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Oxidative modification of neurofilament-L and neuronal cell death induced by the catechol neurotoxin, tetrahydropapaveroline

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HIGHLIGHTS

- THP induced formation of high molecular weight NF-L aggregates.
- The carbonyl compound and dityrosine were formed in the THP-treated NF-L.
- Treatment of C6 astrocyte cells with THP led to the reduction in cell viability and increased reactive oxygen species generation.
- Exposure of C6 astrocyte cells to THP lead to intracellular modification of NF-L.

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ABSTRACT

Tetrahydropapaveroline (THP), which is an endogenous neurotoxin, has been suspected to be associated with dopaminergic neurotoxicity of L-DOPA. In this study, we examined oxidative modification of neurofilament-L (NF-L) and neuronal cell death induced by THP. When disassembled NF-L was incubated with THP, protein aggregation was increased in a time- and THP dose-dependent manner. The formation of carbonyl compounds and dityrosine were observed in the THP-mediated NF-L aggregates. Radical scavengers reduced THP-mediated NF-L modification. These results suggest that the modification of NF-L by THP may be due to oxidative damage resulting from the generation of reactive oxygen species (ROS). When THP exposed NF-L was subjected to amino acid analysis, glutamate, proline and lysine residues were found to be particularly sensitive. We also investigated the effects of copper ions on THP-mediated NF-L modification. At a low concentration of THP, copper ions enhanced the modification of NF-L. Treatment of C6 astrocyte cells with THP led to a concentration-dependent reduction in cell viability. When these cells were treated with 100 μM THP, the levels of ROS increased 3.5-fold compared with control cells. Furthermore, treatment of cells with THP increased NF-L aggregate formation, suggesting the involvement of NF-L modification in THP-induced cell damage.

1. Introduction

Tetrahydropapaveroline [THP; 1-(3,4-dihydroxybenzyl)-1,2,3,4-tetrahydro-6,7-isoquinolinediol] (Fig. 1), which is a metabolite, has been detected in the urine of Parkinson's disease (PD) patients on L-DOPA medication (Sandler et al., 1973). Previous studies have shown both *in vitro* and *in vivo* that THP is toxic to dopaminergic neurons under conditions. It has also been reported that THP causes necrosis in human dopaminergic neuroblastoma SH-SY5Y cells (Maruyama et al., 2000) and apoptosis in C6 glioma cells (Soh et al., 2003). THP-treated PC 12 cells exhibited increased intracellular accumulation of reactive oxygen species (ROS) (Shin et al., 2004; Park et al., 2007).

Neurofilament (NF) proteins have been implicated in the pathogenesis of several neurodegenerative disorders. Recent progress in PD research indicates that PD might be caused by protein aggregation due to aberrant protein folding or disturbed protein degradation (Riess et al., 2000). Lewy bodies (LBs) are cytoplasmic inclusions that are present consistently and with greatest frequency in neurons of the substantia nigra and locus ceruleus of patients with PD (Forno, 1986). NF proteins have been identified immunohistochemically as the major protein components of the LB filament (Pollanen et al., 1993). Neurofilaments are composed of three subunits, which have been termed light (NF-L), medium (NF-M), and heavy (NF-H) (Nixon and Lewis, 1986; Nixon and Shea, 1992) and these three subunits have been identified in LB (Hill et al., 1991).

Abnormal accumulation of neurofilaments in neurons is associated with other neurodegenerative diseases, such as Alzheimer's disease (Shepherd et al., 2002) and amyotrophic lateral sclerosis

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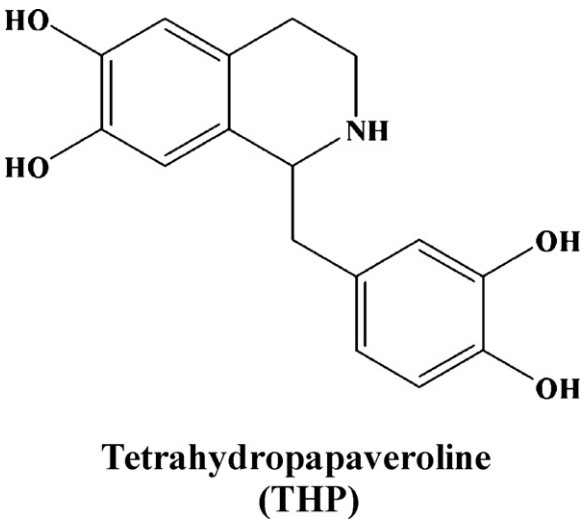

Fig. 1. The structure of THP. THP [1-(3,4-dihydroxybenzyl)-1,2,3,4-tetrahydro-6,7-isoquinolinediol] is (http://www.mondofacto.com/facts/dictionary) formed in brain and liver from dopamine and L-DOPA.

(ALS) (Collard et al., 1995). It has been reported that the overexpression of mouse NF-L led to a severe loss of neurons in the parietal cortex and the ventrobasal thalamus with age (Ma et al., 1999). Other studies have shown that transgenic mice overexpressing NF-L by a factor of 4 displayed neuron degeneration and neuron loss, which resembles the pathology of ALS (Xu et al., 1993). More recently, mass spectrometry has revealed that neuronal staining using the glial fibrillary protein antibody was caused by a cross-reaction with NF-L (Middeldorp et al., 2009) and implied that NF-L could accumulate in some tangle-like structures in Alzheimer brains. However, the factor and mechanisms resulting in NF aggregation are still unknown. In the present study, we report the oxidative modification of NF-L and cell death induced by THP and possible involvement of protein modification.

2. Materials and methods

2.1. Materials

Tetrahydropapaveroline [THP; 6,7-dihydroxy-1-(3,4-dihydroxybezyl)-1,2,3,4-tetra-hydroisoquinoline)], urea, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetra acetic acid (EGTA), *N*-acetyl-L-cysteine, glutathione, thiourea, urate, diethylenetriaminepentaaetic acid (DTPA), diethyldithiocarbamic acid (DDC), penicillamine (PA), 2,4-dinitrophenyl hydrazine (DNPH) and dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma (St. Louis, MO, USA). Chelex 100 resin (sodium form) was obtained from Bio-Rad (Hercules, CA, USA). All solutions were treated with Chelex 100 resin to remove trace amounts of transition metal ions.

2.2. Expression and purification of NF-L

A full-length cDNA clone of mouse NF-L in a pET-3d vector was a generous gift from Dr. Beckman (University of Alabama). To express the recombinant protein, the vector was transformed into *Escherichia coli* (BL21). Protein expression was performed as previously described (Kang, 2007). Bacteria were grown in Luria broth supplemented with 1 mM isopropyl β-D-thiogalactopyranoside beginning at an OD 600 nm reading of 0.8. After incubation at 37 °C for 3 h, bacteria were harvested by centrifugation (4000 × *g* for 10 min at 4 °C), and resuspended in standard buffer (50 mM MES, 170 mM NaCl, 1 mM DTT, pH 6.25). The cells were lysed using a French press at a pressure of 20,000 pounds per square inch and centrifuged at 8000 × *g* for 15 min at 4 °C. The supernatant was incubated for 3 h at 37 °C and then was centrifuged at 100,000 × *g* for 20 min at 25 °C. The pellets containing the aggregated NF-L proteins were washed twice with standard buffer before they were dissolved in urea buffer (25 mM Na-phosphate, pH 7.5, 6 M urea, 1 mM EGTA and 1 mM DTT). The sample was loaded onto a diethylaminoethyl (DEAE)-sepharose column and the column was washed with urea buffer. The column was eluted with a linear 25–500 mM phosphate gradient in urea buffer and 1 ml of NF-L was eluted between 300 and 360 mM phosphate. These fractions were pooled and either used directly or stored at −80 °C for later experiments. The protein concentration was determined using the bicinchoninic acid (BCA) method (Smith et al., 1985).

2.3. Oxidation of protein

Oxidative modification of NF-L (0.4 mg/ml) was carried out by incubation of the protein with THP in 10 mM potassium phosphate buffer (pH 7.5) at 37 °C. After incubation of the reaction mixtures, the mixtures were placed into Vivaspin ultrafiltration spin column (Sartorius Stedim Biotech, Goettingen, German) and centrifuged at 13,000 rpm for 1 h. The column was washed with Chelex 100 treated water and centrifuged for 1 h at the same speed. This step was repeated four times. The filtrate was freeze dried and dissolved in phosphate buffer.

2.4. Analysis of modified protein

After treatment with different concentrations of THP for various periods, samples of the reaction mixtures were diluted with a concentrated sample buffer (0.25 mM Tris, 40% glycerol, 0.01% bromophenol blue). An aliquot of each sample was subjected to SDS-PAGE analysis as described by Laemmli (1970), using a 12% acrylamide slab gel. The gels were stained with 0.15% Coomassie Brilliant Blue R-250.

2.5. Detection of protein carbonyl compound

The carbonyl content of the proteins was determined using spectrophotometric assays, as described elsewhere (Reznick and Packer, 1994). Both native and oxidized proteins were incubated with 10 mM 2,4-DNPH in 2.5 M HCl for 1 h at room temperature. After incubation, 20% TCA was added to the sample and the tubes were left in an ice bucket for 10 min and then centrifuged for 5 min with a tabletop centrifuge to collect the protein precipitates, after which the supernatants were discarded. Another wash was performed using 10% trichloroacetic acid (TCA), and the protein pellets were mechanically broken using a pipette tip. Finally, the pellets were washed 3 times with ethanol–ethyl acetate (1:1) (v/v) to remove any free DNPH. The final precipitates were dissolved in 2 ml of a 6 M guanidine hydrochloride solution and left for 10 min at 37 °C with general vortex mixing. The carbonyl content was calculated from the absorbance at 370 nm using an extinction coefficient ε of 22,000 M^{-1} cm^{-1}.

2.6. Detection of O,O′-dityrosine

The reactions to detect O,O′-dityrosine were carried with NF-L (0.4 mg/ml) and THP in 10 mM potassium phosphate (pH 7.4). The fluorescence emission spectrum of the sample was then monitored between 340 and 500 nm (exitation, 325 nm) using Spectrofluorometer SMF 25 (Bio-Tek Instruments, Milano, Italy).

2.7. Amino acid analysis

Aliquots of modified and native NF-L preparations were hydrolyzed at 110 °C for 24 h after the addition of 6 N HCl. Since acid hydrolysis destroys tryptophan, the tryptophan content of oxidized and native NF-L preparations was determined by means of alkaline hydrolysis as described previously (Hugli and Moore, 1972). The amino acid content of acid and alkaline hydrolysates was determined by HPLC separation of their phenylisothiocyanate-derivatives using a Pico-tag free amino acid analysis column and 996 photodiode array detector (Waters, USA).

2.8. Cell culture

C6 astrocyte cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and antibiotics (100 unit/ml penicillin and 100 μg/ml streptomycin) at 37 °C, under humidified conditions of 95% air and 5% CO_2.

2.9. Measurement of THP-induced cytotoxicity

A 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) assay was used to determine the viability of the cells treated with THP. The cells were treated with various concentrations of THP (10–200 μM) for 24 h. The absorbance was measured at 540 nm using a Multiskan MCC/340 ELISA microplate reader (Labsystems Oy, Helsinki, Finland) and cell viability was defined as the percent of untreated control cells.

2.10. Immunoblot analysis

The protein (20 μg) loaded in wells of 12% sodium dodecyl sulfate-polyacrylamide gel and then performed electrophoresis. The resolved proteins were electro-transferred to a nitrocellulose membrane, which was then blocked with 5% non-fat dry milk in PBS. The membrane was probed with a mouse NF-L antibody (1:500; Sigma–Aldrich, St. Louis, MO, USA), followed by incubation with goat anti-mouse immunoglobulins (dilution 1:10,000; Sigma–Aldrich, St. Louis, MO, USA). The bound antibodies were then visualized by enhanced chemilluminescence according to the manufacturer's instructions (Amersham, Franklin Lakes, NJ, USA).