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Long-term heat shock proteins (HSPs) induction by carbenoxolone improves hallmark features of Parkinson's disease in a rotenone-based model

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ABSTRACT

Protein aggregation and dysfunction of ubiquitin proteasome system (UPS) have been implicated in Parkinson's disease (PD) pathology for a long time. Heat shock proteins (HSPs) have neuro-protective effects in PD as they assist in protein refolding and targeting of irreparable proteins to UPS. To realize their benefits in a chronically progressing disease like PD, it is imperative to maintain slightly up-regulated levels of HSPs consistently over a longer period of time. Here, we evaluate the possible beneficial effects of HSP inducer carbenoxolone (cbx) in a rotenone-based rat model of PD.

Simultaneously with rotenone, a low dose of cbx (20 mg/kg body weight) was administered for five weeks to male SD rats. Weekly behavioral analysis along with end-point evaluation of HSPs, UPS activity, apoptosis, and oxidative stress were performed. The activation of heat shock factor-1 (HSF-1) and up-regulation of HSP70, HSP40, and HSP27 levels in mid-brain following cbx administration resulted in the reduction of α-synuclein and ubiquitin aggregation. This decrease seems to be mediated by reduction in protein carbonylation as well as up-regulation of UPS activity. In addition, the decrease in apoptosis and oxidative stress following HSP upregulation prevented the decline in tyrosine hydroxylase (TH) and dopamine levels in mid-brain region, which in turn resulted in improved motor functions.

Thus, persistent HSP induction at low levels by cbx could improve the PD pathophysiology.

1. Introduction

One of the characteristic pathological hallmarks of Parkinson's disease is the occurrence of intracellular protein aggregates known as Lewy bodies (Ferrer, 2009). Lewy bodies are considered detrimental to neurons as they impose spatial constrains and may sequester other normal proteins required for cellular functions (Patterson and Höhfeld, 2008). Though many components have been identified, α-synuclein comprises the major fraction of Lewy bodies (Cookson, 2009). Misfolding and aggregation of α-synuclein is triggered by several factors like overexpression, point mutations and oxidative stress (Thomas and Beal, 2011). Processes like mitochondrial dysfunctions, dopamine metabolism and neuro-inflammation that lead to excessive generation of free radicals, introduce several modifications in the proteins such as S-nitrosation, methionine sulfoxidation and carbonylation (Dalle-Donne et al., 2006). Under conditions of mild oxidative stress, these oxidatively damaged proteins can be efficiently degraded by ubiquitin proteasome system (UPS) (Grimsrud et al., 2008). However, excessive modifications of proteins like α-synuclein make them resistant to degradation leading to their aggregation (Dalle-Donne et al., 2006).

A testimony to this fact is the co-localization of ubiquitin in Lewy bodies (Sampathu et al., 2003; Liu et al., 2002) pointing towards the futile attempts made by UPS for the degradation of misfolded α-synuclein aggregates. Chronic and excessive surge of oxidatively damaged and misfolded proteins as observed in PD overwhelms the UPS. In fact, UPS dysfunction is one of the main pathological factors that underlie neurodegeneration process. Decline in proteolytic activity of the 20S core of proteasome has been reported in PD brains (McNaught and Jenner, 2001).

Abbreviations: PD, Parkinson's disease; UPS, Ubiquitin proteasome system; HSPs, Heat shock proteins; Cbx, Carbenoxolone; HSF-1, Heat shock factor-1; TH, Tyrosine hydroxylase; RIPA, Radio immunoprecipitation assay; PMF, Post mitochondrial fraction; DAB, Diaminobenzidine; BCIP, 5-Bromo-4-chloro-3-indolyl phosphate; NBT, Nitrobluetetrazolium; AMC, 7-Amino-4-methylcoumarin; TCA, Trichloroacetic acid; MDA, Malondialdehyde; TBA, Thiobarbituric acid; LPO, Lipid peroxidation; DOPAC, 3,4-Dihydroxyphenylacetic acid; HVA, Homovanillic acid; MAO-B, Monoamine oxidase-B; PGA-1, Prostaglandin A-1.

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Additionally in the substantia nigra region of brain in PD patients, decline in the expression of subunits in the 20S core particle as well as 19S and 11S proteasomal regulators has also been observed (McNaught et al., 2003). This suggests that the 26S proteasomal complex exhibits both structural and functional deficits during PD. Importance of UPS dysfunction in PD etiology can be gauged by the fact that mutations in its components UCHL-1 and parkin have been associated with inherited forms of PD (Dawson and Dawson, 2003). Thus, PD can be viewed as a protein conformational disorder where the accumulation of proteins occurs due to misfolding and concomitant inability of UPS to remove these misfolded aggregates.

Neurons employ several measures to remove the misfolded proteins or aggregates. One of the first approaches is to refold the proteins with the help of molecular chaperones or heat shock proteins (HSPs), as they are commonly known. HSPs can recognize the exposed hydrophobic segments in misfolded/unfolded proteins (Aridon et al., 2011) and hence act as first line of defense against the misfolded proteins. Besides refolding, HSPs can function to reduce the protein aggregates by shielding the exposed interaction surfaces of misfolded proteins (Auluck et al., 2002) to prevent their ordered assembly (fibrillation) and non-specific interaction with other vital proteins in the cells. When the molecular chaperons are unable to repair misfolded proteins, they target them to the UPS or lysosomes for removal (Mclean, 2008). HSPs, associated co-chaperones and regulatory components of UPS like 19S complex work in tight coordination to remove the misfolded proteins (Esser et al., 2004). However, in PD, the activity of UPS and levels of HSPs become insufficient to meet the increased need for proper disposal of misfolded proteins (Aridon et al., 2011) and result in accumulation of oxidatively damaged proteins (Anderson, 2004). Thus, HSPs up-regulation can be a potentially beneficial approach in PD that can act to reduce the proteolytic stress.

Numerous studies have shown that HSPs induction can reduce the α-synuclein aggregation *in vitro* (Luk et al., 2008; Klucken et al., 2004; McLean et al., 2002) as well as *in vivo* (Auluck et al., 2002). On the other hand, some suggest that HSPs can reduce the toxicity of these aggregates but do not affect the aggregation process (Luo et al., 2010). In addition to the direct effects on misfolded proteins, HSPs possess anti-apoptotic activity (Neef et al., 2011) and hence can rescue the dopaminergic neurons from apoptosis. Irrespective of the mechanism, beneficial effects of HSPs induction were observed in all of these studies. In humans, PD is a chronically progressing disease that may span a decade or more. Thus, for any beneficial effects, HSP inducers will need to be given for a long term. Moreover, since inducible HSPs have a short half-life it is necessary to maintain their level consistently over a longer period. Present study looks into the possible neuroprotective benefits of HSPs induction in a chronically progressing rotenone induced model of PD. Here, we use a drug named carbenoxolone (cbx), a semisynthetic derivate of glycyrrhizic acid, that activates heat shock factor-1 (HSF-1) (Kawashima et al., 2009), which in turn stimulates induction of various HSPs (Ali et al., 2010). Simultaneous administration of cbx with rotenone for five weeks slows down the neurodegenerative process and leads to the improvement in motor functions in the rat model of PD indicating its prophylactic potential in treatment of PD.

2. Material and method

2.1. Animals and drug treatments

Male Sprague–Dawley (SD) rats in the weight range of 230–260 g were procured from the Central Animal House of Panjab University. Animals were kept under ambient conditions of temperature and humidity with 12-h day/light photoperiod. All the experimental protocols adhered to the guidelines of ethics committee for the use of experimental animals of Panjab University, which are in accordance to the NIH guidelines.

Animals were randomly divided into four groups-control, rotenone, rotenone + cbx and cbx with 13 animals per group. Control group was given daily injection of 0.2 ml saline (intra peritoneal) and 0.2 ml sunflower oil (subcutaneous) which were vehicles for cbx and rotenone respectively. Rotenone group was administered rotenone suspended in sunflower oil at the dose of 2 mg/kg body weight (Thakur and Nehru, 2013). In the rotenone + cbx group, along with rotenone, cbx (20 mg/kg body weight) was given by intra peritoneal injection. Cbx group was given cbx alone in the manner described above. All drug treatments continued for five weeks. Animals were subjected to behavioral testing and body weight analysis every week. After five week, animals were sacrificed by cervical dislocation, mid-brains dissected out and immediately frozen at −80° C till further use. However, biochemical analysis was done immediately following sacrifice. The animals undergoing histological analysis were perfused with chilled PBS followed by 4% paraformaldehyde.

2.2. Western blot

For western blot analysis, mid-brain tissue was isolated and homogenized in RIPA buffer. PMF was extracted and protein content was estimated using Bradford reagent. Samples were run on 15% SDS-PAGE for α-synuclein and ubiquitin and 10% SDS-PAGE for other proteins. Proteins were transferred onto the nitrocellulose membrane and transfer monitored quantitatively by Ponceau S staining. After blocking with 2% BSA for 2 h, membranes were incubated with antibodies against β-actin (1:1000), TH (1:1000), PSMA-1 (1:1000), HSP90 (1:1000), HSP40 (1:1000), HSP27 (1:1000), HSF-1 (1:1000) (Sigma, St. Louis, MO); HSP70 (1:500), caspase-9 (1:500), caspase-3 (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were developed using alkaline phosphatase conjugated anti-rabbit or anti-mouse secondary antibody (1:10,000) (Genei, Bangalore, India). Quantification of the immunoblots was done with the help of Image J software (NIH). All the intensities were normalized to that of loading control, β-actin.

To detect the high molecular weight aggregates of α-synuclein, the sample were subjected to differential detergent extraction according to the protocol described by Tapia-González et al. (2011). The mid-brains were homogenized in the buffer A (10 mM Tris–HCl, pH 7.6; 0.15 M NaCl, PMSF) containing 1% Triton X-100. Homogenates were centrifuged at 15,000 × *g* at 4 °C for 30 min and supernatant used as the Triton-X soluble fraction. The pellet was redissolved in the buffer B containing 0.1% SDS (10 mM Tris–HCl, pH 8.0; 0.15 M NaCl, 1% Triton X-100, 0.5 sodium deoxycholate). It was centrifuged again at 15,000 × *g* at 4 °C for 30 min and supernatant separated to be used as SDS-soluble fraction. The pellet was redissolved and boiled in the buffer C (3% SDS and 5% β-mercaptoethanol) and used as the SDS-insoluble fraction. All these fractions were subjected to western blot as described previously.

2.3. Immunohistochemistry (IHC)

For TH, ubiquitin and α-synuclein IHC, rats were anesthetized with diethyl ether and fixed by transcardial perfusion with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). The brains were removed and fixed in the same solution for at least 24 h. After the brains get hardened, coronal section was cut at −4 mm from the bregma to get substantia nigra (Paxinos and Watson, 2004). Following this, the brains were embedded in paraffin wax (58°–60 °C) according to the standard protocol (Pearse, 1968). 5-μM serial sections were cut on microtome. IHC was performed according to the standard protocol. Briefly, sodium citrate buffer (pH 6.0) was used as antigen retrieval solution while blocking was done using 2% BSA solution. Sections were incubated in the primary antibodies (1:1000) for 2 h and the corresponding secondary antibody (1:10,000) for another two hours in a moist chamber at 37 °C. Sections were visualized with DAB for α-synuclein and BCIP/NBT for TH and ubiquitin. Eosin was used as counterstain. Quantitation of ubiquitin and α-synuclein expression as well as counting of TH positive neurons was done with the help of Image J software (NIH).

2.4. Proteasome activity assay

Proteasome activity was assessed using 20S proteasome activity assay kit (Chemicon International Inc, CA, USA) according to the manufacturer's protocol. Briefly, samples were homogenized in TSDG buffer [10 mM Tris, 25 mM KCl, 1 mM dithiothreitol (DTT), 1 mM NaN3, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 2 mM ATP; pH 7.5]. Further, homogenized samples were cleared by centrifugation at 14,000 rpm at 4 °C for 20 min. Protein concentrations in the supernatants were determined using Bradford reagent. An aliquot containing 20 μg protein was mixed with assay buffer and appropriate substrate followed by incubation at 37 °C for 30 min. Three different substrates conjugated with AMC (7-amino-4-methylcoumarin) were used to evaluate the different type of peptidase activity of proteasome. These substrates were (i) substrate-A (ZLLE-AMC) for caspase-like activity, (ii) substrate-B (Suc-LLVY-AMC) for chymotrypsin-like activity and (iii) substrate-C (BOC-LRR-AMC) for trypsin like activity. Fluorescence emitted by cleavage of substrates with the release of AMC was read at excitation/emission wavelengths of 380/460 nm using Perkin Elmer fluorescent spectrometer. Enzymatic activity was normalized with protein concentration and expressed as percent of control.