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Research Paper

Nrf2 activation by tauroursodeoxycholic acid in experimental models of Parkinson's disease

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ABSTRACT

Parkinson's disease (PD) is a progressive neurological disorder, mainly characterized by the loss of dopaminergic neurons in the substantia nigra *pars compacta*. Although the cause of PD remains elusive, mitochondrial dysfunction and severe oxidative stress are strongly implicated in the cell death that characterizes the disease. Under oxidative stress, the master regulator of cellular redox status, nuclear factor erythroid 2 related factor 2 (Nrf2), is responsible for activating the transcription of several cytoprotective enzymes, namely glutathione peroxidase (GPx) and heme oxygenase-1 (HO-1). Nrf2 is a promising target to limit reactive oxygen species (ROS)-mediated damage in PD.

Here, we show that tauroursodeoxycholic acid (TUDCA) prevents both 1-methyl-4-phenylpyridinium (MPP^+)- and α-synuclein-induced oxidative stress, through Nrf2 activation, in SH-SY5Y cells. Additionally, we used C57BL/6 male mice treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to elucidate the effect of TUDCA in this *in vivo* model of PD. *In vivo*, TUDCA treatment increases the expression of Nrf2, Nrf2 stabilizer DJ-1, and Nrf2 downstream target antioxidant enzymes HO-1 and GPx. Moreover, we found that TUDCA enhances GPx activity in the brain.

Altogether, our results suggest that TUDCA is a promising agent to limit ROS-mediated damage, in different models of PD acting, at least in part, through modulation of the Nrf2 signaling pathway. Therefore, TUDCA should be considered a promising therapeutic agent to be implemented in PD.

1. Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease affecting approximately 0.3% of the entire world population (Massano and Bhatia, 2012). PD is a severe progressive disorder characterized by the loss of dopaminergic neurons in the substantia nigra *pars compacta* (SNpc) and depletion of dopamine in the striatum, as well as by the presence of intracytoplasmic inclusions of aggregated proteins designated by Lewy bodies (Przedborski, 2005; Nagatsu and Sawada, 2006; Thomas and Beal, 2007).

Although the leading cause of dopaminergic cells loss remains elusive, it seems to be associated with interconnected mechanisms of cell damage, with particular focus on mitochondrial dysfunction and severe oxidative stress (Jenner, 1999; Lev et al., 2003; Keane et al., 2011).

The brain is considered the most vulnerable organ to oxidative stress and oxidative damage. In fact, the brain consumes more oxygen than any other organ, and contains relatively low levels of antioxidant enzymes, compared to other tissues, as well as high amounts of phospholipids, which are highly vulnerable to oxidative changes (Dias et al., 2013; Gaki and Papavassiliou, 2014). Increased mitochondrial activity as well as age-associated mitochondrial dysfunction results in increased reactive oxygen species (ROS) generation, which may overwhelm the endogenous antioxidant mechanisms (Betarbet et al., 2002; Moon and Paek, 2015). In fact, different *postmortem* analyses in PD brains revealed

Abbreviations: GPx, glutathione peroxidase; HO-1, heme oxygenase-1; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; Nrf2, nuclear factor erythroid 2 related factor 2; PD, Parkinson's disease; SNpc, substantia nigra *pars compacta*; TUDCA, tauroursodeoxycholic acid.

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decreased GSH levels in SNpc, increased byproducts of lipid peroxidation, such as 4-hydroxyl-2-nonenal, carbonyl modifications of soluble proteins, and DNA and RNA oxidation products (Perry et al., 1982; Perry and Yong, 1986; Yoritaka et al., 1996; Alam et al., 1997; Floor and Wetzel, 1998; Jenner, 1998; Zhang et al., 1999). Consequently, neuronal cell death in PD may occur due to an imbalance between mitochondrial ROS and endogenous ROS scavengers (Winklhofer and Haass, 2010; Keane et al., 2011). To maintain a proper physiological redox balance, cells are endowed with a wide variety of endogenous antioxidant enzymes (de Vries et al., 2008; Tufekci et al., 2011). The expression of several of these cytoprotective enzymes is activated, upon ROS exposure, by the transcription factor, nuclear factor erythroid 2 related factor 2 (Nrf2), the master regulator of cellular redox status (de Vries et al., 2008; Holmström et al., 2016; Carvalho et al., 2016a). Once activated, Nrf2 binds the antioxidant response element (ARE), located in the promoter or enhancer regions of antioxidant and cytoprotective genes (Williamson et al., 2012; Zhang et al., 2013). Among the different Nrf2 downstream targets, the most important are glutathione peroxidase (GPx), heme oxygenases (HO), superoxide dismutase (SOD), catalase, peroxiredoxins (Prx), nicotinamide adenine dinucleotide phosphate (NADPH): quinone oxidoreductases (NQOs), GSH synthesis enzymes, and thioredoxins (Trx) (Trachootham et al., 2008; de Vries et al., 2008). Accordingly, the overexpression of these proteins under neurotoxic conditions prevents hydrogen peroxide accumulation and lipid peroxidation, and consequently neuron loss (Wang et al., 2003). Importantly, several studies, including *postmortem* studies in PD brains, and toxin-based animal models, have implicated Nrf2 deregulation in the pathogenesis of PD (Tufekci et al., 2011; Ramsey et al., 2007; Wang et al., 2014). In fact, Nrf2 knockout mice displayed increased susceptibility to different neurotoxins and dopaminergic neuronal depletion (Burton et al., 2006; Jakel et al., 2007; Chen et al., 2009).

Tauroursodeoxycholic acid (TUDCA) is an endogenous bile acid, produced at very low levels in humans, that crosses the blood-brain barrier, with no associated toxicity (Keene et al., 2002; Rodrigues et al., 2002). Growing evidence suggests that it plays an important protective role in both *in vitro* and *in vivo* models of neurological disorders. Importantly, TUDCA is a U.S. Food and Drug Administration-approved drug used in humans to treat primary biliary cirrhosis. Interestingly, it was demonstrated that TUDCA has the ability to prevent ROS production, and to attenuate mitochondrial toxicity and prevent apoptosis (Rodrigues et al., 2000; Keene et al., 2002; Fonseca et al., 2016). TUDCA has anti-apoptotic effects in experimental models of Alzheimer's disease by modulating amyloid β-peptide-induced apoptosis, as well as synaptic toxicity, and attenuates amyloid precursor protein processing and amyloid-β deposition (Solá et al., 2003; Ramalho et al., 2004, 2006; Nunes et al., 2012). Moreover, TUDCA prevents striatal degeneration, and ameliorates locomotor and cognitive deficits in the 3-nitropropionic acid rat model of Huntington's disease (Keene et al., 2002). Very recently, TUDCA efficacy was demonstrated in humans with amyotrophic lateral sclerosis (Elia et al., 2016). We also showed that TUDCA prevents 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced ROS generation and dopaminergic cell death *in vivo*, although the upstream antioxidant mechanisms triggered by TUDCA in this mice model of PD are still incompletely characterized (Castro-Caldas et al., 2012).

In this work we evaluated the ability of TUDCA to modulate the Nrf2 pathway, using experimental models of PD. Here we demonstrate that Nrf2 may be a promising target for TUDCA to limit ROS-mediated damage in PD, potentially leading to interesting therapeutic approaches.

2. Material and methods

2.1. Animal treatments

All animal experiments were carried out in accordance with the institutional, Portuguese and European guidelines (Diário da República, 2ª série N° 121 of 27 June 2011; and 2010/63/EU European Council Directive), and methods were approved by the Direcção Geral de Alimentação e Veterinária (DGAV, reference 021943) and the Ethical Committee for Animal Experimentation of the Faculty of Pharmacy, University of Lisbon.

Twelve-week-old C57BL/6 male mice were purchased from Harlan (Spain) and were housed under standardized conditions, on a 12 h light/dark cycle with free access to a standard diet and water *ad libitum*.

TUDCA and MPTP (Sigma Aldrich) were dissolved in saline and administered intraperitoneally (i.p.). TUDCA was daily injected (1 injection/day), for 3 consecutive days, at a dose of 50 mg/kg body weight, and MPTP was administered at a single dose of 40 mg/kg body weight, as previously described (Castro-Caldas et al., 2012). Mice were divided in 5 groups: i) mice that received saline (Control group); ii) mice treated with TUDCA for 3 consecutive days, and sacrificed 6 h after the last TUDCA injection; iii) mice injected with MPTP and sacrificed at 1, 3 or 6 h after MPTP administration; iv) mice that received daily injection of TUDCA beginning on day 1, followed by i.p. administration of MPTP on day 3, 6 h after the last TUDCA injection, and sacrificed 1, 3 or 6 h after MPTP administration; and v) mice injected with MPTP followed by TUDCA injection 1, 3 or 6 h after neurotoxin administration, and sacrificed on day 3, 6 h after the last TUDCA injection.

After treatment, mice were decapitated, under anesthesia with sodium pentobarbital (50 mg/kg, i.p.), and the entire midbrain region, containing the SNpc, and the whole striatum were dissected as previously described (Castro-Caldas et al., 2009).

Time course studies were carried out in, at least, 3 independent experiments with groups of 3 mice per condition. Previous data showed that the evaluated parameters did not change in control animals through the time course and, therefore, to avoid increasing the number of animals, controls were sacrificed together with TUDCA-treated animals.

2.2. Cell culture conditions and cell treatments

In vitro studies were carried out using the human neuroblastoma SH-SY5Y cell line, obtained from American Type Culture Collection. SH-SY5Y cells (passage 18–20) were maintained in minimum essential medium (MEM) and Ham's F-12 mixture (1:1) supplemented with 15% fetal bovine serum (FBS), 1% non-essential amino acids, 2 mM L-glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin (Gibco, ThermoFisher Scientific), at 37 °C in a humidified atmosphere of 5% CO_2.

Twenty-four hours after plating, the medium was changed and cells were immediately treated with 100 μM TUDCA or vehicle for 12 h. After treatment with TUDCA, cells were incubated with 1 mM 1-methyl-4-phenylpyridinium (MPP^+) for different time points. Controls exposed to vehicle were always included.

2.3. SH-SY5Y cells overexpressing wild-type α-synuclein

A stable SH-SY5Y cell line with inducible expression of human WT α-synuclein was generated as previously described (Vekrellis et al., 2009). Cells were maintained in RPMI 1640® medium supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), 250 μg/mL G418 and 50 μg/mL hygromycin B (ThermoFisher Scientific). The expression of α-synuclein was switched off by the addition of 2 μg/mL doxycycline (Dox). Stock cultures were kept in the presence of Dox. Overexpressing α-synuclein cells were maintained for 12 days in culture in the absence of Dox. Overexpression of α-synuclein was confirmed by Western blot analysis using an antibody against α-synuclein (BD Transduction Laboratories).

Twenty-four hours after plating SH-SY5Y cells overexpressing α-synuclein (DIV 12), the medium was changed and the cells were immediately treated with 100 μM TUDCA or vehicle for 24 or 48 h.

Controls exposed to vehicle were always included.