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Attenuation of 6-hydroxydopamine (6-OHDA)-induced nuclear factor-kappaB (NF-κB) activation and cell death by tea extracts in neuronal cultures

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Abstract

Antioxidant and anti-inflammatory therapy approaches have been in the focus of attention in the treatment of neurodegenerative Parkinson's and Alzheimer's diseases where oxidative stress has been implicated. Tea extracts have been previously reported to possess radical scavenger, iron chelating and anti-inflammatory properties in a variety of tissues. The purpose of this study was to investigate potential neuroprotective effects of tea extracts and possible signal pathway involved in a neuronal cell model of Parkinson's disease. We demonstrated highly potent antioxidant-radical scavenging activities of green tea (GT) and black tea (BT) extracts on brain mitochondrial membrane fraction, against iron (2.5 μM)-induced lipid peroxidation. Both extracts (0.6–3 μM total polyphenols) were shown to attenuate the neurotoxic action of 6-hydroxydopamine (6-OHDA)-induced neuronal death. 6-OHDA (350 and 50 μM) activated the iron dependent inflammatory redox sensitive nuclear factor-κB (NF-κB) in rat pheochromocytoma (PC12) and human neuroblastoma (NB) SH-SY5Y cells, respectively. Immunofluorescence and electromobility shift assays showed increased nuclear translocation and binding activity of NF-κB after exposure to 6-OHDA in NB SH-SY5Y cells, with a concomitant disappearance from the cytoplasm. Introduction of GT extract (0.6, 3 μM total polyphenols) before 6-OHDA inhibited both NF-κB nuclear translocation and binding activity induced by this toxin in NB SH-SY5Y cells. Neuroprotection was attributed to the potent antioxidant and iron chelating actions of the polyphenolic constituents of tea extracts, preventing nuclear translocation and activation of cell death promoting NF-κB. Brain penetrating property of polyphenols may make such compounds an important class of drugs for treatment of neurodegenerative diseases.

Keywords: Oxidative Stress; Parkinson's disease; Iron; 6-hydroxydopamine; Nuclear factor-κB; Green tea

1. Introduction

The etiology of nigrostriatal dopamine neuron death in idiopathic Parkinson's disease (PD) is not known. A number of reports from our laboratory, as supported by others, point to the presence of ongoing oxidative stress (OS) and inflammatory processes occurring selectively in the substantia nigra pars compacta (SNPC) of Parkinsonian brains [1–4]. This includes proliferation of reactive microglia with highly significant increase in cytokines TNF-α, interleukin (IL)-1 and IL-2 [5, 6], along with an increase in the levels of iron within the reactive microglia and the melanin containing dopamine (DA) neurons [1, 7–9]. The increase of iron in the reactive microglia and melanin containing DA neurons of SNPC may explain the oxidative stress-induced activation of the redox-sensitive transcription factor, nuclear factor-κB (NF-κB) [10], recently reported in Parkinsonian brains [11]. The studies of Lin et al. [12] using liver macrophages, and our own [13] with rat brain microglia in cell culture have provided evidence for direct participation of chelatable-iron in NF-kB activation and its prevention with iron chelators L-1, desferal and the DA agonist apo-

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Abbreviations: BT, Black Tea; EMSA, Electromobility Shift Assay; EGCG, (-)-Epigallocatechin-3-gallate; GT, Green Tea; NB SH-SY5Y cells, Human neuroblastoma SH-SY5Y cells; 6-OHDA, 6-hydroxydopamine; IL, Interleukin; LDH, Lactate Dehydrogenase; MPTP, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NF-κB, nuclear factor-κB; OS, Oxidative Stress; PD, Parkinson's Disease; ROS, Reactive Oxygen Species; SNPC, Substantia Nigra Pars Compacta; TBARS, thiobarbituric acid reactive substances.

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morphine (APO). The role of reactive oxygen species as second messengers in NF-kB activation and its inhibition with antioxidants such as N-acetyl cysteine [14], vitamin E [15] and aspirin [16], and iron chelators [12], is well established. Thus, the observed elevation of iron in the reactive microglia and DA neurons of Parkinsonian SNPC [3], may initiate OS via Fenton reaction promotion of reactive oxygen species (ROS), resulting in transcription factor NF-kB activation [13].

The approaches to neuroprotection in PD reflect the current concepts of the etiology of the disease. Antioxidant strategies, and iron-chelating/anti-inflammatory strategies have been at the focus of attention [17]. Indeed, iron chelators, desferrioxamine [18] or APO [19, 20] were highly effective to induce neuroprotection *in vivo* and *in vitro*. Recent reports have revealed that polyphenols may be neuroprotective in neuronal primary cell cultures: the polyphenol flavanol epicatechin was shown to attenuate neurotoxicity induced by oxidized low-density lipoprotein in mouse-derived striatal neurons [21]. Moreover, the Ginkgo biloba extract, known to be enriched with flavonoids, has been shown to protect hippocampal neurons from nitric oxide or beta-amyloid derived peptides-induced neurotoxicity [22, 23]. (-)-Epigallocatechin-3-gallate (EGCG), the major polyphenol of GT solid fraction, as well as theaflavin-3,3′-digallate, polyphenol from BT, were shown to inhibit LPS-induced inflammatory TNF-α [24] and iNOS [25–27] mRNA expression and production in macrophages, apparently via reduction of the activity of NF-κB. The bioflavonoid silymarin was found to suppress both NF-κB-DNA binding activity and its dependent gene expression induced by okadaic acid in the hepatoma cell line HepG2 [28]. Thus, it is likely that the neuroprotection exerted by polyphenols may involve regulation of NF-κB activity.

We have recently shown that GT extract and its major catechin component (EGCG), exerted *in vivo* neuroprotective activities and prevented the depletion of striatal dopamine and tyrosine hydroxylase content and activity caused by the parkinsonism inducing neurotoxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in C57/BL mice [29]. In order to confirm and extend the *in vivo* studies, we investigated the neuroprotective effect and regulation of NF-kB nuclear activation by GT and BT extracts in rat pheochromocytoma (PC12) and human neuroblastoma (NB) SH-SY5Y cell cultures, using the neurotoxin 6-hydroxydopamine (6-OHDA), known to induce dopaminergic neurodegeneration via OS (for review see [30]). The possible mutual relationship between neuroprotection and nuclear events will be discussed.

2. Materials and methods

2.1. Materials

Dried GT and BT extracts are from Indonesia, ART. 85007 and 90177, respectively. (Plantextrakt GmbH & Co. KG, Vestenbergsgreuth, Germany) and were obtained from Wissotzky company. Horseradish peroxidase conjugated antibody against mouse Fab and DNA intercalating fluorescent marker Hoechst 33258 were purchased from Sigma Chemical Co., USA. Polyclonal antibody against NF-κB subunit p65 was from Santa Cruz Biotechnology, USA. Rhodamine fluorescent labeled secondary antibody was purchased from Jackson Immunoresearch Lab. Inc., PA, USA. Other chemicals and reagents were of the highest analytical grade and were purchased from local commercial sources.

2.2. Tea extracts

Dried tea extract powders were used from the same stock for every preparation of tea solutions in each experiment, thus minimizing the possible variations in the concentration of the active components that occur between extract preparations. We confirmed that both extracts displayed very similar polyphenol concentrations around 20% of the powdered extracts as determined spectrophotometrically with phosphomolybdic phosphotungstic acid reagent (Narr Ben et al., 1996) and quercetin as a standard.

2.3. Mitochondrial isolation and lipid peroxidation assays

Rat brain mitochondrial homogenates were prepared essentially as previously described [31]. The homogenate buffer consisted of 10 mM Tris-HCl buffer, pH 7.5, 0.25 M sucrose, 2 mM EDTA (sodium salt) and 2% bovine serum albumin. The crude mitochondrial fraction was prepared from the brains by differential centrifugation in 10 mM Tris-HCl and stored at −70° Lipid peroxidation was determined by the formation of thiobarbituric acid reactive substances (TBARS) as described [31]. GT and BT extracts were dissolved prior to use in the reaction buffer and added to the samples before starting the reaction with $FeSO_4$/ascorbate (2.5 μM/25 μM).

2.4. Cell cultures

Rat PC12 cells were grown at 37°, in a humid 5% CO_2, 95% air environment, in a growth medium containing Dulbecco's modified Eagle's Medium (DMEM, GIBCO, BRL) supplemented with glucose (1 mg/mL) streptomycin/penicillin (100 U/mL), sodium pyruvate (110 mg/L), 5% fetal calf serum and 10% horse serum. NB SH-SY5Y cells were grown similarly, but using high glucose concentration (4.5 mg/mL) DMEM and fetal calf serum (10%) only.

2.5. MTT and LDH tests for cell viability

PC12 and NB SH-SY5Y cells were detached by vigorous washing, centrifuged at 200xg for 5 min and resuspended in DMEM with low serum content (2% of total amount contained in regular growth medium), to reduce false positive cross-reaction that may arise from lactate dehydrogenase

(LDH) contained in serum. The cells were placed in microtiter plates (96 wells) at a density of 1.5–2 × 10^4 cells/well and were allowed to attach for 24 hours before treatment. One row contained medium only for background substraction. BT and GT extracts were added 15 min before insult with 6-OHDA, for a subsequent 24 h. The plates were centrifuged at 250xg for 10 min and 100 μL of medium were transferred to a new plate for LDH analysis using a cytotoxicity detection kit (Boehringer, Mannheim, Germany). The absorption was determined in a Perkin-Elmer Dual Wavelength Elisa-Reader at OD_{532} nm and the background readings were automatically subtracted.

In parallel, the remaining cells were washed with fresh medium and subjected to MTT test as previously described [20]. The absorption was determined in a Perkin-Elmer Dual Wavelength Eliza-Reader at λ = 570/650 nm after automatic subtraction of background readings. The results are expressed as percentage of the untreated control.

2.6. Preparation of nuclear extract

The extraction and isolation of nuclear fraction were performed based on the method of Dignam et al. [32] with some modifications: cells (2 × 10^6) were seeded onto 100 mm plates in culture medium with full serum for 48 h. The medium was then replaced with 10 mL fresh medium containing 2% serum for an additional 24 h (subconfluent culture). 6-OHDA was added to NB SH-SY5Y and PC 12 cells (50 and 350 μM, respectively) for different time intervals. When required, GT extract was added 15 min before addition of 6-OHDA. At the end of incubation, the plates were immediately transferred onto an ice-cold tray, the medium was aspirated and 3 mL ice-cold PBS supplemented with EDTA-free mixture of protease inhibitors, (Complete™, Boehringer, Mannheim, Germany) were added. The cells were scraped with a rubber policeman, centrifuged for 15 sec at 14,000xg, at 4° and the pellet was washed with 1 mL ice-cold PBS + protease inhibitor mix and transferred to 1.5 mL polypropylene tubes. The pellet was resuspended in 2 cell pellet volumes (volume prior to the initial volume of buffer A) of ice-cold buffer A (10 mM Hepes, pH = 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.5% Np-40), supplemented with protease inhibitor mixture. After pipetting the homogenates 15–20 times, the tubes were incubated for 5 min on ice to promote lysis. The samples were then centrifuged for 20 sec at 14,000xg, at 4° and the supernatant, consisting of the cytosolic fraction, was immediately frozen for further analysis. The pellet was washed once with ice-cold buffer A and once with buffer A without Np-40. The homogenates were centrifuged as described above. The pellet was resuspended in 0.5 cell pellet volumes of cold buffer C (20 mM Hepes, pH = 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, and protease inhibitors mix). The tubes were vortexed for 20 min at speed 1, centrifuged for 20 sec at 14,000 g, and the supernatants containing the nuclear extracts, placed in fresh tubes. Then, an equal volume of cold buffer D (20 mM Hepes, pH = 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, and protease inhibitors mix) was added. The samples were aliquoted into 3 tubes, immediately frozen on dry ice and kept at −70°. Protein concentration of both fractions was determined with Bradford reagent (Sigma Chemicals Co., USA), at 595 nm.

2.7. Electrophoretic mobility shift assay (EMSA)

20 μg of nuclear protein were mixed with 1–2 ng [α-^{32}P]dCTP end labeled double-stranded NF-κB oligonucleotide (~10,000 cpm), top strand: 5′-CAACGGCAGGG GAATTCCCCTCTCCTTGGTT-3′, bottom strand: 5′-AAGGAGAGGGGAATTCCCCTGCCGTTGGGTT-3′, in binding buffer (10 mM Tris pH 7.5, 1 mM EDTA, 1 mM DTT, 5% glycerol, 1 mg/mL salmon sperm DNA, 1 mg/mL poly (dIdC), 0.05% NP-40). The tubes were incubated at room temperature for 20 min. Specificity of binding was examined by competition with an excess of unlabeled oligonucleotide. The DNA/protein complexes were electrophoresed on 4% non-denaturing polyacrylamide gel in TBE buffer, pH 8.0 and run at 120 V for 2.5 hours at room temperature. Dried gels were autoradiographed overnight at −70°. Quantification of bands was accomplished by the densitometry program Bio-Profil (Vilber Laurmat, France).

2.8. Immunocytochemistry

NB SH-SY5Y cells were placed on 8 wells chamber slides (8 × 10^4 cells/well) in medium with low serum content (1/3 of growth medium) and allowed to attach for 48 hours before treatment. At the end of treatment the slides were washed three times with ice-cold PBS and fixed with 4% formaldehyde in PBS for 10 min at RT. After three additional washes, permeabilization was performed in 1% FCS + 0.5%Triton x-100 in PBS, for 15 minutes at room temperature. The slides were incubated with polyclonal antibody against NF-κB subunit p65 at a dilution of 1:50, in Triton x-100/FCS in PBS for 2 hours at RT with shaking. After four washes immunoreactive NF-κB was revealed by a 30 min incubation with Rhodamine fluorescent labeled secondary antibody diluted 1:50. Nuclei were visualized by the DNA intercalating fluorescent marker Hoechst 33258 (0.4 μg/mL). The labeling was analyzed by fluorescence microscopy.

2.9. Image and data analysis

Cytoplasmic or nuclear staining for NF-κB were counted on each stained section by a computer-based quantitative analysis of the area of interest (analySIS, SIS GmbH).

2.10. Statistics

One-way analysis of variance followed by the Tukey test, or student *t*-test, were performed using the scientific

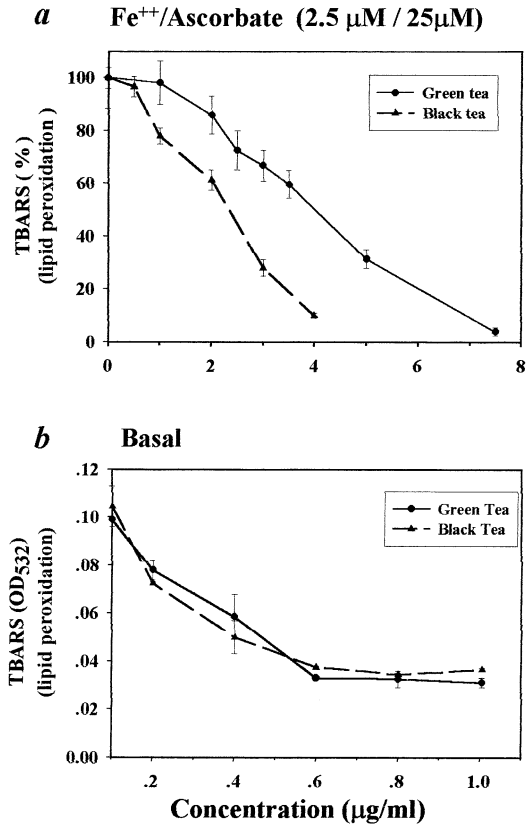

Fig. 1. Inhibition of brain mitochondrial membrane lipid peroxidation by green tea and black tea extracts. A; TBARS formation was promoted by $FeSO_4$ (2.5 μM)/ascorbic acid (25 μM) using rat brain mitochondrial preparation. IC_{50}s for GT and BT are 4 and 2.3 μg/mL, equivalent to 2.44 and 1.40 μM total polyphenols, respectively. B; Basal production of TBARS in the absence of $FeSO_4$. The IC_{50}s for both GT and BT extracts are 0.24 μM total polyphenols. The data are the mean ± SEM of a representative experiment conducted in triplicates and repeated at least 3 times.

statistic software *GraphPad Instat*™ version 2.04. *P* values of less than 0.05 were considered significant.

3. Results

3.1. Antioxidant properties of tea extracts in brain mitochondrial fraction.

Tea and tea polyhenols have been shown to possess antioxidant effects both *in vitro* and *in vivo.* We decided first to determine the radical scavenger activity of GT and BT extracts in brain mitochondrial membrane preparation. For this purpose, the oxidative process was promoted by Fe^{2+}/ascorbate. Both BT and GT extracts displayed high potencies in inhibiting lipid peroxidation as measured by the formation of TBARS, with IC50s of 1.40 and 2.44 μM total polyphenols (2.3 and 4 μg/mL extract), respectively (Fig. 1A). In addition, the compounds were equally effective in reducing the basal levels of lipid peroxides, in the absence of Fe^{2+}, from the mitochondrial fraction, IC_{50}s 0.24 μM total polyphenols (Fig. 1B).

3.2. Neuroprotective effects of tea extracts in neuronal cell cultures

The possible protective effect of tea extracts against neurotoxin-induced cell death was assessed in cultures of NB SH-SY5Y and PC12 cells. The cells were pretreated with increasing concentrations of GT or BT extracts (0–60 μM total polyphenols) before addition of 6-OHDA (50 or 350 μM, respectively) for an additional 24h. NB SH-SY5Y cells survival was dramatically reduced by 6-OHDA (13% of control) as assessed by MTT test, while pretreatment with GT or BT extracts doubled and tripled the viability of the cells respectively, when compared to 6-OHDA alone (Fig. 2A). The effects were dose dependent between 0.06 and 6 μM total polyphenols. On the other hand, doses higher than 6 μM promoted damage. The protective effect of BT and GT extracts was also demonstrated by measuring LDH leakage induced by 6-OHDA. The maximum protective polyphenol concentrations of both extracts ranged from 0.6–6 μM, correlating with the effective doses seen with MTT test (Fig. 2B).

A similar picture was also observed with PC12 cells, except that a higher dose of 6-OHDA (350 μM) was required than that used with neuroblastoma cells, indicating that the latter cells are more sensitive to 6-OHDA than PC12 cells (Fig. 2C and D).

3.3. Kinetics of 6-OHDA-induced NF-κB binding activity

The mechanism of 6-OHDA-induced cell damage was investigated by examining NF-κB translocation in PC12 and NB SH-SY5Y cells, using polyclonal antibody against p65 subunit of NF-κB (Fig 3A and B, respectively). Maximal NF-κB nuclear translocation upon exposure to 6-OHDA occurred at 35 and 90 min, respectively.

The activity of NF-κB was assessed in NB SH-SY5Y cells by EMSA. Upon 6-OHDA administration (50 μM) NF-κB binding increases in the nuclear fraction as a function of time reaching its maximum at 90 min, with a concomitant disappearance from the cytoplasm. This effect was reversed after 2 hours (Fig 4A). The bands were quantified by densitometry and the changes are shown in Fig. 4B.

3.4. Effect of green tea extract on NF-κB binding activity in human neuroblastoma SH-SY5Y cell nuclear fraction

Based on the well established antioxidant and anti-inflammatory properties of tea polyphenols, we investigated whether GT can prevent activation of NF-κB. NB SH-SY5Y cells were pretreated for 15 min with GT (0.6 and 3 μM) and then exposed to 6-OHDA (50 μM) for an additional 90 min. 6-OHDA significantly increased NF-κB

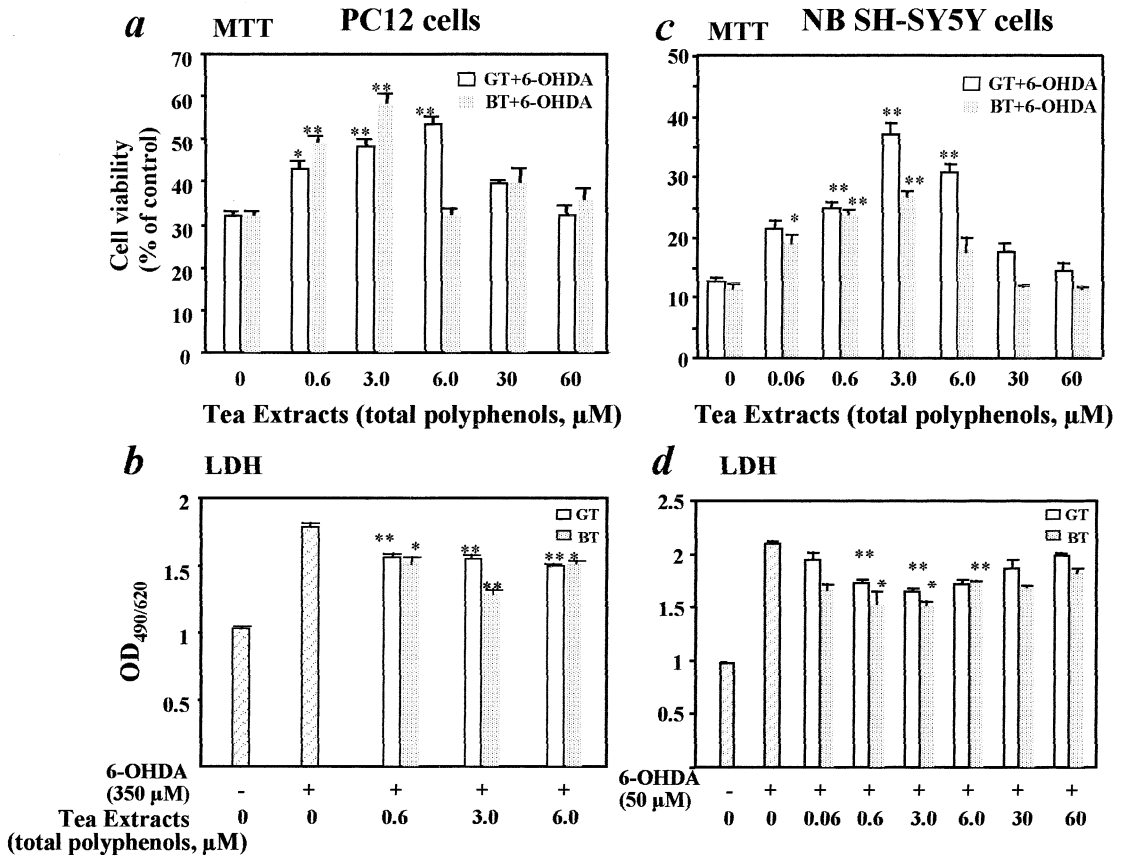

Fig. 2. Protection by green tea and black tea extracts against 6-OHDA-induced neurotoxicity in neuronal cell lines. NB SH-SY5Y or PC12 cells were pretreated with increasing concentrations of tea extracts 15 min before the addition of 6-OHDA (50 or 350 μM, respectively) for 24 hours. A,C; cell viability was assayed with MTT and expressed as percentage of controls (without 6-OHDA). B,D; LDH leakage was determined 24 hours after treatment and expressed as $OD_{490/620}$ units. The results are the mean + SEM, n = 8. one-way ANOVA; $*P < 0.05$, $**P < 0.001$, vs 6-OHDA).

binding activity in nuclear fraction from NB SH-SY5Y cells after a 90 min exposure time (Fig. 5). However, pretreatment with GT extract abolished this activation already at 0.6 μM total polyphenols. Specificity of binding was examined by competition with an excess of unlabeled probe.

3.5. Inhibition of NF-κB nuclear translocation by green tea in cultured human neuroblastoma SH-SY5Y cells

To emphasize these findings, immunofluorescence studies were conducted to assess translocation of the NF-κB protein to the nucleus in cultured NB SH-SY5Y cells. In control, untreated cells the majority of the protein is preferentially located in the cytoplasm, as observed by immunofluorescent NF-κB location and image analysis quantitation (8–10% in the nucleus, Fig. 6B). Upon 6-OHDA treatment NF-κB immunoreactivity was considerably increased in the nucleus (25–30%, Fig. 6E). GT extract (3 μM total polyphenols) considerably inhibited the translocation of NF-κB to the nucleus, most of the protein being localized to the cytoplasm (10–15%, Fig. 6H), confirming the results obtained by EMSA analysis (Fig. 5). These results are further emphasized in Fig. 6C, F and I by superimposition of red NF-κB labeling on the Hoechst blue stain.

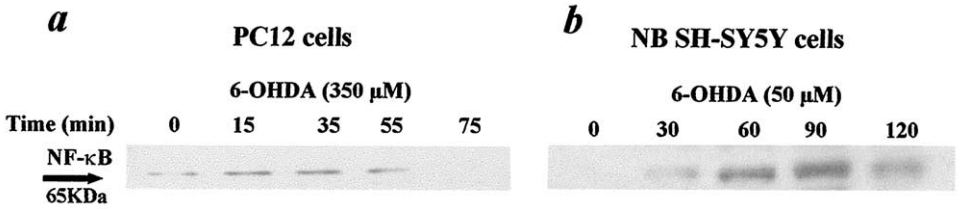

Fig. 3. Immunoblotting of NF-κ B protein in nuclear homogenates. A; PC12 cells were exposed to 6-OHDA (350 μM) for different time intervals. NF-κ B immunoreactivity was detected in the nuclear fraction with a rabbit polyclonal antibody (1:2,000) against the p65 subunit of NF-κ B. B; NB SH-SY5Y cells were treated as in A except that the dose of 6-OHDA used was 50 μM.

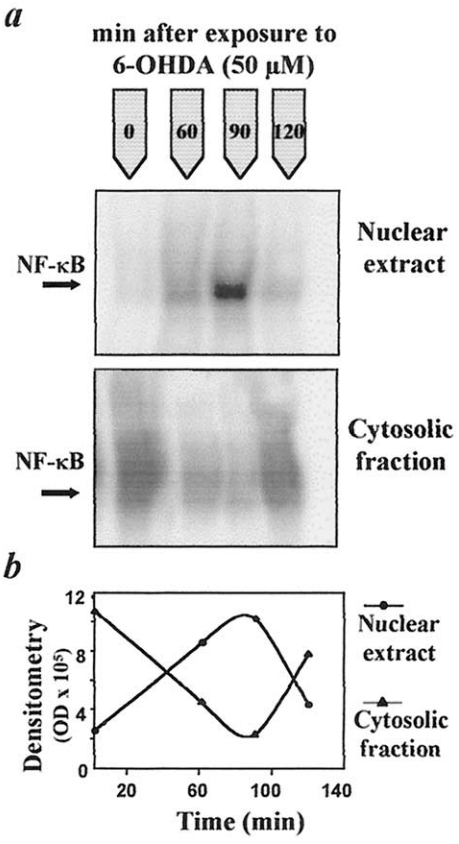

Fig. 4. Kinetics of 6-OHDA-induced NF-κ B translocation in human neuroblastoma SH-SY5Y cells. A; NB SH-SY5Y cells were stimulated with 6-OHDA (50 μM) and the binding of NF-κ B to a end-labeled probe carrying a NF-κ B motif, in nuclear and cytosolic extracts, was assessed by EMSA. B; The bands were quantified by densitometry and the changes are represented in the graph.

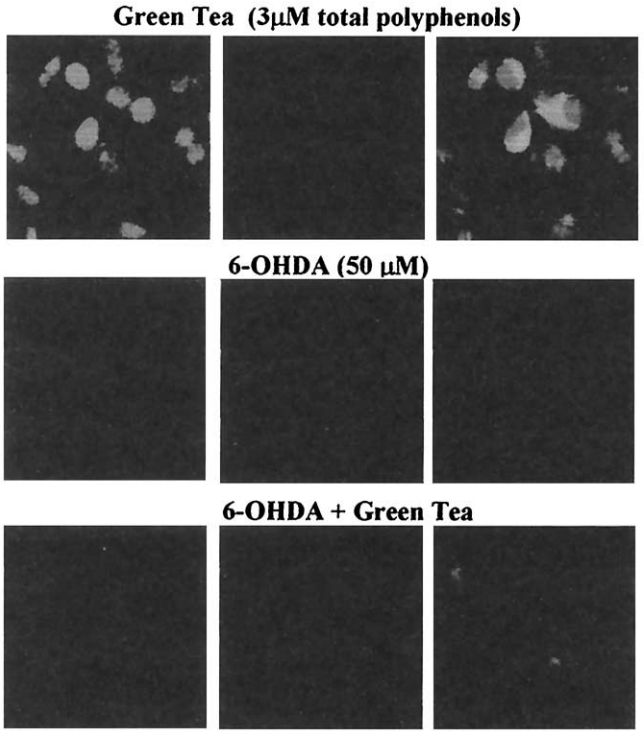

Fig. 6. Immunofluorescence analysis of 6-OHDA-induced NF-κB nuclear translocation in human neuroblastoma SH-SY5Y cells and its prevention by green tea extract. B, E,H; NF-κB was detected by rhodamine immunofluorescence (red) with an antibody against the 65-kDa subunit. A, D, G; nuclei were visualized with the fluorescent intercalating agent Hoechst 33258 (blue). In control cells, that received GT only (3 μM total polyphenols), NF-κB immunoreactivity was preferentially localized in the cytoplasm and appears red (B, green arrows). After the addition of 6-OHDA (50 μM, 90 min), NF-κB was principally detected in the nuclei (yellow arrows, E). Upon pretreatment with 3 μM GT extract polyphenols (15 min before the insult) most NF-κB labeling remained in the cytoplasm (H). C, F, I; superimposition of rhodamine on Hoechst fluorescence emphasizes the presence of the transcription factor either in cytoplasm, nuclei, or both.

4. Discussion

This study is the first to show neuroprotective effects of tea extracts on Parkinsonism inducing neurotoxin 6-OHDA-initiated cell death in culture (PC12 and NB SH-SY5Y cell lines).

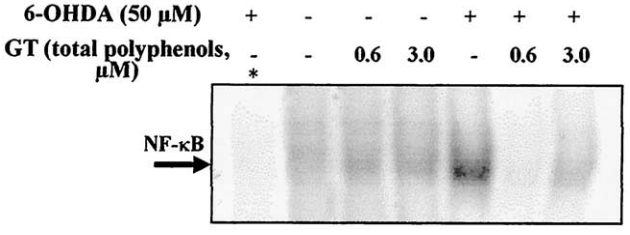

Fig. 5. EMSA analysis of 6-OHDA-induced NF-κB activation in human neuroblastoma SH-SY5Y cells and its prevention by green tea extract in isolated nuclear fraction. NB SH-SY5Y cells were pretreated for 15 min with 1 and 5 μg/mL GT extract (equivalent to 0.6 and 3 μM polyphenols) and subsequently exposed to 6-OHDA (50 μM). Nuclear extraction was carried out 90 min later and the binding of NF-κB to an end-labeled probe carrying a NF-κB motif was assessed by EMSA. *Specificity of binding was assessed by addition of an excess of unlabeled oligonucleotide probe.

The current hypothesis concerning the pathogenesis of idiopathic PD, points to selective OS that expresses itself with compatible biochemical changes in SNPC. Therefore, drugs that exhibit free radical-scavenging and iron chelating properties, may serve as potential candidates for the treatment of PD. Indeed, tea extracts and tea polyphenols have been previously reported to possess antioxidant [33–35], and iron chelating properties [36, 37]. In addition, EGCG, the main constituent of GT, was shown to be easily absorbed from the digestive tract and penetrate the brain reaching levels similar to those found in lung, liver, kidney and others [38]. In this study, both GT and BT extracts strongly inhibited lipid peroxidation promoted by iron-ascorbate in brain mitochondrial membranes (IC_{50}: 2.44 and 1.40 μM, respectively). The antioxidant effect of tea extract in brain tissue is supported by previous work using brain synaptosomes, in which the four major polyphenol catechines of GT were shown to inhibit iron-induced lipid peroxidation [36]. Furthermore, both extracts were equally

potent in inhibiting basal lipid peroxidation with IC_{50} of 0.24 μM.

GT and BT exhibited potent neuroprotection against 6-OHDA-induced NB SH-SY5Y and PC12 cell damage in culture, as indicated by MTT and LDH tests. 6-OHDA is a highly reactive substance, which is readily autooxidized and oxidatively deaminated by monoamine oxidase, to give rise to H_2O_2 and ROS [30]. In addition, it releases iron from ferritin [39, 40] and potently inhibits mitochondrial complex I activity [41]. Free-redox iron exacerbates formation of the highly reactive hydroxyl radical, which in turn can cause DNA strand breaks, damage protein residues and initiate lipid peroxidation reactions [42]. These results are supported by our recent findings *in vivo* where both GT extract and EGCG prevented HPTP-induced loss of SN neurons, striated DA levels and reduction of tyrosine hydroxylase content and activity [29]. One possible mechanism underlying the effectiveness of GT and BT tea against 6-OHDA neurotoxicity, may involve its catechol-like structure since it is known that catechol-containing polyphenols are potent radical scavengers and chelators of ferric ion [43]. Indeed, we have previously shown that the catechol derivative DA agonist R-APO, is a potent iron chelator and radical scavenger in brain mitochondrial fraction [31] and protects PC12 cells against toxicity induced by 6-OHDA and H_2O_2 [20]. In addition, the GT polyphenol EGCG also was shown to prevent 6-OHDA and 1-methyl-4-phenylpyridinium-induced PC12 and NB SH-SY5Y cell death (data not shown).

Alternatively, a possible direct scavenging of oxidized 6-OHDA by tea extracts during the 24h incubation period cannot be ruled out. However, the concentrations of GT and BT extracts required for neuroprotection are far lower than those of 6-OHDA used in the assay, thus it may not be a simple stoichiometric reaction. This assumption is also supported by the fact that tea extract concentrations higher than 6 μM total polyphenols could not prevent the toxic effects of 6-OHDA.

The actions of GT and BT extracts are biphasic phenomenon: they protected against 6-OHDA at concentrations up to 6.0 μM total polyphenols, while higher doses promoted damage. A similar biphasic effect was obtained in our *in vivo* studies in mice pretreated with GT followed by MPTP (24 mg/kg) injection for 4 days. Low concentrations (0.5–2.5 mg powdered extract/kg, corresponding to 0.30–1.52 μmoles total polyphenols/kg) were neuroprotective, whereas 6.08 μmoles total polyphenols/kg promoted striatal DA content loss [29]. This bell-shaped pattern is typical of antioxidative drugs, such as vitamin C [44] and R-APO [20], being neuroprotective at few micromolar concentrations, while having pro-oxidant activity at higher doses.

Although a large number of studies have shown a wide spectrum of biological actions for GT and BT polyphenols, including antioxidative, anti-inflammatory and antitumorigenic effects, only few studies have been conducted on the potential effects of BT. Wang et al. [45] showed that administration of BT was comparable to GT as an inhibitor of UVB-induced skin carcinogenesis in mice. A recent report [27] described anti-inflammatory activity of the BT polyphenol, theaflavin-3,3′-digallate against LPS-induced NF-κB activity, through down-regulation of IkB kinase activity in macrophages. In our study, both tea extracts displayed similar potencies as radical scavengers and neuroprotective agents against 6-OHDA-induced neurotoxicity in neuronal cell cultures. The concentrations at which the effects of GT and BT were achieved were relatively low (1–5 μg/mL extract, 0.6–3 μM total polyphenols).

In the present study we have demonstrated that the mechanism of damage by 6-OHDA in neuronal cell cultures involves activation of NF-κB. Oxidative stress-induced ROS generation has been implicated in NF-κB activation [46], though their exact target is still unknown. Therefore, we suggest that the OS produced by 6-OHDA may be responsible for NF-κB increased nuclear translocation and binding activity. Support for this findings comes from Hunot et al. [11] who found a 70 fold increase in NF-κB immunoreactivity in the nucleus of melanized dopaminergic neurons of the SNPC, compared to non-Parkinsonian brains. In the recent years, increasing evidence has accumulated indicating OS and inflammation as common components of several neurodegenerative diseases including elevation in cytotoxic cytokines in the microglia and in the surrounding neurons [47]. This and the increased levels of iron in the reactive microglia and melanin-containing DA neurons of SNPC [9] could be responsible for the activation of NF-κB in PD [13]. This situation resembles the events occurring in peripheral macrophages, in which the iron chelator desferrioxamine inhibited LPS-induced activation of NF-κB [12]. Indeed, we recently reported increased activation of NF-κB in brain striatum of 6-OHDA-treated rats and its prevention by pretreatment with desferrioxamine [48]. This is supported by our recent gene expression analysis following mice MPTP treatment, using cDNA microarray, where the elevated expression of NF-κB p65 subunit mRNA *in vivo*, was prevented by pretreatment by the iron chelator-radical scavenger R-APO [49, 50]. Furthermore, NF-κB has been shown to be activated in animal-induced focal ischemia [51], in rat brain after kainate-induced seizures (for review see [52]), as well as in neurons and astrocytes in the immediate vicinity of the amyloid plaques in brain sections from Alzheimer's disease patients [53]. Whether the activation of NF-κB associated with amyloid deposits is a neurodegenerative event remains to be elucidated.

Activation of NF-κB nuclear translocation and binding induced by 6-OHDA, was prevented by pretreatment with GT. This effect is understandable if we consider the chemical structure of the polyphenols. It is suggested that the galloyl moiety attached to flavan at position 3 and ortho trihydroxyl group in the B ring, are responsible for their strong scavenging activity and iron chelating properties [36], [34]. Thus, we hypothesize that free radical scaveng-

ing action and modulation of intracellular free iron levels by GT, may play a role in inhibition of NF-κB activation.

In spite of the growing data on the involvement of NF-κB in brain neuropathology, there are some reports indicating an important neuroprotective role of constitutive NF-κB expression in cortical and hippocampal neurons [54]. Therefore, whether NF-κB will be either protective or detrimental may depend on the duration, the circumstances and the type of cells. This is presently being investigated in *in vivo* studies.

Although the exact specific cell targets of polyphenol action are still unrevealed, its mechanism of action appears to involve antioxidant-radical scavenging and iron chelating properties. Our data indicate that GT and BT extracts contain potent neuroprotective agents, the full composition of which is not known. The prospects for neuroprotection must consider either the use of combination of different drugs or individual drugs having multipharmacological actions [17]. The variety of compounds present in tea extracts, displaying different actions, make them a potential compound in the treatment of neurodegenerative diseases such as PD and Alzheimer's disease, where OS has been implicated in their pathogenesis. As a consequence we are currently studying the neuroprotective activities of individual and the combination of the purified major GT polyphenols [(-)epigallochatechin-3-gallate, (-)epigallochatechin] and BT (theaflavin-3,3′-digallate) constituents.

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