Full Paper

(+)−Catechin, an Ingredient of Green Tea, Protects Murine Microglia From Oxidative Stress-Induced DNA Damage and Cell Cycle Arrest

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Abstract. Extracts of Chinese herbs have been demonstrated to inhibit oxidative stress in vitro. In this study, we investigated the mechanism of (+)-catechin, isolated from green tea, which preserved murine microglia N9 cells from an oxidative agent tert-butylhydroperoxide (tBHP)-induced cell death. (+)-Catechin augmented the cell survival ratio after exposure to tBHP. Protective action of this drug was more efficacious than that of N-acetylcysteine, which is a putative antioxidant. DNA damage, detected by the Comet assay, was diminished with treatment of the drug. Results of flow cytometric analysis showed that the amount of intracellular 'OH was decreased, and the cell cycle arrest was reversed by down-regulation of p53 phosphorylation after treatment with (+)-catechin. The reduced p53 activity followed the impairment of NF-κB translocation to the nuclear region. Then the phosphorylation of extracellular signal regulated protein kinase, a cell survival facilitative signal, was upregulated at the later stage. Taken together, (+)-catechin inhibited tBHP-induced translocation of NF-κB to improve cellular survival.

Keywords: (+)-catechin, DNA damage, cell cycle arrest, NF-κB, p53

Introduction

Numerous studies have reported increased reactive oxygen species (ROS) and oxidative stress in various neurodegenerative disorders including Parkinson’s disease (PD), Alzheimer’s disease (AD), and amyotrophic lateral sclerosis (ALS) (1 – 3). Studies have demonstrated that redundant intracellular ROS can lead to the destruction of cellular components, including lipid, protein, and DNA, and even causes cell death via apoptosis or necrosis (4). Many kinds of ROS, such as hydroxyl radical (‘OH), superoxide anions (O2−), and peroxynitrite anions (ONOO−), are generated as side-products during oxygen metabolism. Under normal physiological conditions, the generation and clearance of ROS in cells maintain a balanced state with the help of endogenic biological antioxidants, including glutathione, vitamin E, ascorbic acid, and some antioxidative enzymes. In addition, some compounds in the diet are also important antioxidants sources (5, 6).

(+)−Catechin is a flavanol antioxidant extracted from some Chinese herbs: Acacia catechu L., cistus species (7, 8). It is also abundantly contained in tea, especially in green tea (9). Polyphenols of tea have been demonstrated to modulate carcinogen metabolizing enzymes and to act in cancer chemoprevention (10, 11). (+)-Catechin possesses various pharmacological functions including treatment of viral hepatitis, depression UVB-induced skin stress, and inhibition of intestinal tumor (12 – 14). However, the mechanism by which (+)-catechin protects nerve cells from death under conditions of oxidative stress remains unclear.

The oxidative stress activates many signal transduction pathways, but one of the most widely studied pathways is the nuclear factor-kappa B (NF-κB). NF-κB widely exists in the cells and participates in regulation of inflammatory responses, cell survival, and differentiation (15, 16). It is composed of a heterodimer of p50 and p65 in the cytoplasm, and it displays an inactive form when it is bound to its inhibitory unit I-kappa B (I-κB). Under certain stimulus, including oxidizing stress, ultraviolet irradiation, and inflammatory cyto-
kines, I-κB kinases (IKKs) phosphorylate I-κB, and this event results in the release of NF-κB. Subsequently, the released NF-κB translocates into the nuclear region to regulate the expression of numerous target genes.

DNA damage, caused by a variety of stimuli including oxidative stress, arrests the cell cycle to allow damage repair or direct cell death. The p53 protein, the product of the tumor suppressor gene p53, is a regulator of cell cycle progression and apoptosis in many cell lines (17). The mitogen-activated protein kinase (MAPK) cascade is an important signaling modulatory pathway that propagates extracellular stimulation into intracellular responses and regulates cell growth, differentiation, and apoptosis. Extracellular signal regulated protein kinase (ERK), one of the subfamily members of MAPKs, modulates cell survival.

In this study, we chose a widely used oxidative agent tert-butylhydroperoxide (tBHP) to injure murine microglia cells to investigate the antioxidative mechanism of (+)-catechin.

Materials and Methods

Chemicals

(+)-Catechin, isolated from *Thea viridis*, was obtained from Beijing Institute of Biological Products (Beijing, China) (Fig. 1). The purity was measured by HPLC and determined to be above 98%. N-Acetylcysteine (NAC), proteinase K, propidium iodide (PI), 3[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium (MTT), dithiothreitol (DTT), 3,3-diaminobenzidine tetrahydrochloride (DAB), RNase A, tBHP, and ethidium bromide were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Dalian Biological Reagent (Dalian, Liaoning, China). Iscove’s modified Dulbecco’s medium (IMDM) and horse serum were purchased from Hyclone (Logan, UT, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, OR, USA).

Rabbit polyclonal antibodies against NF-κB p65, phospho-IκBa, phospho-p53, p53, murine polyclonal antibodies against phospho-ERK, and horseradish peroxidase-conjugated secondary antibody (goat-anti-rabbit) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The murine microglia cell line (N9) was generously provided from Dr. Wu Chunfu (Department of Pharmacology, Shenyang Pharmaceutical University, Shenyang, China). The cells were cultured in IMDM with 5% FBS, 5% horse serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Cell viability assay

N9 cells were seeded into 96-well plates (NUNC, Roskilde, Denmark) at 1 × 10⁶ cells per well in 100 µl IMDM overnight. After preincubation with tBHP at the given concentrations for 1 h, the cells were treated with various concentrations of (+)-catechin. Viabilities were measured by MTT assay using a Plate Reader (Bio-Rad, Hercules, CA, USA).

DNA strand break assay

The alkaline single cell gel electrophoresis (Comet assay) was performed using a slight modification of the previously described method (18). Briefly, 1 × 10⁶ cells were incubated in each culture flask for 12 h. The cells were pre-treated with or without (+)-catechin for 1 h and then washed and exposed to 0.3 mM tBHP for 30 min. The cells from the control or treated samples were mixed with 80 µl of warm low-melting point agarose (LMP) (1%, 37°C) in a microfuge tube and spread on a glass slide pre-coated with 200 µl of 1% normal-melting point (NMP) agarose. After gelling at 4°C, the cover slips were spread with 1% NMP agarose and kept at 4°C. The slides were immersed in a jar containing freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% Triton X-100, and 10% DMSO). Lysis was done at 4°C for 1 h in the dark, and then washed in a freshly prepared alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na₂EDTA, pH >13). The DNA was electrophoresed at 300 mA, washed twice in a neutralizing buffer (0.4 M Tris, pH 7.5), and then stained with 50 µg/ml ethidium bromide. The cells were photographed using a fluorescent microscope (Leica, Nussloch, Germany). The damage profile of DNA was assessed by scoring of the tail length of the comets randomly observed for at least 100 cells on each slide.

Fig. 1. Chemical structure of (+)-catechin.
Measure of intracellular ROS

Cells (1 × 10^6) were incubated in 100-mm dishes. While being treated with (+)-catechin after exposure to tBHP, the cells were digested and washed with phosphate-buffered saline (PBS) three times. The cells were suspended in fresh medium and stained with 0.5 mM DCFH-DA for 15 min at 37°C and then added to 10 μg/ml PI before measuring in a flow cytometer (Becton Dickinson FACScan; Franklin Lakes, NJ, USA). The cells, PI-negative, were chosen to detect the quantities of intracellular OH. Data were analyzed by using CellQuest software (Becton Dickinson, San Jose, CA, USA).

Flow-cytometric analysis for cell-cycle phase

Following treatment with (+)-catechin after exposure to tBHP, N9 cells, both adherent and floating, were pelleted and washed with PBS three times and stained with 1.0 ml of staining solution, containing 50 μg/ml PI, 1 mg/ml RNase A, and 0.1% Triton X-100 in 3.8 mM sodium citrate; and then they were incubated on ice for 30 min in the dark. The samples were analyzed in the flow cytometer. The CellQuest software was used to analyze the percentage of cells at different phases of the cell cycle.

Preparation of nuclear and cytoplasmic extracts

Extracts of nuclear and cytosol were prepared according to the modified method of Nalini et al. (19). Briefly, after treatment with or without (+)-catechin, 3 – 5 × 10^6 cells were washed with 0.5 ml PBS and suspended in 20 μl of buffer A; containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, and 0.1% NP-40, per 1 × 10^6 cells; allowed swell on ice for 10 min; mixed; and then centrifuged at 12,000 × g at 4°C for 10 min. The nuclear pellet obtained was resuspended in 15 μl of buffer B, containing 20 mM Hepes, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 5 μg/ml leupeptin; incubated on ice for 15 min; and centrifuged at 12,000 × g for 10 min at 4°C. The supernatant, the nuclear extract, was transferred to new tubes. These were either used immediately or stored at −70°C for later use.

Western-blot analysis

Cells were suspended in lysis buffer [50 mM Tris, 5 mM edetic acid, 150 mM NaCl, 0.5% NP-40, 10 μg/ml leupeptin (Sigma), 10 μg/ml aprotinin (Sigma), 1 mM PMSF (Sigma)] on ice for 1 h. Cell lysates were centrifuged at 4,000 × g, at 4°C for 15 min. Protein concentrations in the supernatant were determined by using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). Total cellular proteins were resolved by 10% SDS-PAGE and blotted onto a nitrocellulose membrane. Special anti-p53, anti-phospho-p53, anti-β-actin, anti-C/EBP, anti-NF-κB p65, and anti-phospho-IκBα (Santa Cruz) antibodies were applied to detect those proteins. Anti-rabbit IgG and anti-mouse IgG (Santa Cruz) conjugated with horseradish peroxidase (HRP) were used as secondary antibodies, and DAB (Sigma) as the HRP substrate, was used to visualize proteins.

Statistical analysis

One-way ANOVA followed by LSB test (SPSS 11.0 software; SPSS, Chicago, IL, USA) was used for statistical analyses of the results. Data were expressed as means ± S.D., and they were considered significant at P<0.05.

Results

(+)-Catechin reduced tBHP-induced cytotoxicity

tBHP has been frequently used for induction of oxidative stress in vitro, such as induction of apoptosis on human hepatoblastoma cells (20 – 22). In this study, different doses of tBHP, ranging from 0.1 to 1.0 mM, were tried to determine if they would cause N9 cell death. The cell death ratio reached the plateau at the tBHP dose of 0.6 mM (data not shown), and a half dose of tBHP, 0.3 mM, was chosen to induce cell death. As shown by the results given in Fig. 2, with increasing concentrations of (+)-catechin from 0.13 to 2 mM, the protective effect of (+)-catechin on N9 cells increased significantly after treatment for 24 h, and the viability...
peaked at 64.63% at 1 mM. The protective effect of (+)-catechin was also supported by morphological changes (Fig. 3). Most of the cells showed characteristics of cell death: shrinkage and floatation in the tBHP-exposed group (Fig. 3B), markedly different from the characteristics of the adherent cells in the medium control group (Fig. 3A). After treatment with 0.5 mM (+)-catechin, the majority of cells grew normally (Fig. 3C). To estimate the anti-oxidative effect of (+)-catechin, the viabilities after treatment with (+)-catechin or NAC were analyzed by the MTT method (Fig. 4). The data indicated that (+)-catechin had more potent protective effect than NAC at the doses ranging from 0.25 to 2 mM.

**Effect of (+)-catechin on the intracellular ‘OH**

Redundant intracellular ROS level is associated with DNA damage and protein cross linkage (23). The quantities of intracellular ‘OH were measured using a fluorescent probe, DCFH-DA. DCFH-DA diffuses through the cell membrane and is hydrolyzed by intracellular esterases to non-fluorescent DCFH, which is then rapidly oxidized to highly fluorescent DCF in the presence of ‘OH. The DCF fluorescence intensity is proportional to the amount of ‘OH formed intracellularly. (+)-Catechin markedly reduced tBHP-induced intracellular ‘OH radical increase at 12 h (Fig. 5: A and B).

**Protective effect of (+)-catechin on DNA damage**

Hydrogen peroxide is known to diffuse into the cell nucleus and cause damage in DNA by generating the ‘OH in close proximity to the DNA. Alkaline single cell gel electrophoresis revealed that the number of DNA strand breaks in the cells was significantly higher in the tBHP-exposed group than the (+)-catechin treated one (Fig. 6: A and B). Although the comet tail length of the (+)-catechin treated group increased slightly compared to that in the control group, it also markedly decreased about one fold compared with the tBHP-exposed group.

**Modulation of NF-κB translocation in (+)-catechin treated N9 cells**

It had been reported that the NF-κB pathway is activated by enhanced ROS generation in various cell
To examine the role of NF-κB in tBHP-exposed cells after treatment with (+)-catechin, Western blot analysis was carried out to detect the changes of phosphorylated IκB and the expression of NF-κB p65 (Fig. 7). After treatment with 0.5 mM (+)-catechin for 1, 2, 4, 6, and 10 h, the nuclear and cytoplasmic proportion of NF-κB p65 was examined to confirm nuclear the translocation of NF-κB. The results demonstrated that in the (+)-catechin un-treated group, nuclear NF-κB p65 expression markedly increased and was maintained from 4 to 10 h; meanwhile, cytoplasmic NF-κB p65 decreased slightly. In comparison, after treatment with (+)-catechin, the expression of NF-κB p65 in the nuclear region increased from 2 to 4 h and then began to decline. The total expression of NF-κB p65 showed no significant change. In addition, in the (+)-catechin untreated group, the p-IκB expression was significantly enhanced from 4 to 10 h. However, after treatment with (+)-catechin, it slightly increased at 2 h and then began to decrease from 4 to 10 h. The timepoint of NF-κB translocation coincided with that of IκB phosphorylation. Taken together, these results suggested that (+)-catechin inhibited the translocation of NF-κB by exposure to ROS.

Involvement of p53 and ERK on (+)-catechin reversed cell cycle arrest and death

Current literature on the effect of oxidative stress on the cell cycle reveals that increase in ROS-induced DNA damage is correlated with cell cycle arrest (23). Flow cytometric analysis showed that 45.1% of N9 cells were arrested in the G2/M stage at 12 h, and 7.26% cells were dead as represented by the sub-G1/G0 stage, in the tBHP-exposed group (Fig. 8). However, the percentage of dead cells decreased, and cellular populations in the G2/M stage were reduced to 14.91% in the (+)-catechin-treatment group. In addition, the cells in the S phase increased slightly in this group. The result indicated that (+)-catechin restrained ROS-induced cell cycle arrest and death. The p53 tumor suppressor protein is one of the key regulatory molecules in both the G1 and G2 stage arrest in response to DNA damage (17, 26). Western blot analysis was carried out to detect the changes of p53, phosphorylated 53, and phosphorylated ERK expression after treatment with or without (+)-catechin. As shown in Fig. 9, the expression of p53 showed no significant change within 16 h. However, the phosphorylation of p53 began to decrease in the 0.5 mM (+)-catechin-treated group at 8 h, and it appeared to increase slightly in the untreated group from 10 to 16 h. In addition, phosphorylation of ERK was remarkably enhanced at 10 h in the (+)-catechin-treated group. These results indicated that (+)-catechin blocked the phosphorylation of p53 to reverse cell cycle arrest and increased phosphorylated ERK to protect cell survival.

Discussion

(+)-Catechin, a flavanol compound, widely exists in various foods such as apples, chocolates, and tea. We can intake it by drinking tea because of its high water solubility. Polyphenol compounds of green and black tea had been found to have various bioactivities, includ-
(+) -Catechin has been reported to have the neuropharmacological function of having a synergistic protective effect with resveratrol on beta-amyloid induced cytotoxicity in PC12 cells, and it also has a cytoprotective function against 6-hydroxydopamine induced cell death in primary cultures of mesencephalic cells (27, 28). However, there have been no reports on the effect and mechanism of (+)-catechin against oxidative cell damage in microglia cells. Our present study has demonstrated that (+)-catechin had an effect against tBHP-induced oxidative stress in murine microglia cells.

Abnormally increased ROS is relevant to many diseases such as neurodegenerative disorders, cardiovascular disease, and inflammatory reactions (25, 29). Redundant ROS causes adverse influence on cellular biochemical events, including DNA damage, lipid oxidation, cycle arrest, and apoptosis (4, 30, 31). The percentage of cell death caused by exposure to tBHP obviously decreased after treatment with (+)-catechin. Furthermore, (+)-catechin possessed more potent antioxidative activity compared with NAC at the lower doses. We observed a reduced amount of \(^{\cdot}\)OH after treatment with (+)-catechin. Hydrogen peroxide is known to diffuse into the cell nucleus and injure DNA by generating \(^{\cdot}\)OH (32). DNA damage is one of the fatal damages within cells, if it cannot be effectually repaired. The degree of DNA damage, analyzed by the comet assay, reflects the capacity of the cells to resist \(^{\cdot}\)OH stress and repair single strand breaks. (+)-Catechin significantly decreased tBHP-induced DNA single-strand breakage and repaired the damage. This protec-

![Fig. 6. Protective effect of (+)-catechin against tBHP-induced N9 cell DNA damage. A: After pre-treatment without (b) or with (c) 0.5 mM (+)-catechin for 1 h, DNA strand damage was detected by the comet assay after exposure to 0.3 mM tBHP for 30 min. a: medium control. Changes of nuclei were observed by fluorescence microscopy (×400, ethidium bromide staining). Scale bar represents 10 µm. B: DNA damage level was analyzed as described in the Materials and Methods. **P<0.01, #P<0.01.](image)
tive effect may contribute to its \^OH scavenging ability or activation of antioxidase.

Many chronic diseases have been associated with aberrant activation of NF-\(\kappa\)B, and several therapeutic strategies targeting NF-\(\kappa\)B activation have been considered for the treatment of inflammation and neurodegenerative disorders (33, 34). The widely documented heterodimer is p50/p65 (Rel A) in several NF-\(\kappa\)B subunits. The p65 subunit, but not p50, possesses a transactivating domain, which can bind to the \(\kappa\)B sequence in DNA with a regulatory effect upon transcription. The phosphorylation of IkB is a necessary step in the release and activation of NF-\(\kappa\)B, which translocates to the nuclear region to modulate target gene expression. Many reports demonstrated that \(\text{H}_2\text{O}_2\) causes the dissociation of IkB from NF-\(\kappa\)B, promoting translocation of NF-\(\kappa\)B to the nuclear region (19, 35, 36). Our results prove that a similar step occurs in tBHP-induced NF-\(\kappa\)B activation. Based on the results of Western blot analysis, (+)-catechin blocked the activation of NF-\(\kappa\)B. The changes of NF-\(\kappa\)B p65 and p-IkB\(\alpha\) expression confirmed that (+)-catechin inhibited NF-\(\kappa\)B translocation to the nuclear region in tBHP-exposed N9 cells. It is possible that (+)-catechin acts as an inhibitor of IkB kinases to modulate NF-\(\kappa\)B translocation.

The p53 protein plays an important role in genotoxic stress, growth arrest, and cell death. It also serves as a NF-\(\kappa\)B downstream molecule, which had been confirmed on neurons exposure to DNA damage (37). Translocated NF-\(\kappa\)B may affect p53 phosphorylation by

Fig. 7. The expression of NF-\(\kappa\)B p65, phosphorylated (p-) IkB\(\alpha\), and p-ERK in (+)-catechin treated and untreated N9 cells after exposed to tBHP. The cells were incubated in medium containing 0.5 mM (+)-catechin for the indicated times after exposure to 0.3 mM tBHP for 1 h. The total NF-\(\kappa\)B p65, nuclear (N), and cytoplasmic (C) NF-\(\kappa\)B p65 and p-IkB\(\alpha\) were analyzed by Western blotting.

Fig. 8. Flow cytometric analysis of the cell cycle distribution of (+)-catechin treated N9 cells. Cells were treated with and without (+)-catechin after exposure to 0.3 mM tBHP for 1 h; then the cell cycle stages were analyzed by flow cytometry at 12 h. A: medium control, B: 0.3 mM tBHP exposed without (+)-catechin treatment, and C: 0.3 mM tBHP exposed with 0.5 mM (+)-catechin treatment. Arrowhead (at left) indicates the sub-G\(_0\)/G\(_1\) stage, and arrow (at right) represents the G\(_2\)/M stage.

Fig. 9. Activation of p53 was involved in the cell cycle arrest in N9 cells. The 1 h tBHP-pretreated cells were treated with or without 0.5 mM (+)-catechin for 0, 4, 8, 10, and 16 h. Cell lysates were separated by 12% SDS-PAGE electrophoresis; and p53, phosphorylated (p-) p53, and p-ERK protein bands were detected by Western blot analysis.
regulating the kinase transcription. In addition, it is accepted that the signals of MAPKs are regulated by p53 in oxidative stress (25). In this study, the majority of cells are arrested in the G2/M stage accompanied by upregulation of p-p53 due to oxidative stress. With treatment of (+)-catechin, diminished phosphorylation of p53 and increased p-ERK play an important role in sustaining cell survival. Based on the timepoints observed in Western blot analysis, it was indicated that (+)-catechin obstructed NFκB translocation in an earlier stage, whereafter, phosphorylation of p53 was diminished, and then p-ERK was increased in microglia cells in vitro. In this mechanism, NFκB-xB played a considerable role to modulate its downstream signal activation of p53 and ERK.

Microglia cells are principal immuneocytes in the central nervous system. It plays a critical role in defense against invading microorganisms and in phagocytosis of endogenous toxicants. ROS-mediated stress can influence the immune response of microglia cells in both inflammatory reactions and neurodegenerative disorders. (+)-Catechin protects microglia cells from tBHP-induced DNA lesion by the blockage of NF-xB activation. Subsequently, the decrease of NF-xB activation down-regulates phosphorylation of p53, therefore reversing cell cycle arrest, and promotes ERK activation. Subsequently, the decrease of NF-xB activation of p53 and ERK.

References