Neuroscience Research 114 (2017) 62–69

Contents lists available at ScienceDirect

Neuroscience Research

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

# Lipopolysaccharide-induced functional and structural injury of the mitochondria in the nigrostriatal pathway

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ARTICLE INFO

*Article history:*
Received 8 July 2016
Received in revised form 26 August 2016
Accepted 15 September 2016
Available online 22 September 2016

*Keywords:*
Parkinson's disease
Neuroinflammation
Mitochondria
Lipopolysaccharide
Oxidative stress

ABSTRACT

Accumulating evidence suggests that chronic inflammation plays a role in the progressive dopaminergic neurodegeneration that occurs in Parkinson's disease. It has been hypothesized that inflammation mediates neuronal damage via exacerbation of a vicious cycle of oxidative stress and mitochondrial dysfunction. The bacterial endotoxin, lipopolysaccharide (LPS), induces microglial activation and inflammation driven dopaminergic neurodegeneration. In order to test the hypothesis that LPS-induced inflammatory response might damage mitochondrial structure and function leading to nigral dopaminergic neuron loss, we injected LPS or saline into the striatum of rats. Here, we found that intrastriatal LPS induced deficit in mitochondrial respiration, damage to mitochondrial cristae, mitochondrial oxidation and nitration. Finally, we found significant loss of dopaminergic neurons in the substantia nigra one week after LPS injection. This study indicates that LPS-induced dopaminergic neurodegeneration might be exerted by mitochondrial injury.

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## 1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that affects approximately 1–3% of the population (Bennett et al., 1996; Lang and Lozano, 1998) and currently has no known cure. The primary pathology of the disease is significant loss of the dopaminergic neurons in the substantia nigra (SN) (Hornykiewicz, 1993; Riederer and Wuketich, 1976). Evidence suggests that inflammation may be a driving factor in the etiology of PD since patients have marked gliosis with increased proinflammatory molecules and receptors in their CSF and brain tissue (Boka et al., 1994; Hunot et al., 1996; McGeer et al., 1988; McRae-Degueurce et al., 1988; Mogi et al., 1996). LPS injection causes microglial activation, dopamine depletion, and loss of dopaminergic neurons (Choi et al., 2009) supporting the thought that the dopaminergic neuron is highly susceptible to inflammation-induced oxidative stress (Blesa et al., 2015). An epidemiological study (Chen et al., 2003) and experimental researches (Kim et al., 2004; Teismann and Ferger, 2001; Vijitruth et al., 2006) showed NSAIDs reduces the risk for development of PD.

The mitochondria produce ATP through the electron transport system (Mitchell, 1961) and ATP synthesis is in synchrony with the rate of oxygen consumption in isolated mitochondria. Oxidative phosphorylation is a major source of endogenous ROS, which is generated at both complexes I and III (Nicholls, 2002). Normally, about 1–2% of the oxygen consumed by mitochondrial respiration becomes free radicals (Boveris and Chance, 1973), but a dysfunctional mitochondria can increase free radical production (Celardo et al., 2014). In addition to being a power source to provide cellular ATP, the mitochondria are also the death switch of cells because they play a role in both apoptotic and necrotic cell death (Bernardi et al., 1999). Impaired electron transport system leads to increased oxidative stress and decreased energy supply (Albers and Beal, 2000; Manfredi and Beal, 2000; Schon and Manfredi, 2003), which contributes to neurotoxicity in several mitochondrial associated neurodegenerative conditions (Beal, 2000; Celardo et al., 2014; Cooper and Schapira, 1997; Greenamyre et al., 1999). Impairment in mitochondrial complex I or complex II can induce neuronal death (Beal, 2000; Manfredi and Beal, 2000). In idiopathic PD, there is evidence for a 30–40% decrease in SN mitochondrial complex I function as well as reduced complex I subunits (Hattori et al., 1991). The discovery of several PD related genetic mutations in mitochondria-associated proteins also implicates that the mitochondria play a significant role in the pathogenesis of PD (Bonifati et al., 2003; Dawson and Dawson, 2003; Kazlauskaite and Muqit, 2015).

In previous studies, we demonstrated that intrastriatal LPS caused the dopaminergic neurodegeneration (Choi et al., 2009; Hunter et al., 2009). However, we have not demonstrated ultrastructural damage in the mitochondria of neurons after intrastriatal LPS. We speculate that intrastriatal LPS injection will induce neu-

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http://dx.doi.org/10.1016/j.neures.2016.09.007
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roinflammatory responses and oxidative stress which in turn leads to ultrastructural changes in the mitochondria and subsequent dopaminergic neuronal death. Here, we show that intrastriatal LPS causes oxidative stress and ultrastructural damage in the mitochondria which precedes loss of dopaminergic neurons in the SN.

## 2. Methods

### 2.1. Animals

Three-month-old male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN) and were housed under a twelve hour light–dark cycle with free access to food and water in the Division of Lab Animal Resources at the University of Kentucky. Experimental protocols involved in the animal studies were in strict accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky (approval #: 2006-0053).

### 2.2. Treatments and injections

Rats were randomly selected and grouped for intrastriatal injections with either saline or LPS from *Salmonella minnesota* (Sigma–Aldrich, St. Louis, MO). Rats were anesthetized with sodium pentobarbital (i.p., 50 mg/kg, Abbott Laboratories, N. Chicago, IL) and were positioned in a stereotaxic apparatus. Eight small openings were created in the skull using a dental trephine. The stereotaxic coordinates measured from Bregma were: anterior/posterior +1.0, medial/lateral ±2.0 and ±3.5, and dorsal/ventral −5.5 and −6.0 as well as anterior/posterior −0.5, medial/lateral ±2.5 and ±4.0, and dorsal/ventral −5.0 and −6.5 (Paxinos and Watson, 1998). Next, either 2 μl/site of 0.9% sterile saline or 2 μl/site of LPS solution made from 2 μg LPS/μl sterile saline (32 μg or 48,000 endotoxin units of total LPS) was injected into each site using a 30 gauge 10 μl Hamilton syringe. The rate of injection was 0.4 μl/min and the needle was kept in place for five minutes post injection before slow withdrawal. Following surgery, the rats were kept on a heating pad, 10 ml/kg of subcutaneous sterile saline was given to aid in postoperative recovery, and the rats were assessed for weight loss and changes in body condition until sacrificing. The saline injections continued until the rats became hydrated and ambulatory and a curved syringe was used to inject Vitacal into the corner of their mouth to make sure the animals did not suffer from nutritional withdraw. The rats were also cleaned twice a day for the first a few days following injections because porphyrin staining appeared on their fur around their eyes and nose.

### 2.3. Immunohistochemistry and histology

Animals were euthanized with $CO_2$ and the brains were removed, fixed in 4% paraformaldehyde, and were stored at 4 °C for three days before transferring them to a 30% sucrose 1× PBS solution stored at 4 °C until the tissue was used for sectioning and immunocytochemistry. Every sixth section throughout the entire nigra was processed for immunocytochemical detection (n = 4–5 per group) of the dopaminergic neuronal marker tyrosine hydroxylase (TH, PelFreeze, 1:2000) using a sensitive ABC–peroxidase method previously described (Lu et al., 2000). Free-floating brain sections were washed in 1× PBS and blocked for one hour. After blocking, they were incubated overnight at 4 °C with the primary antibody solution. Next, they were washed in wash buffer and incubated with the appropriate biotinylated secondary antibody (1:1000) for one hour. Sections were rinsed in 1× PBS and incubated for one hour with the ABC kit (Vector Laboratories, Inc., Burlingame, CA). Peroxidase activity in the tissue sections was visualized using 0.05% diaminobenzidine (Sigma–Aldrich, St. Louis, MO) as a substrate. All sections were mounted on glass slides, dehydrated and coverslipped using Permount (Fisher Scientific, Fair Lawn, NJ). The stained sections were used to determine the extent of dopaminergic neuronal cell damage or microglial activation. For Nissl-staining, adjacent SN sections were mounted on gelatinized slides, stained with cresyl violet, dehydrated in ascending alcohol concentrations, and coverslipped using Permount.

### 2.4. Cell counting

The total number of TH-positive dopaminergic neurons or OX-6 positive activated microglia, present in the SN seven days post LPS was estimated using the optical fractionator method for unbiased cell counting. The optical fractionator method of cell counting combines the optical dissector with fractional sampling, and is unaffected by the volume of reference or the size of the counted elements (West, 1993). The cell counts were performed using a computer-assisted image analysis system consisting of a Zeiss Axioskop2Plus photomicroscope equipped with a Sony DXC-390 video camera, a MS-2000 computer-controlled motorized stage (Applied Scientific Instrumentation, Eugene, OR), a DELL GX260 workstation, and the Bioquant Image Analysis software (R&M Biometrics). Every sixth section throughout the entire SN was counted on both the left and right sides to yield the estimated total number of SN dopaminergic neurons or activated microglia. Each section was viewed at low power (×10 objective) to outline the SN, and the outlines of the compacta region were determined in the TH-stained sections by the distribution of the dopaminergic neurons and by referencing well-established landmarks (Hagg and Varon, 1993). Only the cells visualized within one focal plane and with clearly visualized TH-positive cell bodies were counted at high power (×40 objective). After all of the cells were counted, an estimate of the total numbers of neurons or activated microglia in the SN was calculated by the module The number of neurons in sterile saline injected rats was used to calculate the percentage of surviving neurons in the LPS-injected rats. The number of OX-6 positive microglia determined the amount of activated microglia.

### 2.5. Mitochondria isolation and respiration

This method has been previously described with some minor modifications (Sullivan et al., 2003, 2000). All procedures were performed on ice throughout the protocol. The brains were rapidly dissected out on ice and the striatum and nigral regions were isolated quickly and carefully using a rat brain matrix. Three whole rat nigra had to be pooled to make one nigral mitochondrial sample while a single whole striatum was used for one striatal sample. All the tissues were immediately homogenized using a glass dounce homogenizer with isolation buffer containing 215 mM mannitol, 75 mM sucrose, 0.1% BSA, 1 mM EGTA and 20 mM HEPES. The pH was adjusted to 7.2 (both the stock HEPES and the buffer itself with KOH). Next, the mitochondria were isolated using differential centrifugation. The homogenate was spun at 4 °C for five minutes at 1300 × *g* and the supernatant was transferred into a new tube. The loose pellet was resuspended in an isolation buffer with EGTA and was spun again at 1300 × *g* for five minutes. The resulting supernatant was transferred to a new microcentrifuge tube, topped off with the isolation buffer containing EGTA, and was spun at 13,000 × *g* for ten minutes. The supernatant was discarded and the pellet was resuspended in 500 μl of isolation buffer with EGTA. A nitrogen cell disruption bomb (Parr Instrument Company Moline, IL, USA model 4639) cooled to 4 °C was used to burst the synaptoneurosomes as the resuspended mitochondrial samples were placed inside the nitrogen cell bomb at 1000 psi for five minutes (Brown et al., 2004). Using isolation buffer with EGTA, the sam-

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