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Modulatory effects of sodium salicylate on the factors affecting protein aggregation during rotenone induced Parkinson's disease pathology

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ABSTRACT

Sodium salicylate (SS) confers neuroprotection in various models of Parkinson's disease (PD) but the mechanisms behind its protective actions are not clear. PD pathology is multifactorial involving numerous processes such as protein aggregation, dysfunction of protein degradation machinery and apoptosis. Detailed evaluation of effects of SS on these processes can provide an insight into the mechanism of neuroprotection by SS in PD pathology.

In a rotenone (2 mg/kg b.w.) based rat model of PD, SS (100 mg/kg b.w.) was administered in conjunction. Drug treatments continued for 5 weeks after which various analyses were conducted using mid-brain tissue. IHC analysis revealed a decline in the aggregation of α-synuclein and ubiquitin with SS supplementation. These effects might be mediated by the elevation in HSF-1, HSP-40, and HSP-27 expression following SS co-treatment. This HSP upregulation helped in the improvement in proteasome activity as well as expression. Further, IHC analysis revealed that SS co-treatment prevented the activation of astrocytes caused by rotenone. Since astrocytes are involved in maintenance of glutathione (GSH) homeostasis, it resulted in a concomitant improvement in the GSH levels. As a result, decrease in apoptosis as indicated by caspase-9 and caspase-3 expression as well as TUNEL assay was also observed in the SS conjunction group.

Our results indicate that besides being a known free radical scavenger and anti-inflammatory compound, SS can provide neuroprotection by differently upregulating the HSPs and reducing the protein aggregation burden.

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1. Introduction

Sodium salicylate is a widely used drug that has been shown to have beneficial effects in numerous disorders affecting the central nervous system. Among these disorders, the protective effects of SS have been very strongly projected in the Parkinson's disease (PD). Numerous studies in the MPTP and 6-OHDA based models of PD (Ferger et al., 1999; Mohanakumar et al., 2000; Sairam et al., 2003) have conclusively shown its neuroprotective ability. The major mechanism implicated in neuroprotective effects of SS is free radical scavenging (Mohanakumar et al., 2000). In addition, anti-inflammatory properties of SS have also been shown to be important for its protective actions (Thakur and Nehru, 2013). However, none of these studies has explored the effects of SS on the protein aggregation associated with PD.

The formation of protein aggregates called Lewy bodies is one of the important pathological factors that contribute to the selective neuronal death associated with PD (Moore et al., 2005). α-Synuclein is the key component of Lewy bodies (Surguchov, 2008, 2013) which gets misfolded and attains an abnormal β-sheet rich structure (Takalo et al., 2013) ultimately leading to aggregation (Cookson, 2009). The misfolding is triggered by numerous factors such as oxidative stress, gene mutations and failure of protein quality control machinery (Takalo et al., 2013). The aggregated synuclein can itself cause the inhibition of ubiquitin proteasome system (UPS) directly (Cook and Petrucelli, 2009). To combat against the misfolded proteins and associated toxicity, cells recruit

Abbreviations: 6-OHDA, 6-hydroxydopamine; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; COX-2, cyclooxygenase-2; DTNB, 5,5-Dithiobis 2-Nitrobenzoic acid; ETC, electron transport chain; GFAP, glial fibrillary acidic protein; HSP, heat shock proteins; HSF-1, heat shock factor-1; iNOS, inducible nitric oxide synthase; NBT, nitrobluetetrazolium; PBS, phosphate buffer saline; PBST, phosphate buffer saline tween; PD, Parkinson's disease; PMF, post mitochondrial fraction; ROS, reactive oxygen species; TCA, trichloroacetic acid; MPTP, methyl-4-phenyl-1,2,3,6 tetrahydropyridine.

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the molecular chaperones or HSPs. They recognize solvent-exposed hydrophobic segments of non-native polypeptides and assist them in proper folding and assembly into their native conformations (Aridon et al., 2011). When the molecular chaperones are unable to repair misfolded proteins, they target these proteins to the UPS or lysosomes for removal (Mclean, 2008). Several families of HSPs such as HSP-70 (Klucken et al., 2004) and HSP-27 (Stetler et al., 2009) function in conjunction to prevent either the aggregation (Auluck et al., 2002) or block the apoptosis (Neef et al., 2011). However, the levels of HSPs become insufficient to handle the chronic stress conditions during PD (Aridon et al., 2011).

Recent evidence suggests that neurons can release the protein aggregates into the extracellular space where they are taken up by the neighboring glial cells (Zhang et al., 2005). Since these aggregates are highly immunogenic, they instigate the activation of glial cells resulting in the release of reactive oxygen species, reactive nitrogen species and proinflammatory cytokines (Hirsch et al., 2012; Lee et al., 2010). Excessive production of these products leads to the oxidative stress and burdens the anti-oxidant defenses of the neurons (Andersen, 2004). The most affected among them is the glutathione (GSH), which has an important role in the shielding the thiol groups of various proteins from oxidation (Martin and Teismann, 2009). The GSH depletion seen in PD is not only due to its increased utilization to combat oxidative stress but also stem from its compromised synthesis. This happens due to the activation of astrocytes, which is the cell type involved in regulating GSH homeostasis in brain (Barreto et al., 2011). As astrocytes have higher concentration of GSH inside them in comparison to neurons, they release it into extracellular space where it is cleaved into di-peptides by the γ-glutamyltranspeptidase enzyme residing on the outer membrane of astrocytes (Rappold and Tieu, 2010). These di-peptide precursors are then taken up by the neighboring neurons for GSH synthesis inside them (Rappold and Tieu, 2010). However, the GSH secreting function is hampered when astrocytes are activated (Ricci et al., 2009). Loss of GSH results in even more oxidative modifications and subsequent misfolding of various proteins (Martin and Teismann, 2009). These misfolded proteins further enhance the neuroinflammation. In this way, a continuous feed-forward cycle of cellular damage sets in. Loss of GSH also results in the release of cytochrome-c from mitochondrial inner membrane and activation of apoptotic pathway (Marí et al., 2009). Resultant death of dopaminergic neurons then manifests in the various clinical symptoms associated with disease (Dauer and Przedborski, 2003).

Thus, protein aggregates not just exert direct cytotoxicity but also influences other processes such as proteasomal function and neuroinflammation. Since all these mechanisms contribute significantly to the PD pathogenesis, we evaluated the effects of SS over these processes in a rotenone-based model of PD.

2. Materials and methods

2.1. Animals and drug treatments

Male Sprague Dawley (SD) rats, 3 months in age, in the weight range of 230–250 g were used in the studies. All the animals were procured from the Central Animal House of Panjab University, India. They were kept under the ambient conditions of temperature and humidity with a 12-h day/light photoperiod. Animals were provided with standard food and water *ad libitum*. All the experimental protocols pertaining to animal handling were approved by ethics committee for the use of experimental animals of Panjab University, India and were in accordance with NIH guidelines.

Rats were randomly divided into four groups with 11 animals in each group. To induce the PD-like symptoms, rotenone (Sigma, St. Louis, MO) was injected subcutaneously to the animals at the dose of 2 mg/kg body weight (Nehru et al., 2008). Rotenone was suspended in the sunflower oil and vortexed thoroughly before injection. The injection volume was 1 ml/kg of rat body weight. To study the neuroprotective effects of SS (CDH, India) another group was given the conjunctive treatment of SS along with rotenone. SS dissolved in normal saline was given by intraperitoneal injection at a dose of 100 mg/kg body weight. One group was administered SS alone. Another group served as vehicle control and was given subcutaneous injection of 0.2 ml sunflower oil and intraperitoneal injection of 0.2 ml normal saline. All the drug treatments were given everyday for the duration of 5 weeks. After completion of the study duration, animals were sacrificed by cervical dislocation. Four animals in each group were used for the histological processing. For the other seven animals, brains were immediately dissected to isolate mid-brain and frozen in −80 °C till further use. A portion of all these midbrains was used for the glutathione assays, which were done immediately following sacrifice. A portion of freshly isolated midbrains from six animals was used for the proteasome analysis. Using the remaining tissue, Western blot analysis was performed using tissue from three animals per group.

Considering the possible gastrointestinal side effects of 100 mg/kg SS dosage, the stomachs of all the animals used in the study were also thoroughly examined under a dissection microscope for the presence of any ulcers or injuries at the time of sacrifice. No gross morphological changes were observed (data not shown).

2.2. Biochemical studies

2.2.1. GSH estimation

The method of Ellman (1959) was used to analyze the reduced glutathione content. Mid-brain tissue was homogenized in 0.2 M phosphate buffer (pH 8.0) and centrifuged at 10,000*g* for 30 min to get post-mitochondrial fraction (PMF). 25% TCA was added to the PMF to precipitate proteins and briefly centrifuged at 1500*g* to remove the precipitate. It was followed by addition of 5,5-Dithiobis 2-Nitrobenzoic acid (DTNB). The thiol groups react with DTNB to form 2-nitro-5mercapto benzoic acid, which can be read spectrophotometrically at 412 nm.

2.2.2. GSSG estimation

For this, the total glutathione content was measured by the method of Zahler and Cleland (1968). PMF was incubated with dithioerytheritol at room temperature. Dithioerytheritol is used to reduce all the thiol groups and the resulting monothiols can be assayed at 412 nm with DTNB in the presence of arsenite. Reduced GSH was subtracted from the total glutathione content to get the value for oxidized glutathione.

2.3. Histology

For tissue fixation, rats were deeply anesthetized using diethyl ether. Afterwards, they were perfused with ice-cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. The brains were dissected and immersed in paraformaldehyde for a period of 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) and embedded in paraffin wax (58°–60 °C) according to the standard protocol (Pearse, 1968). Serial sections were cut (5 μM thickness) by a hand-operated microtome.

2.3.1. Immunohistochemistry (IHC)

Brain sections were deparaffinized in xylene and hydrated through a graded series of alcohol. For antigen retrieval, slides were incubated in boiling sodium citrate buffer (pH 6.0) for 15 min. Afterwards slides were incubated with 2% BSA in PBS for

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