Published in final edited form as:

Neuroreport. 2012 December 05; 23(17): 989–994. doi:10.1097/WNR.0b013e32835a4ea4.

Resveratrol attenuates L-DOPA-induced hydrogen peroxide toxicity in neuronal cells

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Abstract

A variety of polyphenol antioxidant compounds derived from natural products have demonstrated neuroprotective activity against neuronal cell death. The objective of this study was to investigate the effect of resveratrol (RESV) and bioflavonoids in attenuating hydrogen peroxide (H₂O₂)-induced oxidative stress in neuronal cells. H₂O₂ levels were increased by the addition of L-3,4-dihydroxyphenylalanine (L-DOPA) to cultured dopaminergic SKNSH cells. H₂O₂ was monitored by peroxyfluor-1, a selective H₂O₂ optical probe. To examine the neuroprotective effects of RESV and bioflavonoids against L-DOPA, we cotreated RESV, quercetin, or (–) epigallocatechin gallate with L-DOPA and monitored for H₂O₂ levels. The combination of RESV and L-DOPA was 50% more effective at reducing H₂O₂ levels than the combination of quercetin or epigallocatechin gallate with L-DOPA. However, the combination of each antioxidant with L-DOPA was effective at preserving cell viability.

Keywords

bioflavonoids; hydrogen peroxide; L-DOPA; peroxyfluor-1; resveratrol

Introduction

Prevention of oxidative stress in neurons with low molecular weight antioxidants such as ascorbate (vitamin C), α -tocopherol (vitamin E), and coenzyme Q have been proposed as a protective therapy in various neurodegenerative diseases [1,2]. Neurons readily take up these antioxidants; however, if Fe³⁺ ions become available, for example in the damaged brain, these antioxidants can reduce it to the redox active Fe²⁺. When endogenous H₂O₂ reacts with reduced iron (Fe²⁺), hydroxyl radicals are formed by the Fenton reaction [3]. Hydroxyl radicals are the most destructive-free radical promoting lipid peroxidation and DNA damage [4].

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Conflicts of interest

There are no conflicts of interest.

Increasing evidence suggest neuroprotective effects of carotenoids and flavonoids, yet there is limited evidence for their importance as antioxidants in the central nervous system because of their ability to cross the blood–brain barrier (BBB) [5]. Some phenolic molecules can cross the BBB such as the flavonoid (–) epigallocatechin gallate (EGCG), as indicated in animal studies [6]. Nonflavonoid polyphenols such as resveratrol (RESV), received much attention owing to its ability to cross the BBB and act as a powerful antioxidant to ameliorate neuronal cell death [7].

Many studies have tested the toxic effect of L-3,4-dihydroxyphenylalanine (L-DOPA) or its derivatives on clonal catecholaminergic cell lines [8]. This toxicity has been attributed to free radicals generated by increased dopamine (DA) turnover and/or by autooxidation of L-DOPA and DA. Conventional effects of exogenous L-DOPA results in the decarboxylase-catalyzed conversion of L-DOPA to DA in nigral DAergic neurons [9]. The main enzymatic metabolite of L-DOPA is DA; however, L-DOPA can autoxidize in the extracellular space through a nonenzymatic process leading to a number of toxic products including quinones [10]. Inside the cell, L-DOPA is quickly converted to DA by amino acid decarboxylase (AADC), the ‘rate-limiting’ enzyme for DA synthesis from exogenous L-DOPA. DA is then stored in vesicles where it is protected from oxidation by low pH. A small amount of DA is present as a free cytoplasmic fraction that is susceptible to autooxidation and to metabolic degradation. DA is normally metabolized by mitochondrial monoamine oxidase (MAO)[11] producing H_2O_2 as a by-product. When excessive DA accumulates after L-DOPA treatment, DA can autoxidize like L-DOPA and form a number of highly reactive derivatives through both enzymatic and nonenzymatic reactions promoted by transition metals, thus generating deleterious radical species [12].

The present study examines whether increase of H_2O_2 in the dopaminergic human neuroblastoma tumor cell line (SKNSH) cells can be ameliorated with flavonoid polyphenols quercetin (QUERC) and EGCG as well as the nonflavonoid polyphenol RESV. L-DOPA treatments of dopaminergic SKNSH cells were used as a cellular model in studying increases in H_2O_2 and subsequent toxicity to cells. We used a selective H_2O_2 optical probe, peroxyfluor-1 (PF1) [13], to detect H_2O_2 as the major contributor toward oxidative stress. We found that cotreatment of RESV with L-DOPA attenuated H_2O_2 levels and subsequent cytotoxicity, whereas cotreatment of bioflavonoids with L-DOPA attenuated cytotoxicity alone.

Materials and methods

SKNSH cell culture

The human neuroblastoma tumor cell line, SKNSH, established in 1970 from bone marrow biopsy was previously described as having catecholaminergic characteristics and therefore served as a useful immortalized cell line for our studies [14]. This is a biologically heterogeneous tumor that undergoes spontaneous regression or differentiation into neuronal morphology. Cells per 10 cm^2 cell culture dish were routinely plated at 5×10^5 in MEM supplemented with 2mM L-glutamine, 1% penicillin/streptomycin, 1mM sodium pyruvate, 10% fetal bovine serum, and 1mM MEM nonessential amino acids. Cells were cultured in a monolayer at 37°C under 5% CO_2 and 95% air. Medium was changed every 3 days and

passed until they reached 80% confluency. Cells were differentiated by using differentiating media, which consisted of changing the media to MEM (no phenol red) supplemented with 2mM L-glutamine, 1% penicillin/streptomycin, 1mM sodium pyruvate, 5% fetal bovine serum plus 10 μ M all-*trans*-retinoic acid (Sigma-Aldrich, St Louis, Missouri, USA) for 10 days. Culture media and reagents were purchased from Invitrogen (Grand Island, New York, USA).

Immunostaining

Primary antibodies used for immunofluorescence were: tyrosine hydroxylase (TH, ab6211; Abcam, Cambridge, Massachusetts, USA) and MAO-B (ab88510; Abcam). Secondary antibodies were: Cy5 anti-rabbit IgG (ab6564; Abcam) and alexa Fluor 488 anti-mouse IgG (A21121; Invitrogen). Differentiated SKNSH cells were plated at 5×10^4 cells/well on poly-L-lysine (0.1 mg/ml; Sigma-Aldrich) coated coverslips (BD Biosciences, Bedford, Massachusetts, USA) in a six-well plate. The cultures were fixed using 3.7% paraformaldehyde, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS (PBS-TX) and blocked with 1% BSA in 0.1% PBS-TX (blocking buffer). Cells were treated with primary antibodies against TH (1: 1000) or against (MAO-B (1: 1000) in blocking buffer overnight at 4°C. Secondary antibodies were incubated in blocking buffer for 1 h at room temperature followed by PBS washing. Cells were treated with Hoescht 33342 (10 μ g/ml; Invitrogen), washed with PBS and then mounted with slow fade Gold antifade reagent (S36936; Invitrogen) to visualize using a Zeiss Axiovert 200M fluorescence microscope equipped with a Zeiss Axiocam MRM camera (Carl Zeiss MicroImaging Inc., Thornwood, New York, USA). Cell images were processed using NIH image acquisition software, ImageJ 1.440 (National Institutes of Health, Washington, District of Columbia, USA).

Drug administration and H₂O₂ measurements

All drugs and enzyme inhibitors were purchased commercially from Sigma-Aldrich: L-DOPA (D9628), RESV (R5010), (–) EGCC (E4143), QUERC (Q4951), pargyline (PARG) HCl (P8013), and NSD1015 (54880). Dosing regimens were chosen based on pilot studies, which showed maximum viability at 10 days differentiation. Two days before all experiments, differentiated cells were incubated in low tyrosine differentiation media (LTDM) comprising all ingredients except L-tyrosine. Amino acids, inorganic salts, and vitamins for LTDM were purchased from Sigma-Aldrich. Media was then changed to contain H₂O₂ (100 μ M), L-DOPA (50 μ M), L-DOPA (50 μ M)+PARG (100 μ M), L-DOPA +NSD (100 μ M), or vehicle (LTDM) and incubated for 2 h at 37°C. Cultures treated with L-DOPA (50 μ M), L-DOPA+ RESV (10 μ M), L-DOPA+QUERC (100 μ M), and L-DOPA +EGCG (100 μ M) were incubated for 24 h at 37°C. Drug concentrations were chosen based on initial studies of H₂O₂ and L-DOPA toxicity in SKNSH (data not shown). After drug treatment, the cells were aspirated and washed with LTDM. PF1 was loaded onto SKNSH-treated cells for a final concentration of 5 μ M in PBS. Real-time fluorescence microscopy was used to reveal the generation of H₂O₂ after 30 min of PF1 incubation at room temperature, in the dark. Treated cells were imaged with an Olympus IX50 B202 fluorescence microscope equipped with an Olympus digital camera (Olympus America, Center Valley, Pennsylvania, USA). Cell images were processed using NIH image acquisition software, ImageJ 1.440. Fluorescence spectroscopy data for % PF1 emission for

each drug group ($n=3$) listed above were acquired on a QuantaMaster Luminescence spectrofluorometer and data was analyzed using FeliX32 software (Photon Technology International, Birmingham, New Jersey, USA).

Cell viability measurements

Dosing regimens for L-DOPA, RESV, QUERC, and EGCG were similar to above. Cultures were treated with L-DOPA (50 μ M), L-DOPA+RESV (0–10 μ M), L-DOPA+QUERC (0–100 μ M), or L-DOPA+EGCG (0–100 μ M) and incubated at 37°C for 24 h. Cell number was determined using the colorimetric reagent MTS (CellTiter 96 AQueous One Solution; Promega, Madison, Wisconsin, USA). MTS reagent was added (1: 5 ratio) and incubated for 4 h. Absorbances were analyzed on a Thermo Lab Systems Obsys MR plate reader at 490 nm (Thermo Lab Systems, Grand Rapids, Ohio, USA).

Statistical analysis

The significance of differences in mean values between the experimental groups was determined by Tukey's multiple comparison tests. Statistical significance was set at P value less than 0.05 for all experiments and analyzed using GraphPad Prism software (GraphPad Software, La Jolla, California, USA).

Results

SKNSH comprises two distinctly different cell types. One is a large epithelioid cell, which resembles human fibroblasts, flattened and stretched out. This is the first kind to be attached in new cultures (Fig. 1a). As the culture matures, the smaller dense cells with delicate processes accumulate. With the addition of 10 μ M RA to SKNSH cells, the culture's processes extend from smaller nuclei to form a more neuronal morphology (Fig. 1b). To ascertain the relevance of SKNSH cells for dopaminergic properties, we stained differentiated cells for the enzyme that catalyzes the rate-limiting step in DA synthesis, TH. Cotreatment with Hoescht 33342, a vital nuclei stain, indicates that TH is present in almost all SKNSH differentiated cells (Fig. 1c). We also stained for MAO-B, which is responsible for DA metabolism and yields H_2O_2 as a by-product. We found that MAO-B is prevalent in SKNSH cells as is shown with positive immunostaining (Fig. 1d).

We monitored H_2O_2 production using PF1, a selective cell-permeable optical probe that has a high specificity for H_2O_2 over all other reactive oxygen species [13]. To circumvent background fluorescence, 2 days before each experiment SKNSH cells were treated with a specifically formulated differentiation media that eliminated L-tyrosine (LTDM). LTDM was used to more closely mimic the concentration of brain L-tyrosine *in vivo* [15] and was intended to reduce tyrosine conversion to L-DOPA by TH. We found a dramatic decrease in background fluorescence upon treating with the formulated LTDM, otherwise stated as low-tyrosine vehicle (LT VEH)-treated cells (Fig. 2a and b). When SKNSH cells were exposed to 100 μ M H_2O_2 , PF1 signal was significantly increased compared with LT VEH (Fig. 2c). SKNSH cells loaded with PF1 were previously exposed to various combinations of drugs for up to 2 h (to preserve cell viability) and fluorescence was imaged in live cells. L-DOPA (50 μ M) produced approximately 40% higher increase in PF1 emission compared with LT VEH

(Fig. 2d and k). When L-DOPA was cotreated with the AADC inhibitor NSD1015 (NSD), PF1 emission was decreased by approximately 30% (Fig. 2e and k). PARG, an MAO-B inhibitor, also attenuated PF1 emission by approximately 30% when cotreated with L-DOPA (Fig. 2f and k).

SKNSH cells were incubated with 50 μ M L-DOPA for 24 h and then imaged with PF1 (Fig. 2g). The number of cells had been reduced at 24 h of incubation because of the toxicity of L-DOPA; however, the remaining cells produced a higher PF1 emission than LT VEH (Fig. 2k). H_2O_2 formation is apparent at 2 h of L-DOPA treatment (Fig. 2d), yet the cells begin to die between 2 and 24 h. Therefore, 24 h treatment with 50 μ M L-DOPA produced an appropriate amount of detectable H_2O_2 for our recovery studies with antioxidants. Next, we cotreated 50 μ M L-DOPA with each antioxidant and then detected H_2O_2 levels after 24 h. We found that 10 μ M RESV+L-DOPA decreased PF1 emission by approximately 75% compared with L-DOPA alone (Fig. 2h and l). Cotreatment of L-DOPA with 100 μ M QUERC or 100 μ M EGCG did not attenuate H_2O_2 formation remarkably (Fig. 2i, j and l).

Given their potential antioxidant properties in neuronal cells [16], we sought to determine whether all three polyphenolic antioxidants protect dopaminergic cells from L-DOPA-induced toxicity. Each polyphenol antioxidant was cotreated with 50 μ M L-DOPA for 24 h. RESV inhibited L-DOPA-induced toxicity with an IC_{50} of 1.5 μ M (Fig. 2m). Cell viability for L-DOPA alone was $9\pm0.4\%$. Viability was increased to $54.8\pm4.5\%$ in the presence of 1 μ M RESV. 10 μ M RESV increased viability to $78.5\pm2.2\%$. Although QUERC and EGCG were not as effective as RESV at inhibiting H_2O_2 formation in the presence of L-DOPA, both flavonoids were effective at inhibiting L-DOPA-induced toxicity (Fig. 2n). 100 μ M QUERC increased cell viability to $68.8\pm6.7\%$ and 100 μ M EGCG increased cell viability to $62.9\pm8.5\%$. QUERC and EGCG inhibited L-DOPA-induced toxicity with IC_{50} values of 4.25 and 8.130 μ M, respectively. Both flavonoids appear to protect SKNSH cells from L-DOPA-induced cell death; however, not by mechanisms mediated by H_2O_2 .

Discussion

There is a large body of evidence supporting that L-DOPA is a precursor to hydroxyl radical generation *in vitro* [17–21]. Our results found that after treatment of L-DOPA, there is an increase in H_2O_2 formation leading to cell death in the dopaminergic SKNSH cells. Specifically, DA neurons can metabolize L-DOPA into DA in a manner dependent on AADC [9], whereas the formation of H_2O_2 from the subsequent metabolism of DA by MAO-B, creates the pro-oxidant environment necessary to kill DA cells. In addition to bioflavonoid polyphenolic antioxidants EGCG and QUERC, the nonflavonoid polyphenolic antioxidant RESV protected dopaminergic cells from L-DOPA-induced cell death. The formation of H_2O_2 is decreased in the presence of the AADC inhibitor NSD1015 and the MAO-B inhibitor PARG suggesting that H_2O_2 is the predominant source and/or precursor of cell death by L-DOPA toxicity. We show that L-DOPA treatment leads to a detectable increase in H_2O_2 formation and that L-DOPA is capable of producing H_2O_2 by decarboxylation to DA and subsequent oxidative deamination of DA by MAO-B. Although our results suggest that administration of L-DOPA is toxic by H_2O_2 production through DA

metabolism (Fig. 2a–f), we have not ruled out the possibility that L-DOPA is toxic by H_2O_2 production through autoxidation [10].

Pilot studies with SKNSH cells were used as a basis for exhibiting characteristics of dopaminergic phenotype (Fig. 1). SKNSH cells also have dopaminergic and neuronal features, such as dopamine- β -hydroxylase activity [14], AADC activity [22], microtubule-associated protein 2 (MAP2) expression [23], and L-DOPA uptake through L-amino acid carriers [24]. Collectively, SKNSH cells function as DA neurons and are phenotypically similar to mature DA neurons *in vivo*; therefore, SKNSH cells serve as an appropriate model of the dopaminergic neuron and is suitable for the study of L-DOPA-induced toxicity and furthermore, antioxidant protection.

RESV's cellular mechanism of antioxidant activity is not yet clearly defined [7]. In addition, both RESV and the bioflavonoids are considered polypharmacological agents. Several studies suggest that RESV can directly scavenge free radical damage, whereas other studies imply that RESV protects cell viability indirectly by upregulating endogenous antioxidant defenses. Both bioflavonoids are well-known free radical scavengers and most likely protect SKNSH cell viability through mechanisms downstream of H_2O_2 . On the basis of their structures, all three polyphenolic antioxidants have multiple phenol rings with several hydroxyl groups. RESV has three hydroxyl groups stretching from two phenol rings, whereas QUERC has five hydroxyl groups and EGCG has eight (Fig. 3). Usually the antioxidant and reactive oxygen species scavenging activity of phenolic compounds are dependent on the number of hydroxyl groups; therefore, the antioxidant activity of RESV should appear to be poorer than QUERC or EGCG. This leads us to believe that RESV may directly or indirectly inhibit MAO activity in neuronal cells and subsequently decrease the H_2O_2 formed by L-DOPA (Fig. 3). There is no precedence in the literature for QUERC or EGCG influencing MAO activity. The bioflavonoids most likely inhibit cell death by an unrelated mechanism such as quenching toxic radicals formed downstream of H_2O_2 using the Fenton reaction [3]. We have not ruled out the possibility that bioflavonoids have synergistic effects with PF1 thus altering the potential outcomes of H_2O_2 formation. There is precedence in the literature for the cellular metabolism of QUERC altering its redox potential [25]. Future experiments to investigate the metabolites of each antioxidant with PF1 are necessary. Further comparison of these three antioxidants and their influence on MAO activity is also warranted to understand their complete molecular mechanisms under this paradigm. The chemical influence of L-DOPA and DA autoxidation metabolites with all three polyphenolic antioxidants is required to elucidate the precise mechanism by which they preserve cell viability. Future studies in primary neuron culture with the nutritional support of glial cells may alter the effects of these antioxidants considering how extensively they are metabolized *in vivo*.

Conclusion

This study reports the use of a selective H_2O_2 optical probe for the intracellular detection of H_2O_2 increases because of L-DOPA administration in SKNSH cells and the neuroprotective effect of RESV when coadministered. Together, these observations may have important implications for clinical strategies in addressing the toxic effects of neurodegeneration.

Acknowledgments

The authors thank Dr Maria Matos and Dr Adrian Whitty for critical reading of the manuscript. They also thank Yi Luan for contributing to the synthesis of peroxyfluor-1. Scott E. Schaus received NIH grant (R01 GM078240).

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Fig. 1.

Differentiation and characterization of SKNSH cells. (a) SKNSH cells in growth medium at first passage (passage 1). (b) SKNSH cells incubated with 10 μ M retinoic acid for 10 days (+RA). (c) TH immunostaining with TH primary, secondary antibodies, and Hoescht nuclei staining (+TH). (d) MAO-B immunostaining with MAO-B primary, secondary antibodies, and Hoescht nuclei staining (+MAO-B). Scale bar=40 μ m. MAO-B, monoamine oxidase B; TH, tyrosine hydroxylase.

Fig. 2.

Effect of L-DOPA on H₂O₂ formation in SKNSH cells. H₂O₂ levels were determined by PF1 conversion to fluorescein. (a) Cells are imaged with PF1 in vehicle treated (VEH) or with (b) low tyrosine differentiating media for 2 days (LT VEH). (c) Fluorescence was detected in cells that were exposed to H₂O₂ for 2 h. (d) Fluorescence in cells that were exposed to L-DOPA, (e) L-DOPA+NSD1015 or (f) L-DOPA+PARG for 2 h before loading with PF1. (g) Fluorescence in cells that were exposed to L-DOPA, (h) L-DOPA+RESV, (i) L-DOPA+QUERC, or (j) L-DOPA+EGCG for 24 h before loading PF1. Scale bar=30 μm. (k) Percentage of PF1 emission in cells untreated (LT VEH) and treated with H₂O₂, L-DOPA, L-DOPA+NSD1015, and L-DOPA+PARG. Tukey's multiple comparison test; $n=3$; * $P<0.05$; ** $P<0.001$; *** $P<0.0001$. (l) Percentage of PF1 emission in cells untreated (LT VEH) and treated with L-DOPA, L-DOPA+RESV, L-DOPA+QUERC, and L-DOPA+EGCG. Tukey's multiple comparison test; $n=3$; * $P<0.0001$. (m) Effect of RESV and bioflavonoids on L-DOPA-induced cytotoxicity. Cell death was measured with the MTS cell viability assay. Percentage of cells cotreated with L-DOPA+RESV ($IC_{50}=1.5 \mu M$, $n=8$). (n) Percentage of cells cotreated with L-DOPA+QUERC ($IC_{50}=4.25 \mu M$, $n=8$) or L-DOPA

+EGCG ($IC_{50} = 8.130 \mu M$, $n=8$). EGCG, epigallocatechin gallate; L-DOPA, L-3,4-dihydroxyphenylalanine; LT VEH, low-tyrosine vehicle; PARG, pargyline; PF1, peroxyfluor-1; QUERC, quercetin; RESV, resveratrol.

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Fig. 3.

Model of changes induced by increased L-DOPA+RESV, +QUERC, +EGCG. L-DOPA is converted to dopamine (DA) by the enzyme amino acid decarboxylase (AADC). DA is metabolized by monoamine oxidase B (MAO-B) to form H₂O₂ as a by-product. Redox active iron (Fe²⁺) initiates Fenton chemistry in the presence of H₂O₂. As a result, hydroxyl radicals form and promote cell death. RESV serves as a potent antioxidant to interact directly or indirectly with MAO-B and decrease H₂O₂ to promote cell viability. Both QUERC and EGCG promote cell viability by scavenging-free radicals. EGCG, epigallocatechin gallate; L-DOPA, L-3,4-dihydroxyphenylalanine; QUERC, quercetin; RESV, resveratrol.