Experimental Neurology 238 (2012) 29–37

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

L-DOPA is incorporated into brain proteins of patients treated for Parkinson's disease, inducing toxicity in human neuroblastoma cells in vitro

Sandra W. Chan [a], Rachael A. Dunlop [a], Anthony Rowe [b], Kay L. Double [c], Kenneth J. Rodgers [a,d,*]

[a] The Cell Biology Group, The Heart Research Institute, 7 Eliza Street, Newtown, Sydney, NSW 2042, Australia
[b] Faculty of Pharmacy, University of Sydney, Australia
[c] Neuroscience Research Australia and the University of New South Wales, Australia
[d] School of Medical and Molecular Biosciences, University of Technology Sydney, Australia

ARTICLE INFO

Article history:
Received 15 December 2010
Revised 23 August 2011
Accepted 26 September 2011
Available online 1 October 2011

Keywords:
L-DOPA
Protein
Incorporation
Parkinson's disease
L-tyrosine

ABSTRACT

Levodopa (L-DOPA), a close structural analogue of the protein amino acid L-tyrosine, can substitute for L-tyrosine in protein synthesis and be mistakenly incorporated into newly synthesised proteins in vitro. We show that L-DOPA-containing proteins are present in the brain in L-DOPA-treated Parkinson's disease patients and accumulate in specific brain regions. In vitro studies demonstrate that substitution of L-tyrosine residues in proteins with L-DOPA causes protein misfolding and promotes protein aggregation in SH-SY5Y neuroblastoma cells resulting in the appearance of autofluorescent bodies. We show that the presence of L-DOPA-containing proteins causes profound changes in mitochondria and stimulates the formation of autophagic vacuoles in cells. Unlike L-DOPA, which is toxic to cells through its ability to generate radicals, proteins containing incorporated L-DOPA are toxic to SH-SY5Y cells by a mechanism independent of oxidative stress and resistant to antioxidants. These data suggest that the accumulation of L-DOPA-containing proteins in vulnerable cells might negatively impact on cell function.

Introduction

Parkinson's disease (PD) is characterised neuropathologically by the loss of dopaminergic neurons in the substantia nigra and a deficiency in the neurotransmitter dopamine (DA) as well as the deposition of α-synuclein in abnormal intracellular inclusions throughout the nervous system (Dickson et al., 2009). L-DOPA (3,4-dihydroxyphenylalanine), the direct precursor of DA, was first used therapeutically more than 40 years ago and is still the most commonly used and effective agent for the symptomatic treatment of PD. In vitro studies have shown that L-DOPA is toxic to dopaminergic neurons (Basma et al., 1995; Pardo et al., 1995) and have raised the possibility that L-DOPA could have a negative impact on neuronal function in vivo. Indeed, a recent study has shown progressive peripheral nerve deficits with increasing exposure to L-DOPA in prospectively-studied patients with PD (Toth et al., 2010). This adds to data from the ELLDOPA study which showed that while the Unified PD rating scale (UPDRS) scores were better in L-DOPA treated patients than in the placebo-treated group, the subset of L-DOPA treated patients in which neuroimaging techniques were used showed a decrease in striatal DA transport binding, indicating a reduction in dopaminergic neuron function or number (Fahn et al., 2004). These studies suggest that L-DOPA treatment has broader effects on the brain than merely replacing dopamine.

Since L-DOPA readily undergoes oxidation to reactive species in vitro, oxidative stress in dopaminergic neurons has been considered to be a potential mechanism of any L-DOPA toxicity in vivo (Basma et al., 1995; Migheli et al., 1999). Animal studies investigating L-DOPA neurotoxicity have reported conflicting results (Blunt et al., 1993; Lyras et al., 2002), as have large-scale human trials (Fahn, 2006; Fahn et al., 2004; Muller et al., 2004; Olanow et al., 2004). What is clear, is that the acute in vitro toxicity reported for L-DOPA is not supported by data from animal and human studies. It is well established that oxidative stress can be an artefact of cell culture and is enhanced by components of culture medium (Clement et al., 2002). In addition, most in vitro studies are carried out in atmospheric oxygen which is more than fourfold the oxygen concentration present in the brain (Ndubuizu and LaManna, 2007), thus the level of oxidative stress in most cell studies is not representative of that found in vivo. In the present study we investigate the misincorporation of L-DOPA into cell proteins, an aspect of L-DOPA's intracellular behaviour that has been neglected to date.

L-DOPA (also known as 3-hydroxytyrosine) is a close structural analogue of the protein amino acid L-tyrosine (Fig. 1). The structural similarity of these two molecules is such that L-DOPA can be incorporated into cell proteins in place of L-tyrosine. This phenomenon occurs via a protein synthesis-dependant pathway, where the amino and carboxyl

Abbreviations: DMEM, Dulbecco's minimal essential medium; DA, dopamine; FCS, foetal calf serum; L-DOPA, 3,4-dihydroxyphenylalanine; SOD, superoxide dismutase; TCA, trichloroacetic acid; TEM, transmission electron microscopy.

* Corresponding author at: School of Medical and Molecular Biosciences, University of Technology, Harris Street, Sydney, NSW 2007, Australia. Fax: +61 2 9550 3302.
E-mail address: kenneth.rodgers@uts.edu.au (K.J. Rodgers).

0014-4886/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2011.09.029

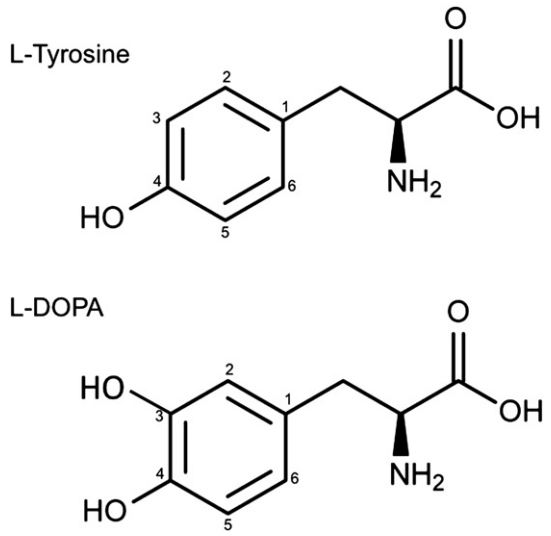

Fig. 1. Schematic representation of the chemical structures of L-tyrosine and L-DOPA. L-DOPA (3-hydroxytyrosine) has an identical chemical structure to the protein (canonical) amino acid L-tyrosine except for the additional hydroxyl group (OH) on carbon 3 of the aromatic ring.

groups participate in peptide bonding so that L-DOPA is inserted into the polypeptide backbone of newly synthesised proteins (Rodgers et al., 2002, 2004). We have demonstrated that L-DOPA-containing proteins are present in lymphocytes from L-DOPA-treated patients (Rodgers et al., 2006). This suggests that neuronal cells may also incorporate L-DOPA into newly synthesised proteins with structural and possible functional implications.

L-DOPA does not selectively target any particular protein but can be randomly incorporated into all newly synthesised proteins depending on the ratio of L-DOPA to L-tyrosine in the cell (Rodgers et al., 2002, 2004). Replacing L-tyrosine residues in proteins with L-DOPA can result in protein misfolding (Ozawa et al., 2005) and aggregation (Dunlop et al., 2008); aggregate-prone proteins would therefore be intrinsically more sensitive to the substitution of L-tyrosine with L-DOPA than other proteins. The post-mitotic status of neurons means these cells are highly susceptible to non-native proteins (Lee et al., 2006), thus a progressive accumulation of modified L-DOPA-containing proteins in these cells over many years of L-DOPA treatment may contribute to some cellular dysfunction in PD. In the present studies we demonstrate the presence of modified proteins containing L-DOPA in the PD brain and explore the functional implications of this phenomenon using in vitro systems.

Materials and methods

Materials

Dulbecco's Modified Eagles Medium (DMEM)/Ham's Nutrient Mixture F12 (DMEM/F12) and Eagle's Minimal Essential Medium (EMEM) deficient in L-tyrosine were purchased from JRH Biosciences (Lenaxa, Kansas, USA). The human dopaminergic neuroblastoma cell-line SH-SY5Y was obtained from the European Collection of Cell Culture (ECACC). L-tyrosine, L-DOPA, D-DOPA, catalase (bovine liver), superoxide dismutase, nitro blue tetrazolium and amido black were obtained from Sigma Chemical Co. (Sigma-Aldrich, Castle Hill, NSW, Australia). L-DOPA [3-^{14}C] (51.0 mCi/mmol) was obtained from Amersham Pharmacia Biotech Australia Pty. Ltd. (Castle Hill, NSW, Australia). BCA protein assay reagent was from Pierce Biotechnology (Rockford, IL, USA). Water was from a Milli Q 4 stage system (Millipore-Waters, Lane Cove NSW, Australia). Chemicals for electron microscopy were sourced from ProSciTech (Kirwan, Queensland, Australia) unless stated otherwise. Other chemicals, solvents and chromatographic materials were AR or HPLC grade.

Extraction of proteins from human brain tissue

Frozen brain tissue was obtained from the Sydney Brain Bank, which has ethics clearance from the University of New South Wales to collect brain tissue from consenting brain donors for ethically approved research projects. The research was approved by the South Eastern Sydney Area Health Service, Sydney, Australia. Frozen tissue (0.2–0.5 g) from the anterior cingulate cortex, motor cortex, occipital cortex and substantia nigra of 5 PD patients (4 males and 1 female, average age 78) who were treated with L-DOPA for between 5 and 13 years and a five control individuals with no history of neurological disease or L-DOPA treatment (4 males and 1 female, average age 76) were sampled. Proteins were extracted using a modification of the protocol of Ericsson et al. (2007); frozen tissue was further snap frozen in liquid nitrogen, and powdered proteins were then extracted into in 0.1 M Tris buffer, pH 6.8 containing 2% SDS. Proteins were then isolated by TCA precipitation and prepared for HPLC analysis as described below.

Cell culture

SH-SY5Y cells were maintained in 750 cm^2 flasks and subcultured into 6 well plates for incorporation and uptake studies. Cells were cultured in DMEM/F12 medium supplemented 15% foetal calf serum (FCS), 2 mM L-glutamine, penicillin and streptomycin (100 units/ml and 100 μg/ml respectively) and non-essential amino acid solution (Sigma-Aldrich, Castle Hill, NSW, Australia) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For electron microscopy, 2.5×10^5 cells in 0.5 ml were cultured on inserts in 24-well plates (BD, North Ryde, NSW, Australia).

BCA assay

The protein content in samples was measured using the bicinchoninic acid (BCA) assay (Smith et al., 1985). A standard curve was constructed using serial dilutions of bovine serum albumin (BSA) in the same buffer as the samples, and protein concentrations calculated from the standard curve. Absorbance at 562 nm was measured using a Sunrise™ absorbance reader (Tecan Trading ClontARF, Queensland, Australia).

Electrophoresis, protein transfer and redox staining

Cells were harvested using trypsin/EDTA (1:250, pH 7.0) washed three times in PBS and recovered by centrifugation. The cell pellet was then lysed in 0.1% Triton X-100, the lysate centrifuged at 14,000 rpm for 20 min, and protein supernatants collected. Supernatant samples were incubated with NuPAGE loading buffer with reducing agent at 95°C for 5 min, then loaded onto a 4–12% Bis–Tris Gel (NuPAGE®, Novex®, Invitrogen, Thornton, NSW, Australia) and separated using in the XCell SureLock™ Mini-Cell system (NuPAGE®, Novex®, Invitrogen, Thornton, NSW, Australia). 20 μg protein was loaded to each lane. Gels were run in NuPAGE® MES SDS running buffer on a Bio-Rad Power Pac 300 at 100 V for 5 min, followed by 5 min at 150 V and 200 V until the dye front reached the end of the gel (30–60 min).

Proteins were transferred onto PVDF membranes overnight using a Towbin buffer system (30 V at 4 °C). Membranes were washed in water 5 times and incubated in nitro blue tetrazolium (0.24 mM) in alkaline glycinate buffer (2 M potassium glycine buffer, pH 10) (Paz et al., 1991). Colour was allowed to develop for up to 30 min in the dark. Membranes were then de-stained in 100% methanol, and re-stained for total protein in 0.1% amido black. Images were obtained using a Umax Powerlook 1120 scanner.