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Impaired hepatic function and central dopaminergic denervation in a rodent model of Parkinson's disease: A self-perpetuating crosstalk?

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ABSTRACT

In Parkinson's disease (PD), aside from the central lesion, involvement of visceral organs has been proposed as part of the complex clinical picture of the disease. The issue is still poorly understood and relatively unexplored. In this study we used a classic rodent model of nigrostriatal degeneration, induced by the intrastriatal injection of 6-hydroxydopamine (6-OHDA), to investigate whether and how a PD-like central dopaminergic denervation may influence hepatic functions. Rats received an intrastriatal injection of 6-OHDA or saline (sham), and blood, cerebrospinal fluid, liver and brain samples were obtained for up to 8 weeks after surgery. Specimens were analyzed for changes in cytokine and thyroid hormone levels, as well as liver mitochondrial alterations. Hepatic mitochondria isolated from animals bearing extended nigrostriatal lesion displayed increased ROS production, while membrane potential ($\Delta\Psi$) and ATP production were significantly decreased. Reduced ATP production correlated with nigral neuronal loss. Thyroid hormone levels were significantly increased in serum of PD rats compared to sham animals while steady expression of selected cytokines was detected in all groups. Hepatic enzyme functions were comparable in all animals. Our study indicates for the first time that in a rodent model of PD, hepatic mitochondria dysfunctions arise as a consequence of nigrostriatal degeneration, and that thyroid hormone represents a key interface in this CNS-liver interaction. Liver plays a fundamental detoxifying function and a better understanding of PD-related hepatic mitochondrial alterations, which might further promote neurodegeneration, may represent an important step for the development of novel therapeutic strategies.

1. Introduction

Parkinson's disease (PD) is one of the most common and devastating neurological disorders in the elderly, and is principally characterized by the degeneration of dopaminergic neurons of the *substantia nigra pars compacta* (SNc) projecting to the striatum. Although PD is the prototypical movement disorder, numerous non-motor functions are also affected in PD patients or PD animal models [1,2]. The splanchnic district, in particular, is significantly involved; gastrointestinal dysfunctions [3–5] represent one of the most common PD non-motor symptoms. In this frame, a potential involvement of the liver in the cascade of events triggered by the central dopaminergic deficit has been occasionally proposed [6], but the issue has never been fully investigated.

The liver is responsible for the body's mainstay protein metabolism, synthesis and degradation, and possesses fundamental detoxifying functions. It is highly connected with the CNS and evidence indicates that the autonomic system plays an important role in regulating hepatic functions [7]. Alterations of liver detoxification capacity and mitochondrial oxidative phosphorylation have been observed in PD [8]. Moreover, detoxification of medications and toxins is less efficient in PD patients compared to healthy individuals, thus possibly reflecting altered liver function [9]. Impaired capacity of hepatic P450 subsystems have been observed in late but not early onset PD

Abbreviations: PD, Parkinson's disease; 6-OHDA, 6-hydroxydopamine; CSF, cerebrospinal fluid; ROS, reactive oxygen species; $\Delta\Psi$, mitochondrial membrane potential; SNc, substantia nigra pars compacta; TH, Tyrosine Hydroxylase; DA, dopamine; DAT, dopamine transporter; ATP, adenosine triphosphate; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; HPA, hypothalamic–pituitary–adrenal pathway; AST, aspartate aminotransferase; ALT, transaminases-alanine aminotransferase; AP, alkaline phosphatase; CNS, central nervous system; HPA, hypothalamic–pituitary–adrenal; T3, triiodothyronine; GSH/GSSH, glutathione/glutathione disulfide

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patients [10,11] suggesting that hepatic alterations are not involved in the etiology of the disease but may emerge as a consequence of central neurodegenerative processes. Recent data indicate that brain dopaminergic systems represent an important center regulating hepatic cytochrome P450 activity [6,12,13]. Complex interactions may, therefore, link liver function to the efficiency of the nigrostriatal system, which represents the main source of dopamine in the CNS.

The development of animal models of PD has considerably improved our understanding of the cellular and biochemical mechanisms involved in the onset and progression of the disease. In particular, loss of nigrostriatal dopaminergic neurons can be induced by the intracerebral administration of the neurotoxin 6-OHDA in rats. Six-OHDA is a hydroxylated analog of dopamine and its neurotoxicity largely depends on its incorporation in dopaminergic neurons via selectively and actively uptake by the dopamine transporter (DAT) [14,15]. In this model unilateral injection of 6-OHDA in the rat striatum results in extensive loss of striatal dopaminergic terminals and a progressive retrograde degeneration of dopaminergic neurons in the ipsilateral SNc [16]. The unilateral 6-OHDA model has been extensively used to investigate various aspects of PD pathophysiology, as well as to test innovative therapeutic strategies [16]. We have recently shown that a massive reduction in fecal output, reminiscent of the constipation seen in PD patients, is present in unilateral 6-OHDA-lesioned animals [17], thereby supporting the use of the "hemi-parkinsonian rat" as a model to assess PD-symptoms affecting the splanchnic district. The central aim of this study was, therefore, to evaluate whether and how changes in hepatic mitochondrial function occur in the presence of on-going nigrostriatal degeneration caused by intrastriatal injection of 6-OHDA.

2. Materials and methods

2.1. Animals, surgery, sacrifice and tissue processing

Male Sprague–Dawley rats (Charles River), weighing 200–225 g at the beginning of the experiment, were housed two per cage, at 20–22 °C on a 12-h light–dark cycle, with food and water *ad libitum*. Animals were left in the housing facilities for at least one week, before the beginning of the experiments. All procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local Animal Care Committee.

Rats (n = 46) were anesthetized with sodium-thiopental (50 mg/kg) and placed in a stereotaxic frame (Stoelting), with the incisor bar positioned 3.3 mm below the interaural line. Animals received an intrastriatal injection of 6-OHDA as described before [18] and were sacrificed at different time points (24 h, 4 and 8 weeks) after the neurotoxic insult. At the time of sacrifice animals were anesthetized (50 mg/kg of sodium-thiopental) and 100–200 μL of cerebrospinal fluid (CSF) was withdrawn from the cisterna magna. Rats were then decapitated, trunk blood was collected and serum aliquots stored in liquid nitrogen. Brains were rapidly removed, and separated in two portions containing the striatum or SNc using a cold Coronal Brain Matrix (2Biological Instruments). Striata from both hemispheres were dissected out, weighted and stored, separately, at −80 °C, while brain coronal portions containing the SNc were stored at −80 °C. At the time of sacrifice, liver from each animal was also removed immediately and processed for isolation of mitochondrial fraction.

2.1.1. Western blot

Striatal and liver samples were homogenized in lysis buffer (Cel-Lytic, Sigma; 1:10, weight:volume) containing protease inhibitors (Roche). Homogenates were centrifuged at 16,000 *g* for 10 min and protein-containing supernatants aliquoted and stored at −80 °C for successive assays. Protein content was quantified using the colorimetric Bicinchoninic Acid Protein method (Sigma).

Striatal and liver samples were separated on a poly-acrylamide gel (4–12% NuPAGE Novex, Invitrogen) and transferred to a nitrocellulose membrane (Invitrogen). Membranes were blocked using a commercial buffer (Odyssey Blocking Buffer, LiCor BioSciences) and incubated overnight at 4 °C with specific primary antibodies against: tyrosine hydroxylase (TH) (Chemicon), β-actin (Santa Cruz) or dopamine transporter (DAT). IRDye800 and IRD700 goat anti-rabbit or anti-mouse secondary antibodies were used for detection (LiCor Biosciences).

2.1.2. Striatal dopamine levels

Quantitative determination of dopamine (DA) was carried out on striatal homogenates using a commercially available competitive ELISA kit (IBL-Hamburg) according to the manufacturer's procedure. All samples were analyzed in duplicates using a spectrophotometer (ELx 808, Biotek) at 450 nm.

2.1.3. Immunohistochemistry

Serial coronal sections (25 μm) were cut throughout the SNc using a cryostat (Leica) picked up on polylysine-coated slides (Thermo Scientific) and stored at −80 °C. Immunohistochemical staining for TH (Chemicon) was carried out as described before [18]. Image analyses were performed by a blinded investigator using an AxioSkop2 microscope (Zeiss) and a computerized image analysis system (AxioCam MRc5, Zeiss) equipped with a dedicated software (AxioVision Rel 4.2, Zeiss). The number of TH-positive neurons was counted bilaterally on every fourth section throughout the SNc. Neuronal survival was expressed as the percentage of TH-positive neurons on the ipsilateral (lesioned) side with respect to the contralateral (intact) side. This approach avoided possible bias due to inter-individual differences. Loss of TH-positive neurons was determined as 100 minus the percentage of surviving neurons.

2.2. Isolation of liver mitochondria

Whole livers (9 g) were washed with ice-cold saline and processed immediately for mitochondria isolation by standard techniques using differential centrifugation [19]. Briefly, minced tissue was homogenized in ice cold medium containing 0.25 M sucrose, 1 mM EDTA, 5 mM HEPES (pH 7.2; all from Sigma, Italy) using a teflon/glass Potter homogenizer (Sartorius). The homogenate was centrifuged at 1000 *g* for 10 min. The supernatant taken up and centrifuged again 10 min at 10,000 *g*. The resulting pellet, resuspended in medium containing 0.25 M sucrose, 5 mM HEPES and centrifuged again 10 min at 10,000 *g*, was kept on ice and used immediately for subsequent determination (see below). Single mitochondrial preparations were obtained for each individual animal (n = 4–6) and protein concentration was determined using the Lowry method [20].

2.2.1. Determination of hepatic mitochondrial ATP production

ATP production was measured using the Perkin Elmer ATPLite kit according to the manufacturer's instructions. Bioenergetics experiments were performed in state 3 in the presence of ADP. Briefly, hepatic mitochondria pellets, obtained from single animals, were re-suspended in phosphate buffer (250 mM sucrose, 5 mM KH2PO4, 1 μM rotenone, pH 7.2) and 25 μM ADP and 6 mM succinate were added. After two minutes suspensions were lysed and ATP production measured [21]. Results represent mean ± S.E of ATP (nmol) produced per mg of protein. Luminescence changes were monitored using a Perkin Elmer Victor 2 luminometer.

2.2.2. Determination of hepatic mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$) was assessed by measuring the uptake of the fluorescent dye rhodamine 123 [22]. Measures were taken from single hepatic mitochondrial preparations