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Nitric oxide synthase inhibitors protect against rotenone-induced, oxidative stress mediated parkinsonism in rats

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ABSTRACT

Rotenone is known to cause progressive dopaminergic neuronal loss in rodents, but it remains unclear how this mitochondrial complex-I inhibitor mediates neurodegeneration specific to substantia nigra pars compacta (SNpc). One of the proposed mechanisms is increased free radical generation owing to mitochondrial electron transport chain dysfunction following complex-I inhibition. The present study examined the role of nitric oxide (NO) and hydroxyl radicals (·OH) in mediating rotenone-induced dopaminergic neurotoxicity. Indications of NO involvement are evidenced by inducible nitric oxide synthase (NOS) over-expression, and increased NADPH-diaphorase staining in SNpc neurons 96 h following rotenone administration. Treatment of these animals with specific neuronal NOS inhibitor, 7-nitroindazole (7-NI) and non-specific NOS inhibitor, *N*-ω-nitro-L-argenine methyl ester (L-NAME) caused reversal of rotenone-induced striatal dopamine depletion, and attenuation of the neurotoxin-induced decrease in the number of tyrosine hydroxylase immunoreactive neurons in SNpc, as well as in apomorphine and amphetamine-induced unilateral rotations. Interestingly, the study also demonstrated the contribution of ·OH in mediating rotenone nigral toxicity since there appeared a significant generation of the reactive oxygen species *in vivo* 24 h following rotenone administration, a copious loss of reduced and oxidized glutathione, and increased superoxide dismutase and catalase activities in the cytosolic fractions of the ipsilateral SNpc area on the 5th day. An ·OH scavenging capacity of 7-NI and L-NAME in a Fenton-like reaction, as well as complete reversal of the rotenone-induced increases in the antioxidant enzyme activities, and the loss in reduced and oxidized glutathione contents in the SNpc supported ·OH involvement in rotenone-induced dopaminergic neurotoxicity. While these results strongly suggest the contribution of both ·OH and NO, resulting in acute oxidative stress culminating in dopaminergic neurodegeneration caused by rotenone, the course of events indicated generation of ·OH as the primary event in the neurotoxic processes.

1. Introduction

Although the neuropathology of Parkinson's disease (PD) is well characterized, molecular mechanisms leading to neuronal loss in the A9, substantia nigra pars compacta (SNpc) region are still elusive. Evidences have accumulated in recent times for a major contribution of reactive oxygen species (ROS), specifically nitric oxide (NO) and hydroxyl radicals (·OH) in the pathophysiology of PD. NO is formed by the action of nitric oxide synthase (NOS), which exists in three major isoforms, the inducible, endothelial and the neuronal. Among these isoforms, inducible NOS (iNOS), which is induced in the reactive glia and endothelial cells under inflammatory conditions, is suggested to be a key mediator of neuronal death in PD (Brown and Bal-Price, 2003). NO and its oxidized product, peroxynitrite can inhibit mitochondrial respiration through oxidizing or nitrosylating a variety of biological molecules (Brown and Cooper, 1994; Clementi et al., 1998), leading to excessive glutamate release and excitotoxic cell death (Bal-Price and Brown, 2001; Brown, 2010; Loihl and Murphy, 1998). In SNpc, increased NOS expression has been shown to be associated with reactive microglia (Eve et al., 1998), and any pharmacological measure that addresses increase in NO could protect neurons against the parkinsonian neurotoxin, MPTP (Chung et al., 2011; Watanabe et al., 2004). Furthermore, either iNOS or neuronal NOS (nNOS) knockout mice have been shown to be resistant to MPTP neurotoxicity (Dawson et al., 1996; Liberatore et al., 1999) by halting microglial activation (Wu et al., 2002). Supporting these findings, pharmacological inhibitors of NOS have been shown to render protection against MPTP- or rotenone neurotoxicity (Aquilano et al., 2008; He et al., 2003; Watanabe et al., 2004).

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Amongst the potent NOS inhibitors used 7-nitroindazole (7-NI), a specific nNOS inhibitor has been shown to protect against MPTP toxicity (Mackenzie et al., 1997; Watanabe et al., 2008), whereas a non-specific NOS inhibitor *N*-ω-nitro-L-argenine methyl ester (L-NAME) failed to protect against the neurotoxicity (Mackenzie et al., 1997). In addition to its nNOS inhibitory potential (Hantraye et al., 1996; Watanabe et al., 2004, 2008) 7-NI also possesses significant monoamine oxidase inhibitory activity (Castagnoli et al., 1997; Herraiz et al., 2008).

Chronic administration of rotenone, a specific and potent inhibitor of complex-I, has been shown to cause behavioral and pathological abnormalities as seen in MPTP model of parkinsonism (Alam and Schmidt, 2002; Betarbet et al., 2000; Fleming et al., 2004). Acute intranigral administration of rotenone has been shown to produce similar, but more progressive neurodegeneration mediated through ·OH (Saravanan et al., 2005; Sindhu et al., 2005), which is comparable to the effects of intracranial administration of MPP^+, the active metabolite of MPTP (Sindhu et al., 2006). Potent superoxide and ·OH scavengers such as melatonin (Saravanan et al., 2007), L-deprenyl (Saravanan et al., 2006), sodium salicylate (Madathil et al., 2012) and quercetin (Karuppagounder et al., 2013) have been shown to be neuroprotective against rotenone mediated dopaminergic neuronal death. *In vitro,* rotenone treatment is reported to induce NO and peroxynitrite production leading to decreased proteasome or calpain activity in cell cultures (Chou et al., 2010; Samantaray et al., 2011). Chronic systemic administration of rotenone in rats has been shown to induce NO production (Bashkatova et al., 2004; He et al., 2003). In the present study we investigated the status of NO and ·OH in SNpc, and the resulting changes in oxidative stress parameters following rotenone infusion into the brain nucleus, and tested neuroprotective actions of two NOS inhibitors, 7-NI and L-NAME.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats (250–300 g) from the Institute colony were used in the present study. The animals were maintained under standard conditions of 12 h light/dark cycles, 22 ± 1 °C temperature and 60 ± 5% humidity. They were provided food and water *ad libitum*. The experimental protocols met the National CPCSEA Guidelines on "Proper Care and Use of Animals in Laboratory Research" (Indian National Science Academy, New Delhi, 2000) and were approved by the Animal Ethics Committee of the Institute. Appropriate care has been taken to minimize the sufferings of the animals, and to limit the number of animals used in the experiments.

2.2. Drugs and chemicals

Rotenone, 7-NI, L-NAME, reduced glutathione (GSH), glutathione disulphide (GSSG), dopamine (DA), pyrogallol, O-pthaldialdehyde, apomorphine, *d*-amphetamine sulphate, EDTA and all the chemicals used for RT-PCR were procured from Sigma Chemicals (St. Louis, MO). Primers were custom synthesized from Sigma Senosys, India. Anti-tyrosine hydroxylase (TH) antibody was procured from Chemicon (Merck, Germany). Chloral hydrate was obtained from Fluka, Germany. NADH, NADPH, acetonitrile, heptane sulphonic acid, O-phosphoric acid, triethylamine and perchloric acid were purchased from SISCO Research Laboratories, Mumbai, India. Rotenone was dissolved in 1:1 polyethylene glycol and DMSO. D-Amphetamine was dissolved in double distilled water and apomorphine in 1% sodium metabisulphite in double distilled water. 7-NI dissolved in DMSO:peanut oil (1:5) and L-NAME in double distilled water were administered in rats (i.p.) for 4 days at 12 h intervals.

2.3. Stereotaxic Surgery

Rats were anesthetized with chloral hydrate (450 mg/kg; i.p.). The animal was placed in the flat skull position on a stereotaxic frame (Stoelting, USA) with incisor bar fixed at 3.5 mm below the interaural line. Rotenone dissolved in DMSO: polyethylene glycol (1:1) was infused (6 μg in 1 μl) into the right SNpc at a flow rate of 0.2 μl/min, employing a microinfusion pump consisting of a Worker Bee and Syringe Pump (BAS, West Lafayette, USA). Following infusion, 5 min were given before retracting the probe for complete diffusion of the drug. The stereotaxic coordinates for SNpc were: Lateral = 0.20 cm; Antero-posterior = 0.53 cm; and Dorso-ventral = 0.75 cm from the Bregma point. Following rotenone infusion, contralateral SN received infusion of vehicle (DMSO: polyethylene glycol), and this side served as sham control. Proper postoperative care was provided till the animals recovered completely. Studies on ·OH generation and reverse transcription-PCR (RT-PCR) experiments to detect NOS mRNA were conducted at 24 and 96 h, for deciphering the initial biochemical events following rotenone infusion. Based on our previous studies (Saravanan et al., 2005), significant biochemical changes were observed 5 day after rotenone infusion and thus animals were sacrificed on day 5 for the following biochemical and histological experiments. Minimum 2 weeks are required to observe any perceptible behavioral dysfunction following rotenone-infusion and thus behavioral study were performed 2 weeks (14th day amphetamine, and 16th day apomorphine-induced rotations) after rotenone infusion. These animals were sacrificed on the 18th day and the brains were perfusion fixed for immunocytochemistry.

2.4. Reverse transcription PCR (RT-PCR)

Total RNA was isolated from SNpc region of frozen brains (from rats sacrificed 24 and 96 h post-surgery) employing GITC extraction method (Chomczynski and Sacchi, 1987). RNA was quantified by measuring the absorbance at 260 nm and the integrity was verified by gel electrophoresis. Total RNA (5 μg) was reverse transcribed in a 20 μl reaction mix consisting of 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTP, 500 ng random hexamer and 200 U of reverse transcriptase at 42 °C for 60 min followed by heat inactivation of reverse transcriptase at 94 °C for 5 min. cDNA thus synthesized was kept at 4 °C and later used for PCR amplification. Generally 25 μl reaction mixture contained 1–4 μl of cDNA, 1× PCR buffer, 0.2 mM dNTP, 10 pmol of primers (R-iNOS1: GTGTTCCACCAGGAGATGTTG, R-iNOS2: CTCCTGCCCGCTGAGTTCGCT, R-actin1: TGCCCATCTATGAGGGTTACG, R-actin2: TAGAAGCATTTGCGGTGCACG), 0.2 mM $MgCl_2$, and 1.5 U Red Taq DNA polymerase. Taq was added after the initial denaturation of 94 °C for 5 min. The PCR (MJ Research, MA) conditions were, 35 cycles of denaturation (30 s at 94 °C), annealing (60 °C for β-actin, 62 °C for iNOS) and extension (72 °C for 45 s) which was followed by 7 min final extension at 72 °C. The PCR products were analyzed using 2% agarose gel containing ethidium bromide.

2.5. NADPH-diaphorase (NADPH-d) histochemistry

NADPH-d histochemistry was used to verify the presence of NOS (Hope et al., 1991). Animals were trans-cardially perfused 5 days after rotenone administration with 0.1 M PBS and fixed with 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose and coronal sections passing through SNpc was washed in 0.1 M PBS for 10 min, and in 0.05 M Tris–HCl; pH 7.6 for 10 min. These