammonium acetate (pH 4.8 with glacial acetic acid) in 44% acetonitrile. The gradient was as follows: 0–4 min 20% B, 4–18 min to 99% B, 18–20 min 99% B, 4 min of pre-wash and back to 20% B for equilibration prior to the next injection. Calibration curves of GSH and GSSG were run with each set of analyses.

Determination of oxysterols in the striatal tissue

In order to determine concentration of oxysterols in striatal tissue, an internal standard (19-hydroxycholesterol) was added to striatal homogenate (200 μL) followed by addition of a mixture of 1 mL hexane: 2-propanol (3:2 v/v), containing 10 ppm butylated hydroxy anisol. The mixture was centrifuged at 18,800 g and the organic phase was separated. The extraction procedure was repeated twice and the combined organic phase was evaporated under nitrogen and samples were kept under argon at –80°C until analysis.

For the analysis of the total amount of oxysterols (free and esterified) the dried extracts were hydrolysed with KOH solution as follows: dried extracts were dissolved in 0.5 mL diethylether (which was treated previously with aluminium oxide to eliminate possible peroxide content) and 0.5 mL KOH (20% in MeOH:DDW 70:30 solution). The solution was mixed for 3 h at room temperature. One millilitre of diethyl ether was added and the pH of the aqueous phase was adjusted to 5 by adding 0.5 mL citric acid solution (20% in DDW). The solution was vortexed and the ethyl ether phase was separated. Another portion of diethylether was added, vortexed and separated. The combined phases of diethylether were dried using Na_2SO_4 and evaporated under nitrogen. The samples were then subjected to the silylating reagent (50 μL of 1, 4-dioxane and 50 μL of N,O-bis(trimethylsilyl) acetamide) and heated to 80°C for 60 min. Gas chromatography (GC)/MS analysis of the silylated mixture was performed by means of high pressure (HP) gas chromatograph, fitted with an HP-5 trace analysis capillary column (column 0.32 mm, I.D. 0.25 mm film thickness, 5% phenyl methyl silicone), with a mass-selective detector, linked to an HP Chem Station data system. Helium was used as the carrier gas, at a flow rate of 0.656 ml/min, pressure 10.4 psi and a linear velocity of 31 cm/s. Samples were detected by GC/MS in total ion monitoring (TIM) mode and 19-OH cholesterol was used as the internal standard (IS). The response factor for each oxysterol under the analytical conditions was calculated from the peak-area ratio [16]. The oxysterols alpha-epoxy and beta-epoxy were selected for analysis as potential products during cholesterol oxidation [17]. Oxysterols were separated and the percentage of each oxysterol was calculated from cholesterol in the same sample [9].

Statistic analysis

In the immunohistochemical study, the statistical analysis was performed using one-way ANOVA. In the microdialysis study, comparison between DA levels, turnover and marker oxidation products between

different animals treatment was performed by two-way ANOVA with repeated measures. GSH and oxysterols comparisons between control and lesioned animals were made using unpaired '*t*' test. Statistical significance level was set at $p < 0.05$.

Results

Extent of dopaminergic lesion

In rats which were subjected to *in vivo* fixation at 5 weeks after i.c.v. injection of three different doses of 6OHDA, a dose-dependent reduction in numbers of TH-positive cells in the substantia nigra pars compacta was seen (Figure 2). The dose of 250 µg 6OHDA caused a 44 ± 5% ($n = 6–8$, $p < 0.0001$) reduction in TH-positive cells and was used in all subsequent experiments. Analysis of striatal tissue DA levels following microdialysis in rats which had received 250 µg 6OHDA showed a 46 ± 6% reduction in left striatal DA content in comparison with control rats ($n = 12–14$, $p < 0.01$).

Measurement of marker oxidation and recovery in in vitro *microdialysis system*

Detailed marker composition and possible marker oxidation products *in vitro*, cell culture and blood samples have already been published by Szuchman et al. [9–11], Khatib et al. [8] and Tavori et al. [18]. In order to determine the recovery and oxidation of the marker in the microdialysis system without tissue exposure, ACSF containing marker (160 µM) and Tween 20 (0.01%) was perfused through a microdialysis probe placed in a vial filled with either water or hydrogen peroxide solution (0.4 mM). The collected dialysates were diluted in acetonitrile (1:4) and the amount of the marker was determined by LC/MS. The recovery of all markers was very high (95 ± 5%), indicating that a negligible amount passed through the dialysis membrane. When hydrogen peroxide was placed in the vial, oxidation of the markers circulating in the probe occurred and the concentration of the oxidized products of the markers (LT-OOH, LT-Epoxy, LTG-OOH and LTG-Epoxy) was higher as the concentration of hydrogen peroxide increased (for example, the ratio of LT-Epoxy to total LT increased in a linear way from 0.58% to 0.98% as the concentration of hydrogen peroxide increased from 10^{-9} M to 10^{-5} M, data not shown). The concentration of LTG-8OXO, however, did not change with hydrogen peroxide exposure. No traces of markers were observed in the vial nor in the striatal tissue itself at the end of all experiments, indicating the markers' lack of ability to cross the microdialysis membrane.

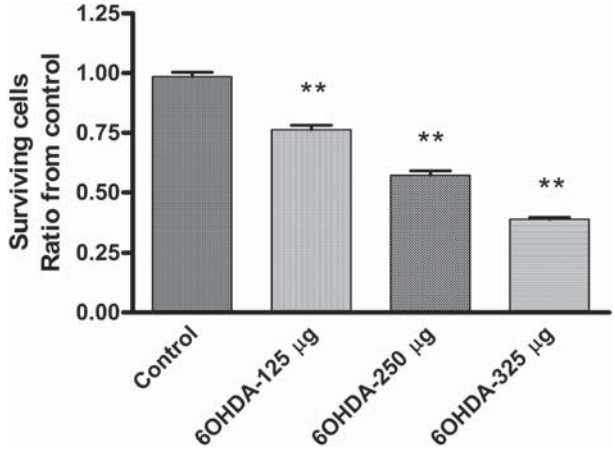

Figure 2. Reduction in TH-positive cells in substantia nigra 5 weeks after i.c.v. administration of different amounts of 6OHDA. The extent of lesions in 6OHDA-treated rats was calculated by dividing the number of TH-positive cells by that in the control rat substantia nigra. Mean values shown ± SEM for $n = 6–8$ rats per group. $^{**}p < 0.01$ vs control by one-way ANOVA.

Measurement of dopamine levels and oxidative stress in striatal extracellular fluid

Striatal microdialysis was performed 5 weeks after the i.c.v. injection of 250 µg 6OHDA ($n = 14$) or saline ($n = 12$). Microdialysate collections (1 h each) were made for 6 h following the 1 h equilibration after insertion of the probe. Constant levels of DA, DOPAC (dihydroxyphenylacetic acid) and HVA (homovanillic acid) were seen over the 6 h period. No significant difference in DA levels was seen between 6OHDA-treated and control rats in the extracellular compartment of the striatum (Figure 3A), but DOPAC and HVA levels were greater in the former. Calculation of the ratio (DOPAC + HVA)/DA levels, an index of DA turnover, showed an increase of ~75% (Figure 3B), indicative of a higher DA release and metabolism in the striatum of partially lesioned animals and, hence, activation of compensatory mechanisms.

The basal oxidative stress of the two groups of animals was assessed according to the production of oxidized markers in the dialysate and is expressed as the ratio of oxidized marker/total non-oxidized marker during the 6 h of perfusion. After the first 3 h of microdialysis, the antioxidant NAC (350 mg/kg) or saline was administered s.c. and microdialysates were collected for an additional 3 h. Four different treatment groups were tested: (i) sham/saline, (ii) sham/NAC, (iii) 6OHDA/saline and (iv) 6OHDA/NAC.

The production of oxidized marker products in rats not treated with NAC was constant over the 6 h collection period (Figures 4 and 5). An increase in the amount of oxidized markers was seen in the 6OHDA-treated rats compared to the saline-treated animals. When comparing the means of the six collection periods for saline and 6-OHDA-treated rats, the hydroperoxide form of LT (LTOOH) was increased in 6OHDA-treated rats by 21 ± 4%

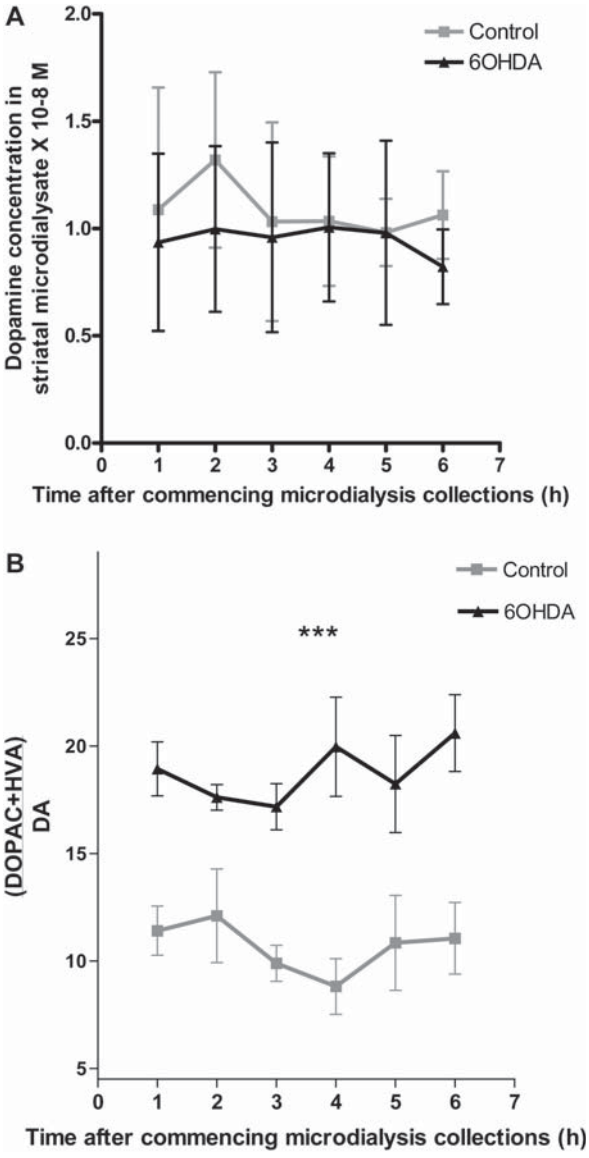

Figure 3. The concentration of DA and DA turnover in striatal microdialysate throughout the 6 h period of dialysate collection. There was no significant difference between control and lesioned animals in DA concentration in the extracellular compartment (A). DA turnover (measured as (DOPAC + HVA)/DA) was increased in the lesioned animals compared to control rats by ~70% (B). $^{***}p < 0.001$ by two way ANOVA. Mean levels shown ± SEM for n = 12–14 rats per treatment.

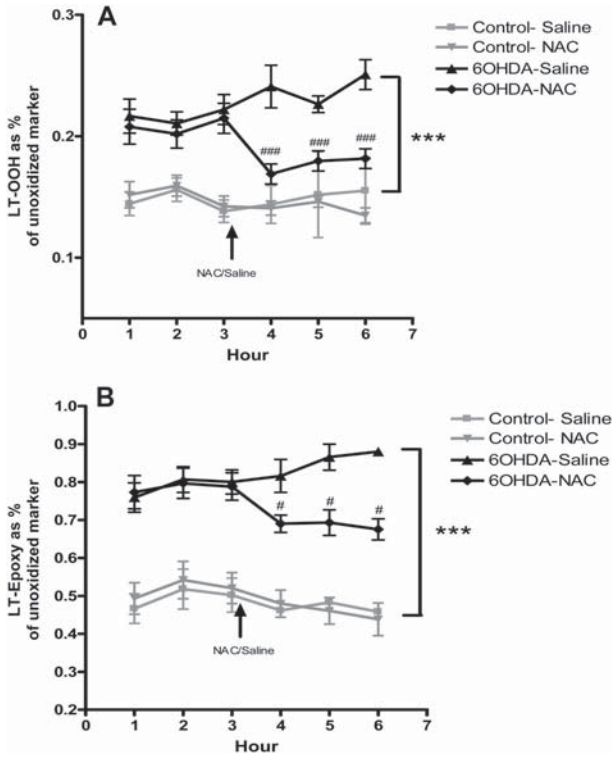

Figure 4. Change in oxidized LT markers during the 6 h of striatal microdialysis expressed as percentage from total marker concentration before and after administration of N-acetyl-cysteine (NAC). (A) Content of the hydroperoxide form of LT (LT-OOH) and (B) Content of the epoxy form of LT (LT-Epoxy). $^{***}p < 0.001$ for difference between control animals (-■-) to 6OHDA treated animals (-▲-) both treated with saline. Three hours after the beginning of the microdialysis, each rat received N-acetyl cysteine (NAC) 350 mg/kg or saline in a single s.c. injection, $###p < 0.001$ and $\#p < 0.05$ for the difference in the amount of oxidized markers after NAC administration in the 60HDA treated rats (-▲-). Mean levels shown ± SEM for n = 6–7 rats per treatment, analysis was performed by ANOVA.

(Figure 4A), the epoxy form of LT was increased by 28 ± 4 % (Figure 4B), the hydroperoxide product of LTG was increased by 37 ± 7% (Figure 5A) and the epoxy product of LTG was higher by 14 ± 3% (Figure 5B, all these increases were highly significant, $p < 0.001$). Systemic administration of the antioxidant NAC significantly decreased the increments in all of these metabolites except that of the epoxy product of LTG. When comparing the means of the three collections before to the three after NAC administration, LT-OOH decreased by 71 ± 5% (Figure 4A), LT-epoxy decreased by 64 ± 3% (Figure 4B) and LTG-OOH decreased by 71 ± 2% (Figure 5A, all of these decrements highly significant, $p < 0.05$). No change in the 8-oxo guanosine form was observed in the 6OHDA treated rats before or after NAC treatment (Figure 5C).

Measurement of reduced and oxidized glutathione and oxysterols in the striatal tissue

In order to determine the level of oxidative stress in striatal tissue, the amount of reduced and oxidized glutathione (GSH and GSSG, respectively), their ratios and the level of oxysterols were measured in striatal homogenate. An increase in the GSH level (37 ± 10%, Figure 6A) and a significant decrease in the GSSG concentration (35 ± 9%, $p < 0.05$) was seen in rats treated with 6OHDA (Figure 6B), resulting in a significantly lower ratio of GSSG:GSH (57 ± 11%, $p < 0.05$, Figure 6C). Levels of oxysterols (alpha and beta epoxy cholesterol) in striatal tissue were also decreased by 51 ± 15% and 57 ± 15%, respectively ($p < 0.05$, Figure 7).

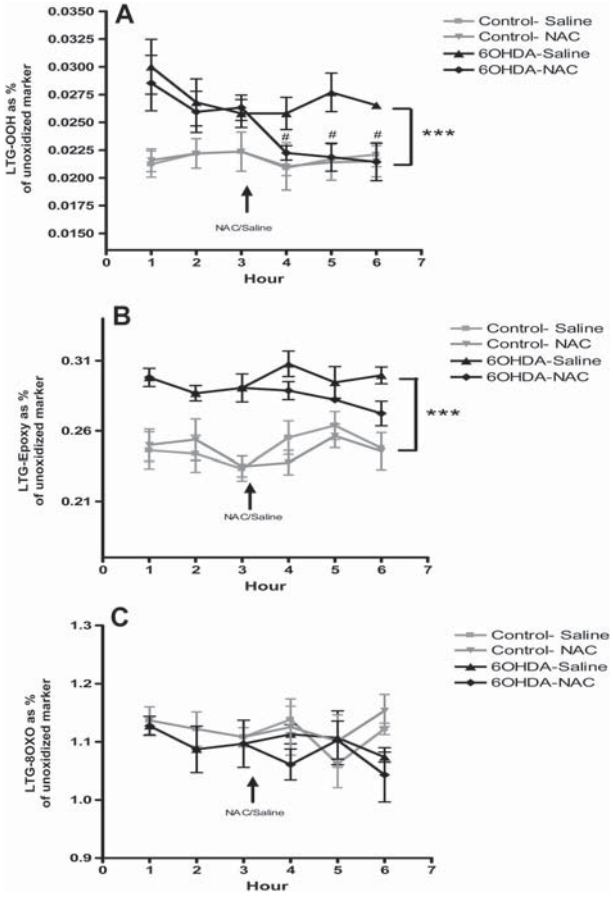

Figure 5. Change in oxidized LTG markers during the 6 h of striatal microdialysis expressed as percentage from total marker concentration before and after administration of N-acetyl-cysteine (NAC). (A) Content of the hydroperoxide form of LTG (LTG-OOH), (B) content of the epoxy form of LTG (LTG-Epoxy) and (C) content of the 8OXO form of LTG (LTG-8OXO). $^{***}p < 0.001$ for difference between control animals (▬) to 6OHDA treated animals (▬) both treated with saline. Three hours after the beginning of the microdialysis, each rat received N-acetyl cysteine (NAC) 350 mg/kg or saline in a single s.c. injection, $\#p < 0.05$ for the difference in the amount of oxidized markers after NAC administration in the 60HDA treated rats (▬). Mean levels shown ± SEM for $n = 6$–7 rats per treatment, analysis was performed by ANOVA.

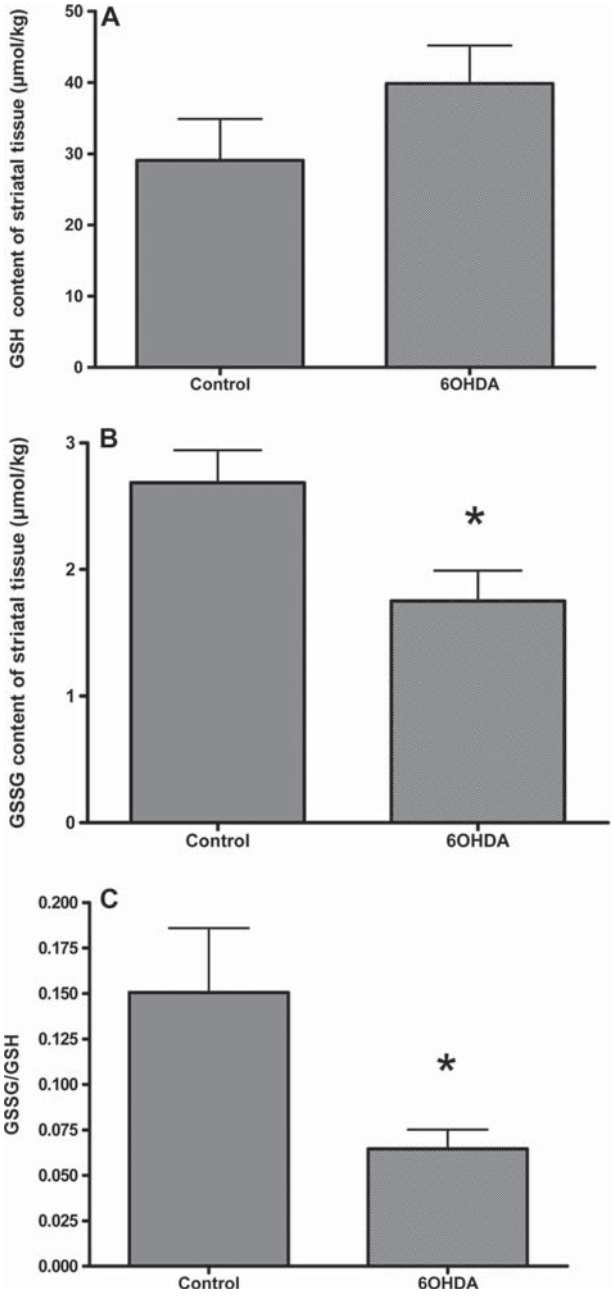

Figure 6. Decrease in GSSG and GSSG/GSH ratio in the striatal tissue of 6OHDA-treated rats. In the striatal tissue of rats treated with 6OHDA, GSH levels were increased by 37 ± 10% (A) and GSSG levels were decreased by 35 ± 9 % (B). As a consequence, a significantly lower ratio (57 ± 11% control values) of oxidized to reduced forms of glutathione (GSSG:GSH) was observed in the 6OHDA-treated animals (C). $^{*}p < 0.05$ for difference from sham-operated rats by Student's *t*-test, mean values ± SEM for $n = 12$–14 rats per group.

Discussion

The LT marker contains linoleic acid connected to tyrosine via an amide bond and the LTG consists of the LT backbone with the carboxylic group in the tyrosine moiety esterified with guanosine via the latter's deoxy-ribose sub-unit. These hybrid markers permit simultaneous assessment of the real time oxidative modification of lipid (linoleic acid), protein (tyrosine) and nucleic acid (guanosine). Synthesis of LT and LTG, their structure and their oxidized species were described in detail by Szuchman et al. [9,10] and Khatib et al [8]. The tyrosine moeity can be attacked by reactive chloride, bromide and nitrogen species, yielding products such as 3-chlorotyrosine, 3,5-dichlorotyrosine, 3-bromotyrosine and 3-nitrotyrosine. Peroxidation of LA gives different hydroperoxides and epoxides, whereas reaction of reactive nitrogen species and chloride species yields in addition to the peroxidation and epoxidation also nitration and chlorination of the linoleic acid moiety on specific positions.

The use of LT constructed from Tyr and LA enabled us to distinguish these oxidized products from those of

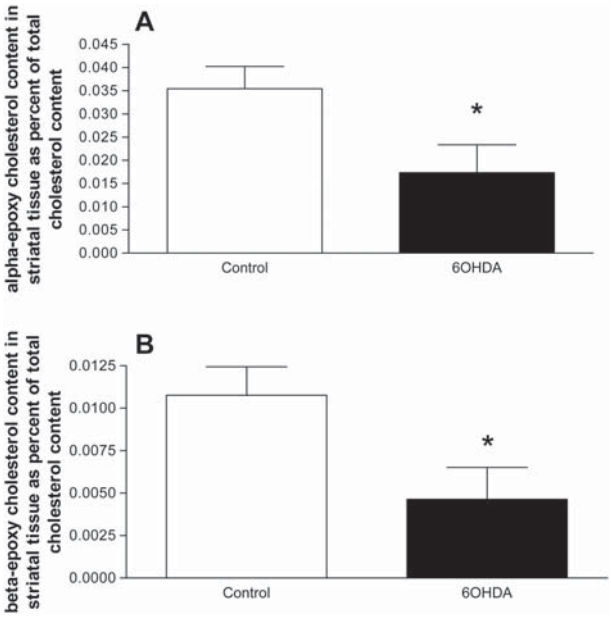

Figure 7. Decrease in endogenous oxysterols in the striatal tissue of 6OHDA-treated rats. In the striatal tissue of rats treated with 6OHDA the level of alpha epoxy cholesterol (A) and beta epoxy cholesterol (B) were significantly lower (51 ± 15% and 57 ± 15%, respectively) than sham-operated rats. $^{*}p < 0.05$ by Student's *t*-test, mean values ± SEM for $n = 12$–14 rats per group.

the endogenous Tyr and LA of the tested sample. In this methodological work our sensitive markers were exposed to the extracellular environment surrounding striatal neurons and accelerated oxidative stress generation was detected, 5 weeks after 6OHDA administration. A marked elevation in oxidative stress (DNA, protein and fatty acid damage) has been shown by different methods and in diverse models of PD [19]; however, the importance of our marker is its sensitivity and its ability to trace a specific fingerprint of oxidation to different ROS\NOS generated *in situ* and to pin-point the oxidative stress as it occurs in the extracellular space .

We report here for the first time application of these novel non-dialysable marker molecules by microdialysis to detect generation of free radicals in real time in the extracellular compartment. The elevation in oxidative stress in the striatum of injured rats was significant in forming four different products of the oxidized markers (LT-OOH, LT-epoxy, LTG-OOH and LTG-epoxy), all of them are products of an attack only on the linoleic acid moiety of the markers, with no oxidation in the 2′-deoxyguanosine and tyrosine sub-units and no nitration nor chlorinated products of the marker either on the tyrosine or on the linoleic acid (see possible nitration and other oxidation products of the synthetic markers sub-units in Szuchman et al. [11]). The generation of LT-OOH, LTG-OOH and their respective epoxy compounds, specifically on the linoleic acid moiety of the markers *in vivo*, and *in vitro* using hydrogen peroxide, suggests that the type of oxidant responsible for this oxidative reaction is probably hydrogen peroxide or other endogenous hydroperoxides in the extracellular compartment which are capable of penetrating the microdialysis membrane. Indeed, the extent and pattern of increase in the marker metabolites in 6OHDA-treated rats was very similar to that seen following exposure of the marker to hydrogen peroxide *in vitro*. Yasuhara et al. [20] found increased levels of 8-OH deoxyguanosine in hemi-Parkinsonian rats, but in their study DA depletion would have been extensive and unilateral and measurement of 8-OH deoxyguanosine levels was performed 2 days after 6OHDA, a time when the effect of the neurotoxin would have been maximal. Other studies have observed increased indexes of oxidative stress in striatal tissue after 6OHDA treatment, such as TBARS, protein carbonyls, 4-hydroxynonenal, but in all cases these were elevated in the first few days after administration of the neurotoxin and subsequently returned to normal levels [21,22].

Administration of a systemic dose of the antioxidant NAC resulted in a significant decrease in the amount of three of the oxidized products, lowering oxidative stress in the striatum of 6OHDA-treated rats almost to the level of the control rats. This reversal of the oxidative changes by NAC reinforces the conclusion that generation of oxidative free radicals in the striatal tissue occurs following partial DA-ergic deafferentation.

In contrast to the increased oxidative stress in the extracellular compartment, a significant reduction in the oxidative stress products GSSG and oxysterols was observed in the striatal tissue itself, together with an increased GSH level. The reduced amount of ROS in the neuronal tissue could be the result either of reduced ROS generation in tissue as opposed to extracellular space or of activation of a compensatory response to an increased extracellular oxidative stress. Such a compensatory response may explain why dopaminergic neurons do not continue to degenerate in this and similar experimental models, generated by single administration of neurotoxin, despite a continued increase in the extra neuronal oxidative stress. Decreased GSH levels were found in striatal tissue 30 days after unilateral 6OHDA lesion to the nigro-striatal pathway in rats [23], but under conditions of near total lesion. In mice [24] GSH levels were decreased shortly after partial 6OHDA lesion by i.c.v. injection, but then increased, and were above normal 28 days after lesion, at a time when DA levels had returned to normal. Response of GSH levels to 6OHDA lesion is therefore highly dependent on the experimental conditions. It is also of interest that GSH levels are reduced in substantia nigra of PD patients [25], however in such patient studies, denervation level is nearly complete and tissues are subject to a long post-mortem delay before assay, which complicates interpretation of this data.

Following partial DA-ergic deafferentation of the striatum, at levels up to 80% depletion of striatal DA

content, compensatory mechanisms are activated which maintain control of movement at normal levels [26]. A part of this compensatory change is an increase in synthesis and turnover of DA, with generation of increased amounts of the oxidized DA metabolites, DOPAC and HVA, indicative of increased DA metabolism by MAO [27–29]. In order to study oxidative stress in this condition, we chose to administer the toxin i.c.v., because of the ease of administration and reproducibility of injection amount by this route. The dose of 250 μg 6OHDA caused ~ 50% depletion of the dopaminergic neurons in the substantia nigra, which was the denervation level we chose in order to model early stage PD. Striatal microdialysis showed that, in keeping with findings from other similar studies, extracellular DA levels are maintained at normal values in rats with this degree of lesion, while generation of oxidized metabolites was increased (Figures 3A and B), confirming the existence of compensatory increased DA turnover in the remaining neurons. Increased compensatory activity by the remaining DA-ergic neurons has been proposed previously to play a potential role in the accelerated DA-ergic degeneration seen in Parkinson's disease [28]. Recently, however, it has been realized that non DA-ergic neuronal pathways may play a part in the compensatory readjustment to partial DA-ergic denervation [27] and overactive DA-ergic pathways are unlikely to play a role in continued DA-ergic degeneration [30]. Indeed, a partial 6OHDA lesion in the rat induces a stable state of denervation, as opposed to the accelerated neuronal loss of PD [31]. Use of high spatial resolution microvoltammetry technique coupled with stimulation of medial forebrain bundle has shown that at physiological stimulation frequencies, similar extracellular fluid DA concentrations are attained in lesioned rats as in controls in response to neuronal activation [32]. Despite the lack of change in mean extracellular DA levels, however, local DA concentrations may well be increased in the region of DA-ergic synapses, but the reduced total number of DA transporter (DAT) sites will lead to a reduction in total reuptake activity, leading to normal extracellular fluid DA levels. Thus, the existence of oxidative stress in this model may be linked to the involvement of the compensation mechanism in the striatum of injured animals and can be the result of lower activity of the DAT, which results in a high local concentration of DA in the synapses of remaining neurons. As described above, excess DA generates free radicals resulting from H_2O_2 production; however, DA also undergoes oxidation via the non enzymatic pathway [33], a process which is accelerated in the presence of Fe^{+2} ions and results in the formation of toxic DA quinones. These two pathways for DA metabolism can lead to elevated levels of oxidative stress in the extracellular space in the vicinity of the remaining DA-ergic axon terminals, although mean DA levels in extracellular fluid, as sampled by microdialysis, is normal.

The central role of oxidative stress in the progression of neurodegenerative diseases in general and PD in particular was shown *in vitro* and in various animal models and the pursuit for a reliable and accurate biomarker has been carried on for a long time. The involvement of imbalanced redox homeostasis and excessive ROS/RNS production in various tissues is generally measured by using endogenous markers of oxidative stress such as 8-oxo 2′-deoxyguanosine (DNA damage), keto proteins (protein alterations) and fatty acid oxidation products (thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA), isoprostanes, etc.). Another approach is the use of salicylic acid as a synthetic probe for scavenging a hydroxyl radical [34]. In a model of near total DA depletion, increased amounts of 2,3- and 2,5-dihydroxybenzoic acid were found in striatal tissue of rats following i.c.v. administration of salicylic acid [35]. Subsequently, no change was found in the same salicylic acid metabolites when salicylic acid was perfused in the striatum by microdialysis [36]. Apart from the fact that DA-ergic denervation was near total in these studies, salicylic acid is a limited indicator for oxidative stress containing only an aromatic ring, its oxidation is not a naturally occurring process in the body and it is limited to monitoring mainly the presence of oxidation products through hydroxyl radical attack, apart from being freely dialysable, as described above (Introduction).

Apart from alteration in activity of the DA-ergic system, increased oxidative stress could result from increased activity of projection neurons from the striatum to other basal ganglia structures such as the external pallidum. The latter nuclei are remote from the microdialysis area, but neuronal tracts leading to them may pass through the microdialysis area and increased neuronal activity in these tracts, i.e. the indirect pathway, whose activity is known to increase following DA-ergic deafferentation [37] may increase production of ROS/RNS.

Our data therefore reveal increased oxidative stress in the extracellular space of the striatum following partial DA-ergic deafferentation, which may be a part of the oxidative stress profile in the Parkinsonian brain. It should be stressed that our system enables study of oxidative stress in real time but only over the short period of microdialysis; measurement of oxidized biomarkers is also of use in evaluating the oxidative stress existing in a model system, but indicates a different aspect, i.e. the effect of the stress in the long-term. Future studies are in progress to further evaluate the ability of LT and LTG to detect oxidative and other attack on endogenous cell components after different pharmacological interventions in the dopaminergic pathways.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Beal MF. Mitochondria, oxidative damage, and inflammation in Parkinson's disease. Ann NY Acad Sci 2003;991:120–131.

[2] Schapira AH. Mitochondrial dysfunction in neurodegenerative disorders. Biochim Biophys Acta 1998;1366:225–233.

[3] Olanow CW. Oxidation reactions in Parkinson's disease. Neurology 1990;40(suppl):32–37; discussion 37–39.

[4] Dexter DT, Sian J, Jenner P, Marsden CD. Implications of alterations in trace element levels in brain in Parkinson's disease and other neurological disorders affecting the basal ganglia. Adv Neurol 1993;60:273–281.

[5] Youdim MB, Stephenson G, Ben Shachar D. Ironing iron out in Parkinson's disease and other neurodegenerative diseases with iron chelators: a lesson from 6-hydroxydopamine and iron chelators, desferal and VK-28. Ann NY Acad Sci 2004; 1012:306–325.

[6] Mayo JC, Sainz RM, Tan DX, Antolin I, Rodriguez C, Reiter RJ. Melatonin and Parkinson's disease. Endocrine 2005;27:169–178.

[7] Halliwell B, Gutteridge JM. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. Arch Biochem Biophys 1986;246:501–514.

[8] Khatib S, Musa R, Vaya J. An exogenous marker: a novel approach for the characterization of oxidative stress. Bioorg Med Chem 2007;15:3661–3666.

[9] Szuchman A, Aviram M, Musa R, Khatib S, Vaya J. Characterization of oxidative stress in blood from diabetic vs. hypercholesterolaemic patients, using a novel synthesized marker. Biomarkers 2008;13:119–131.

[10] Szuchman A, Aviram M, Soliman K, Tamir S, Vaya J. Exogenous N-linoleoyl tyrosine marker as a tool for the characterization of cellular oxidative stress in macrophages. Free Radic Res 2006;40:41–52.

[11] Szuchman A, Aviram M, Tamir S, Vaya J. Cholesterol, linoleic acid or/and tyrosine yield different spectra of products when oxidized alone or in a mixture: studies in various oxidative systems. Free Radic Res 2003;37:1277–1288.

[12] Vaya J, Schipper HM. Oxysterols, cholesterol homeostasis, and Alzheimer disease. J Neurochem 2007;102:1727–1737.

[13] Shachar DB, Kahana N, Kampel V, Warshawsky A, Youdim MB. Neuroprotection by a novel brain permeable iron chelator, VK-28, against 6-hydroxydopamine lession in rats. Neuropharmacology 2004;46:54–63.

[14] Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 4th ed. San Diego: Academic Press; 1998.

[15] Hammermeister DE, Serrano J, Schmieder P, Kuehl DW. Characterization of dansylated glutathione, glutathione disulfide, cysteine and cystine by narrow bore liquid chromatography/electrospray ionization mass spectrometry. Rapid Commun Mass Spectrom 2000;14:503–508.

[16] Vaya J, Aviram M, Mahmood S, Hayek T, Grenadir E, Hoffman A, Milo S. Selective distribution of oxysterols in atherosclerotic lesions and human plasma lipoproteins. Free Radic Res 2001;34:485–497.

[17] Addis PB, Emanuel HA, Bergmann SD, Zavoral JH. Capillary GC quantification of cholesterol oxidation products in plasma lipoproteins of fasted humans. Free Radic Biol Med 1989; 7:179–182.

[18] Tavori H, Aviram M, Khatib S, Musa R, Nitecki S, Hoffman A, Vaya J. Human carotid atherosclerotic plaque increases oxidative state of macrophages and low-density lipoproteins, whereas paraoxonase 1 (PON1) decreases such atherogenic effects. Free Radic Biol Med 2009;46:607–615.

This paper was first published Online on Early Online on 25 March 2010.

[19] Sayre LM, Perry G, Smith MA. Oxidative stress and neurotoxicity. Chem Res Toxicol 2008;21:172–188.

[20] Yasuhara T, Hara K, Sethi KD, Morgan JC, Borlongan CV. Increased 8-OHdG levels in the urine, serum, and substantia nigra of hemiparkinsonian rats. Brain Res 2007;1133:49–52.

[21] Sanchez-Iglesias S, Rey P, Mendez-Alvarez E, Labandeira-Garcia JL, Soto-Otero R. Time-course of brain oxidative damage caused by intrastriatal administration of 6-hydroxydopamine in a rat model of Parkinson's disease. Neurochem Res 2007; 32:99–105.

[22] Smith MP, Cass WA. Oxidative stress and dopamine depletion in an intrastriatal 6-hydroxydopamine model of Parkinson's disease. Neuroscience 2007;144:1057–1066.

[23] Garcia JC, Remires D, Leiva A, Gonzalez R. Depletion of brain glutathione potentiates the effect of 6-hydroxydopamine in a rat model of Parkinson's disease. J Mol Neurosci 2000;14:147–153.

[24] Tanaka K, Ogawa N, Asanuma M. Molecular basis of 6-hydroxydopamine-induced caspase activations due to increases in oxidative stress in the mouse striatum. Neurosci Lett 2006;410:85–89.

[25] Perry TL, Godin DV, Hansen S. Parkinson's disease: a disorder due to nigral glutathione deficiency? Neurosci Lett 1982;33:305–310.

[26] Stricker EM, Zigmond M. Recovery of function after damage to central catecholamine-containing neurons: a neurochemical model for the lateral hypothalamic syndrome. In: Sprague J, Epstein A, editors. Progress in psychobiology and physiological psychology. New York: Academic Press; 1976. p. 121–187.

[27] Brotchie J, Fitzer-Attas C. Mechanisms compensating for dopamine loss in early Parkinson disease. Neurology 2009;72 (7 Suppl):S32–S38.

[28] Zigmond MJ, Abercrombie ED, Stricker EM. Partial damage to nigrostriatal bundle: compensatory changes and the action of L-dopa. J Neural Transm Suppl 1990;29:217–232.

[29] Zigmond MJ, Acheson AL, Stachowiak MK, Stricker EM. Neurochemical compensation after nigrostriatal bundle injury in an animal model of preclinical parkinsonism. Arch Neurol 1984;41:856–861.

[30] Bezard E, Gross CE, Brotchie JM. Presymptomatic compensation in Parkinson's disease is not dopamine-mediated. Trends Neurosci 2003;26:215–221.

[31] Rodriguez M, Barroso-Chinea P, Abdala P, Obeso J, Gonzalez-Hernandez T. Dopamine cell degeneration induced by intraventricular administration of 6-hydroxydopamine in the rat: similarities with cell loss in parkinson's disease. Exp Neurol 2001;169:163–181.

[32] Garris PA, Walker QD, Wightman RM. Dopamine release and uptake rates both decrease in the partially denervated striatum in proportion to the loss of dopamine terminals. Brain Res 1997;753:225–234.

[33] Asanuma M, Miyazaki I, Ogawa N. Dopamine- or L-DOPA-induced neurotoxicity: the role of dopamine quinone formation and tyrosinase in a model of Parkinson's disease. Neurotox Res 2003;5:165–176.

[34] Wan FJ, Tung CS, Shiah IS, Lin HC. Effects of alpha-phenyl-N-tert-butyl nitrone and N-acetylcysteine on hydroxyl radical formation and dopamine depletion in the rat striatum produced by d-amphetamine. Eur Neuropsychopharmacol 2006;16:147–153.

[35] Kostrzewa RM, Kostrzewa JP, Brus R. Dopaminergic denervation enhances susceptibility to hydroxyl radicals in rat neostriatum. Amino Acids 2000;19:183–199.

[36] Nowak P, Szczerbak G, Biedka I, Drosik M, Kostrzewa RM, Brus R. Effect of ketanserin and amphetamine on nigrostriatal neurotransmission and reactive oxygen species in Parkinsonian rats. *In vivo* microdialysis study. J Physiol Pharmacol 2006;57:583–597.

[37] DeLong MR, Wichmann T. Circuits and circuit disorders of the basal ganglia. Arch Neurol 2007;64:20–24.