Research article

Involvement of the mitochondrial apoptotic pathway and nitric oxide synthase in dopaminergic neuronal death induced by 6-hydroxydopamine and lipopolysaccharide

Sarika Singh[1], Sachin Kumar[2], Madhu Dikshit[2]

Divisions of [1]Toxicology and [2]Pharmacology, Central Drug Research Institute (CSIR), India, Lucknow, India

The primary pathology in Parkinson's disease patients is significant loss of dopaminergic neurons in the substantia nigra through multiple mechanisms. We previously have demonstrated the involvement of nitric oxide (NO) in the dopaminergic neurodegeneration induced by 6-hydroxydopamine (6-OHDA) and lipopolysaccharide (LPS) in rats. The present study was undertaken to investigate further the role of NO in the mitochondria-mediated apoptosis of dopaminergic neurons during the early time period after administration of 6-OHDA and LPS. Measurement of dopamine and its metabolites, TH immunolabeling, cytochrome-c release, mitochondrial complex-I and caspase-3 activity assessment was performed in both the 6-OHDA- and LPS-induced experimental models of Parkinson's disease. Significant decreases in dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), tyrosine hydroxylase (TH) immunolabeling and mitochondrial complex-I activity were observed, with increase in cytochrome-c release and caspase-3 activation. Dopmaine and its metabolite levels, mitochondrial complex-I activity and caspase-3 activity were significantly reversed with treatment of the NOS inhibitor, L-NAME. The reduction in the extent of cytochrome-c release responded variably to NOS inhibition in both the models. The results obtained suggest that NO contributes to mitochondria-mediated neuronal apoptosis in the dopaminergic neurodegeneration induced by 6-OHDA and LPS in rats.

Keywords: 6-hydroxydopamine, lipopolysaccharide, nitric oxide, apoptosis, mitochondria, caspase

Introduction

Parkinson's disease is a progressive neurodegenerative disease affecting approximately 1% of the world's population. Several mechanisms including impaired mitochondrial complex-I activity, mitochondrial permeability transition pore (MTP) formation, increased expression of Fas and caspase activity have been

Correspondence to: Dr Madhu Dikshit MSc PhD, Division of Pharmacology and Dr Sarika Singh MSc PhD, Division of Toxicology, Central Drug Research Institute (CSIR), Lucknow 226001, Uttar Pradesh, India. Tel: +91 (0)522 2612411-18; Fax: +91 (0)522 2623405;
E-mail: <madhu_dikshit@cdri.res.in> and <sarika_singh@cdri.res.in>
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Abbreviations: AMC, 7-amino-4–methylcoumarin; BSA, bovine serum albumin; CHAPS, 3[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DOPAC, 3,4-dihydroxyphenylacetic acid; DTT, dithiothreitol; EDTA, ethylenediamine-tetraacetic acid; EGTA, ethyleneglycoltetraacetic acid; 6-OHDA, 6-hydroxydopamine; HVA, homovanillic acid; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; L-NAME, *N*-ω-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MIB, mitochondria isolation buffer; MPP^+, 1-methyl-4-phenylpyridinium; MTP, mitochondrial transition pore; NO, nitric oxide; NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; ROS, reactive oxygen species; SDH, succinate dehydrogenase; TH, tyrosine hydroxylase

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reported in post-mortem brain samples of Parkinson's disease patients.[1–7] Moreover, *in vitro* studies also support a role of apoptosis in the neurotoxin induced death of neurons. 1-Methyl-4-phenylpyridinium (MPP^+) or 6-hydroxydopamine (6-OHDA) in a concentration-dependent manner increased cytochrome-c release into the cytosol, caspase's activity, uncoupling of mitochondrial oxidative phosphorylation and decreased mitochondrial membrane potential in human neuroblastoma SHSY5Y cells, PC12 cells, mesencephalic dopaminergic neurons and in MN9D dopaminergic neuronal cells has also been reported.[3,5,8–15] Bcl-xl, an anti-apoptotic protein, prevented 6-OHDA induced opening of mitochondrial multiple conductance channels, cytochrome-c release and caspase-3 activation in SHSY5Y cells.[16] Infusion of caspase inhibitor offered protection against 6-OHDA-induced dopaminergic neurodegeneration.[17]

Nitric oxide (NO), a free radical molecule, has been identified as a putative neurotransmitter which also modulates various signal transduction pathways in the brain.[18,19] NO and NO-derived nitrogen species also interact with catecholamines and many proteins, modulate their actions, produce more oxidants and free radicals to trigger cytotoxic pathways as well as mitochondrial impairment.[7,20–22] Previous studies from this laboratory demonstrated a time-dependent increase in the nitric oxide synthase (NOS) activity and inducible nitric oxide synthase (iNOS) expression in rat brain striatum at early time points following 6-OHDA- and LPS-induced neurodegeneration.[23,24] Since these changes were specifically observed in the striatum during early time points of neurodegeneration, it was considered worthwhile to explore the involvement of mitochondrial impairment, cytochrome-c translocation and caspase-3 activation at the same time points after 6-OHDA- and LPS-induced neurodegeneration.

Materials and methods

Materials

Aprotinin, ascorbic acid, biotinylated anti-mouse IgG, caspase-3 substrate (AcDEVD-7-amino-4-methyl coumarin), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), anti-donkey cytochrome-c IgG antibody, 3,3′-diaminobenzidine, decylubiquinone, dithiothreitol (DTT), ethylenediamine-tetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), glycerol, 6-hydroxydopamine (6-OHDA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), horseradish peroxidase (HRP) conjugated anti-mouse IgG, leupeptin, lipopolysaccharide (LPS), magnesium chloride, monoclonal tyrosine hydroxylase (TH) anti-mouse IgG antibody, sodium chloride (NaCl), *N*-ω-nitro-L-arginine methyl ester (L-NAME), perchloric acid, pepstatin, Percoll and phenyl methyl sulfonyl fluoride (PMSF) were procured from Sigma Chemical Co. (St Louis, MO, USA). HRP-conjugated anti-sheep antibody was procured from Santacruz (USA) and Neosporin powder was obtained from Burroughs Welcome (India).

Animals

Sprague–Dawley rats (140–160 g) were housed in polypropylene cages and provided with chow pellets and water *ad libitum*. All experiments were performed according to the ethical guidelines of the institute.

Preparation of 6-OHDA and LPS

6-Hydroxydopamine (2 mg/ml) was prepared fresh in 0.2 mg/ml ascorbate saline. LPS (2 mg/ml) was dissolved in saline.[24]

Stereotaxic injection

Rats were anaesthetized with sodium pentobarbitone (35 mg/kg i.p.) and positioned on a stereotaxic instrument (Narishige Instruments, Tokyo, Japan). Unilateral injection of 3 μl 6-OHDA (6 μg) or LPS (6 μg) in right striatum (AP 1.0 mm from bregma, L 2.0 mm and DV 4.5 mm) and right substantia nigra (5.5 mm posterior from bregma, L 1.6 mm and DV 8.0 mm) was administered over a period of ~5 min using a 26-G Hamilton syringe.[25] The same volume of vehicle (ascorbate saline in place of 6-OHDA or saline in LPS-treated group) was administered to control animals. The needle was left at the injection site for an additional 5 min to avoid any reflux. After injection, the skin was sutured and Neosporin powder was sprayed.[23,24]

L-NAME treatment

L-NAME (30 mg/kg, i.p.) was administered 30 min prior to 6-OHDA or LPS injection and was continued daily till the day of sacrifice.[23]

Immunohistochemistry (IHC)

IHC assay was performed as described previously.[24] Rat brain was perfused with phosphate-buffered saline (PBS, pH 7.2) followed by chilled 4% paraformaldehyde in PBS for fixation. Brains were removed and post-fixed in the same fixative overnight followed by cryopreservation in 10%, 20% and 30% (w/v) sucrose in PBS. Sections (15 μm)were cut using a cryostat (Slee Mainz, Germany). Endogenous peroxidase activity was inhibited by incubating the sections in 0.5% H_2O_2 in methanol. To prevent non-specific labelling,

sections were incubated in blocking buffer containing 0.5% bovine serum albumin (BSA), 1.5% normal goat serum and 0.1% Triton X-100.[29] Sections were incubated for 24 h with monoclonal TH (1:300) in the blocking buffer at 4ºC. Sections were washed three times with PBS and incubated in biotinylated secondary antibody (1:200) for 2 h, subsequently with avidin peroxidase complex (1:100) for 1 h and finally with 3,3-diaminobenzidine to develop colour. Sections were transferred onto gelatinised glass slides, dehydrated, and mounted using DPX. Image analysis was done by Leica Q win 500 image analysis software as described earlier.[24] Analyzed values obtained in the lesioned side were expressed as a percentage of the control side.

Estimation of dopamine and its metabolites

Estimation of dopamine and its metabolites was performed according to the method of Barthwal *et al.*[23] Briefly, brain was quickly removed and both right and left striatum were dissected on ice. Striatum was weighed and homogenised separately in 250 µl of 0.1 M perchloric acid and vigorously vortexed for maximal extraction. The samples were centrifuged at 5000 *g* for 20 min at 4ºC, the supernatant was collected, filtered through a 0.2 µm filter and used to measure dopamine (DA) and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) by high performance liquid chromatography (HPLC) using reverse phase-18 column.[26] Detection was performed at +0.85 V using an electrochemical detector (La Chrom L3500). The mobile phase was citrate buffer (87.7 mM) and methanol (87:13, pH = 3.5). Calculations were done by using external standards of dopamine, DOPAC and HVA.[23]

Isolation of mitochondria from the rat brain striatum

Mitochondria were isolated using the method of Rajapakse *et al.*[27] with minor modifications. Both left and right striatum were separately homogenized in 12% Percoll using a Dounce homogeniser with glass pestle. Homogenate was layered on Percoll density gradient (26% and 40%) and centrifuged at 30,000 *g* (LE-80, Beckman USA SW-28 rotor) for 5 min. The top layer containing myelin and other cellular debris was carefully removed and discarded. The light cream-coloured layer containing mitochondria was collected and diluted in cold mitochondrial isolation buffer (MIB; composition: 0.25 M sucrose, 0.5 mM K^+-EDTA, 10 mM Tris-HCl) in the ratio of 1:4 and centrifuged at 15,000 *g* for 10 min. The resulting pellet was washed by suspending in MIB and centrifuging at 15,000 *g* for 5 min. The pellet obtained was re-suspended in 100 µl of MIB. Purity of the mitochondrial fraction was > 95% as assessed by estimation of succinate dehydrogenase (SDH) and lactate dehydrogenase (LDH) activities.

Mitochondrial complex-I activity in homogenate of rat brain striatum

Spectrophotometric assay of nicotinamide adenine dinucleotide co-enzyme-Q (NADH:CoQ) reductase performed in a reaction mixture consisting of 250 mM sucrose, 1 mM EDTA, 50 mM Tris-HCl, 10 µM decylubiquinone, 0.2 mM potassium cyanide (KCN) and 50 µg protein. Reaction was initiated by adding 50 µM NADH (freshly prepared) and the optical density at 340 nm was monitored for at least 5 min. The activity was calculated in nmole/min/mg of protein.[27]

Cytochrome-c Western blotting

Both mitochondrial and cytosolic fractions were isolated by the method described by Fujimura *et al.*[28] Both striata were homogenised separately by douncing 30 times in a glass grinder in seven volumes of cold suspension buffer (20 mM HEPES, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, 2 µg/ml aprotinin, 10 µg/ml leupeptin, 5 µg/ml pepstatin). Homogenates were centrifuged at 750 *g* at 4ºC for 15 min. Supernatant was collected and centrifuged at 8000 *g* for 20 min at 4ºC. Pellet was taken as mitochondrial fraction, while supernatant was collected and centrifuged at 100,000 *g* for 60 min at 4ºC and considered as cytosolic fraction. Protein (20 µg) was separated on 15% polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane. Blocking was done overnight at 4ºC with 5% BSA. Polyclonal cytochrome-c antibody (1:200) was incubated for 3 h and after washing with TBS-T (Tris 25 mM, NaCl 150 mM, Tween 20 [0.1%]) three times, donkey anti-sheep HRP-conjugated IgG was incubated for 2 h. Bands were detected by chemiluminescent ECL-plus detection system and were exposed to the Hyperfilms (Amersham Pharmacia Biotech). The densitometry analysis of blot was done by using NIH Image J Density Analysis software.[29]

Estimation of caspase-3 activity

Caspase-3 activity was measured using the method described by Cid *et al.*[30] Activity was estimated in the homogenate of both left and right striatum at different time points, viz. 6, 12, 24, 48 and at 72 h. after 6-OHDA or LPS injection. Striatum was homogenized

in HEPES buffer containing NaCl 100 mM, DTT 10 mM, EDTA 1 mM, CHAPS 0.1% and 10% glycerol and incubated for 3 h at 4ºC followed by centrifugation at 10,000 *g* for 10 min. Reaction system consisted of 50 μM fluorogenic substrate and 100 μg of protein. Kinetic analysis was performed to measure the fluorescence intensity for 2 h using a fluorimeter (Varian Caryeclipse, USA) at 380 nm (excitation) and 460 nm (emission). Enzyme activity has been reported as pmole of AMC (7-amino-4-methylcoumarin) liberated per minute.

Statistical analysis

Data were analyzed by one-way ANOVA *post-hoc* Dennett's test or Newman–Keuls multiple comparison test. Data are expressed as mean ± SEM. *P*-values less than 0.05 were considered statistically significant.

Results

TH expression in LPS/6-OHDA treated rats

Unilateral injection of LPS or 6-OHDA led to a significant decrease in the TH immunoreactivity in treated rats, while unilateral injection of saline had no significant effect on the TH immunoreactivity. Indeed, TH-positive fibres were significantly reduced after 3 days of 6-OHDA or LPS injection (Fig. 1). The decrease in TH immunolabelling was about 40–50% in both experimental models.

Alterations in dopamine and its metabolite levels

Dopamine and its metabolites (DOPAC and HVA) measured after 3 days of toxin injection were significantly reduced. Dopamine, DOPAC and HVA levels in control rat striatum were 29 ± 2.4 ng/mg, 4 ± 0.3 ng/mg and 2 ± 0.3 ng/mg protein, respectively. The control and vehicle-treated animals did not exhibit any difference in the levels of DA, DOPAC and HVA. However, a significant decrease in levels of DA, DOPAC and HVA in the 6-OHDA/LPS-treated striatum was exhibited in comparison to controls. Moreover, toxin injection in rat brain led to an approximately 40% decrease in dopamine levels. In L-

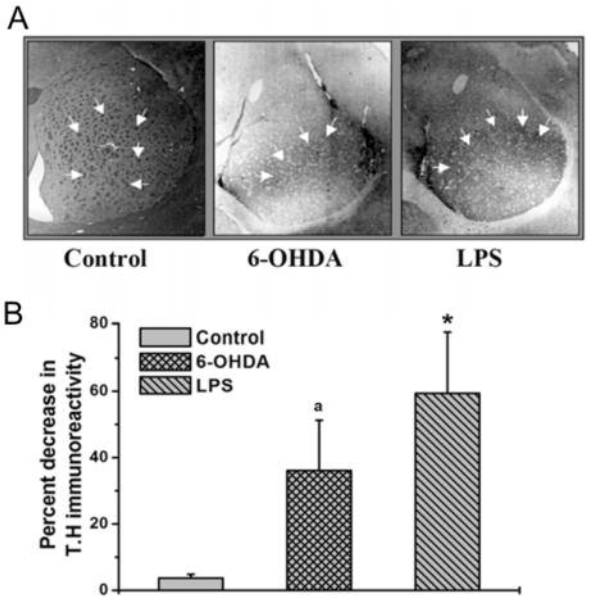

Figure 1 (A) TH immunolabeling shown by arrows, in right rat brain striatum after 3 days of toxin injection. (B) Graphical representation of the TH immunolabeling in the right striatum. Values are mean ± SEM, *n* = 3–5 in each group. [a]*P* < 0.05, **P* < 0.01 in comparison to the respective control by ANOVA Dunnette test

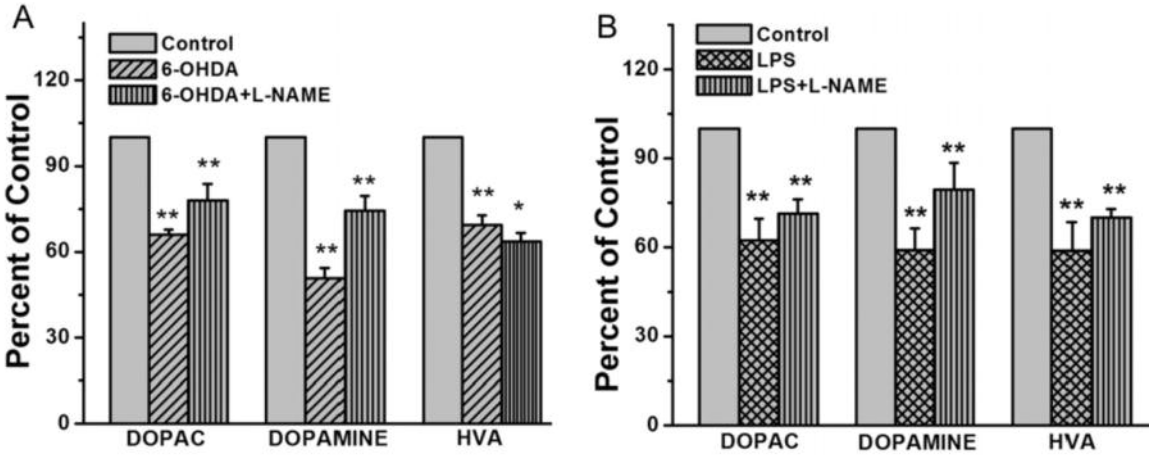

Figure 2 Bar diagram showing decrease in dopamine and its metabolites in rat brain striatum after 3 days of 6-OHDA, 6-OHDA+L-NAME, LPS and LPS+L-NAME treatment. Values are expressed as mean ± SEM, *n* = 3–5 rats in each group. All values were analyzed by one-way ANOVA *post hoc* Dunnette and Newmann–Keuls multiple comparison test. **P* < 0.05 and ***P* < 0.01 in comparison to control (right striatum)

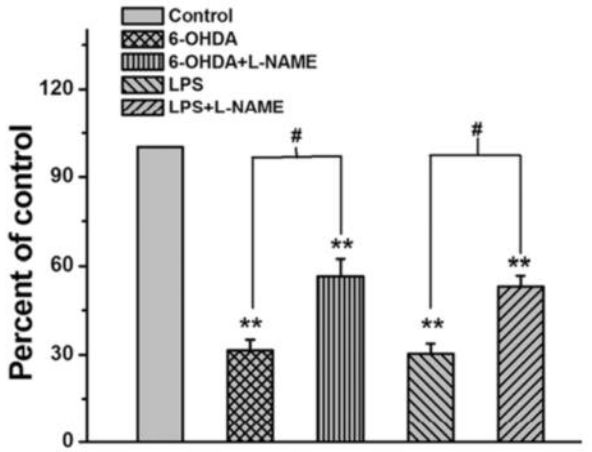

Figure 3 Decrease in mitochondrial complex-I activity in rat brain after 3 days of 6-OHDA, 6- OHDA+LNAME, LPS and LPS+L-NAME treatment. All values are mean ± SEM, **$P < 0.001$ in comparison to control and $^{\#}P < 0.05$ comparison between toxin and toxin + L-NAME treated animals. All values are analyzed by one-way ANOVA *post hoc* Dunnette and Newmann–Keuls multiple comparison test. $n = 3–5$ rats in each group

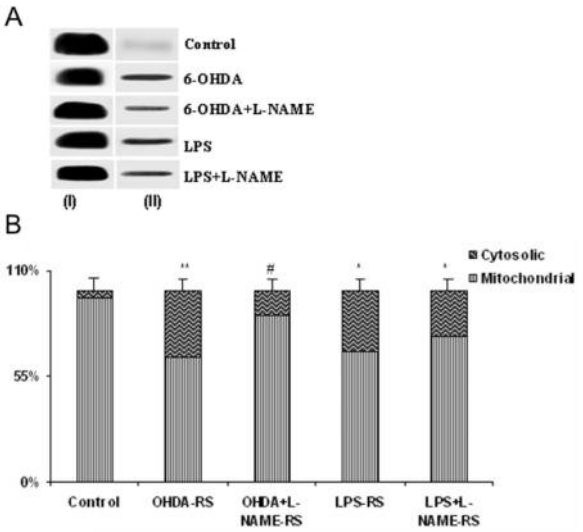

Figure 4 Western blot for cytochrome c in mitochondrial (Lane-I) and cytosolic (Lane-II) fractions of control, toxin injected and toxin + L-NAME treated groups (A). Bar diagram represents the percentage of cytochrome-c in mitochondrial and cytosolic fraction after 3 days of toxin/toxin+L-NAME treatment (B). All values are mean ± SEM, *$P < 0.05$, **$P < 0.01$ toxin vs control and $^{\#}P < 0.01$ toxin vs toxin+L-NAME, analyzed by one-way ANOVA *post hoc* Dunnette and Newmann–Keuls multiple comparison test. $n = 3–5$ rats in each group

NAME-treated rats, the decrease of dopamine and its metabolite levels was reversed up to 25% (Fig. 2). Since the content of dopamine and its metabolites as well as other parameters did not differ significantly in the left and right striatum collected from control vehicle treated rats, the values were pooled together and have been reported.

Change in mitochondrial complex-I activity

The mitochondrial complex-I activity in the striatum of control animals was 44×10^5 nmole/min/mg. Mitochondrial activity of right and left striatum of control animals and left striatum of experimental models was similar and considered as 100%. In both 6-OHDA/LPS-injected animals, the complex-I activity was about 32% of control. In L-NAME treated animals, the activity was reversed and was about 54% of control, indicating partial, but statistically significant, recovery (Fig. 3).

Cytochrome-c release after LPS/6-OHDA treatment

Both 6-OHDA and LPS induced neurodegeneration led to significant release of cytochrome-c in the cytosol, which was almost 30% of the total cytochrome-c content. Cytochrome-c release was much less in the control rat brain striatal cytosol, which could be due to the mechanical disruption during homogenate preparation. L-NAME pretreatment in 6-OHDA-injected animals exhibited significant reduction in cytochrome-c release, while no significant change was observed in LPS-injected rats (Fig. 4).

Caspase-3 activity

Both 6-OHDA- and LPS-injected animals exhibited significant increase in caspase-3 activity in the right striatum. In 6-OHDA-injected rats, the caspase-3 activity was considerably augmented after 24 h, which was further increased up to 72 h. L-NAME pretreatment significantly reduced caspase-3 activity. In LPS-injected rats, increase in caspase 3 activity was observed from 12–72 h, which was always less in comparison to the 6-OHDA-treated group. L-NAME treatment offered significant protection against the increase in caspase-3 activity only at 72 h in LPS-injected rats (Fig. 5). It is pertinent to mention that caspase-3 activity was not detected in control/vehicle treated rats in the conditions used.

Discussion

The present study was undertaken to assess the mitochondrial apoptotic pathway during the early phase of

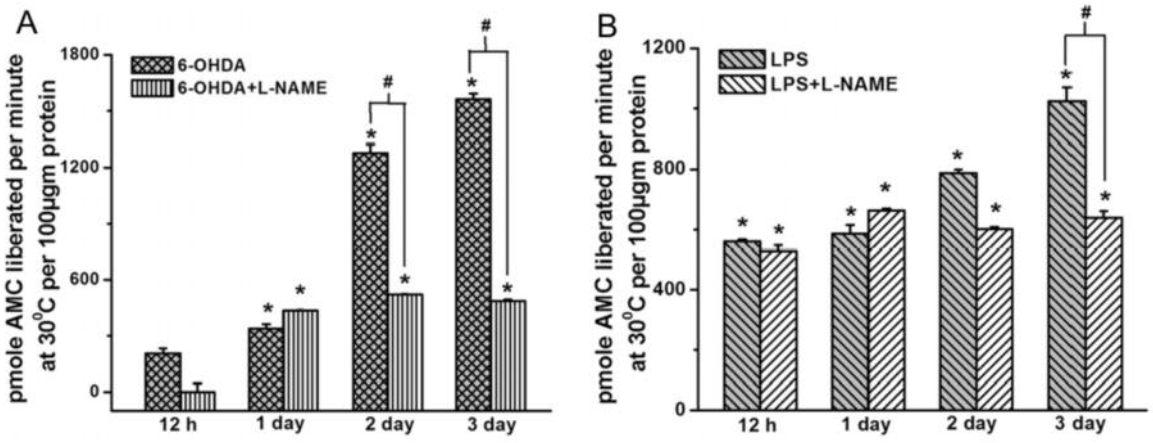

Figure 5 Graphical representation of caspase-3 activity in rat brain striata at different time points after 6-OHDA and 6-OHDA+L-NAME treatments (A) LPS and LPS+L-NAME injection (B). All values are mean ± SEM. ****P* **< 0.05 toxin vs control, #*P* < 0.01 toxin vs toxin+L-NAME by one-way ANOVA *post hoc* Dunnette and Newmann–Keuls multiple comparison test. *n* = 3–5 rats in each group

dopaminergic neurodegeneration, induced by 6-OHDA or LPS injection.[23,24,31] Both 6-OHDA and LPS differ in their mechanism of action to induce dopaminergic neurodegeneration. Intrastriatal 6-OHDA administration can also induce retrograde neuronal damage,[32] while no report was available on LPS-induced retrograde neuronal damage. We observed that only intrastriatal injection of LPS did not lead to progressive neuronal death (data not shown), while LPS injection in striatum as well as in nigra led to progressive neuronal loss. The present study was, therefore, performed by using injection of 6-OHDA or LPS in the striatum as well as in substantia nigra, followed by investigating changes in the striatum, which contains nerve terminals as well as numerous mitochondria. We previously reported augmented iNOS protein expression and nitrite levels with reduced TH expression after 72 h of 6-OHDA and LPS injection in rat brain straiatum.[23,24] The present study was, therefore, undertaken at the same time points to investigate the mitochondria-mediated apoptotic mechanisms in the dopaminergic nerve terminals.

NO-mediated pro- and anti-apoptotic effects and inhibition of mitochondrial complexes, to facilitate the consequent superoxide release, has been reported.[33,34] The excessive release of superoxide contributes to oxidative stress and apoptotic neuronal death.[35] Though impaired mitochondrial complex-I activity has been reported in Parkinson's disease patients[1] and in cell culture studies,[36–38] information on the early time points in the animal models is still lacking. We observed significant increase in the mRNA expression of iNOS in striatum just after 6 h of LPS injection in rat brain striatum and substantia nigra (unpublished data). It was, therefore, obligatory to assess the involvement of mitochondria-associated apoptotic events at the early time points of dopaminergic neurodegeneration.

In the present study, we have found a significant decrease in mitochondrial complex-I activity after toxin injection, which could be partially prevented by L-NAME pretreatment. Mitochondrial impairment along with iNOS expression could lead to the production of toxic $ONOO^-$ that causes mitochondrial swelling, depolarization, calcium release and alteration in permeability transition pore.[39] Decreased mitochondrial activity, mitochondrial membrane potential (MMP), redox imbalance and formation of mitochondrial transition pore (MTP) facilitate the release of cytochrome-c in the cytosol. NO-mediated opening of the mitochondrial membrane pores releases pro-apoptotic proteins of the bcl-2 family and cytochrome-c into the cytoplasm.[40,41]

In the present study, cytochrome-c levels in the cytosolic fractions in the striatum from 6OHDA- or LPS-injected rats were augmented. Decrease in complex-I activity along with increase in the cytosolic cytochrome-c levels suggest the contribution of reduced MMP and formation of MTP during dopaminergic neurodegeneration. L-NAME pre-treatment offered significant protection against 6OHDA-induced cytochrome-c release in the cytosol, while in the LPS model L-NAME did not offer significant protection. Since LPS also enhances TNF-α levels and expression,[42] it might also activate the extrinsic pathway and, consequently, caspase-3 activation. Significant decrease in mitochondrial complex-I activity was found in the LPS model, suggesting involvement of mitochondria-mediated intrinsic apoptotic pathway, but results with L-NAME also indicate the contribution of extrinsic pathway.

The enhanced cytochrome-c release in the cytosol leads to the interaction with apoptotic peptidase activating factor-1 (Apaf-1) and procaspase-9 and activate the procaspase-3.[43] We found a significant increase in caspase-3 activity after 48 h of 6-OHDA injection, which gradually increased till 72 h, in accordance with TH immunoreactivity, suggesting involvement of apoptotic death of the dopaminergic neurons. In LPS-injected rats, caspase-3 activation was significantly more within 12 h of lesion and remained induced up to 72 h. L-NAME treatment offered significant protection in caspase-3 activity at 72 h in both 6-OHDA- and LPS-injected rats.

Conclusions

These results suggest the involvement of mitochondria-mediated apoptotic death of dopaminergic neurons in both models at the early time points of neurodegeneration; however, other factors also seem to be involved in the activation of the terminal executor caspase-3. Significant contribution of NO has revalidated the previous findings from this laboratory, though the mechanisms or extent of its involvement in neurodegeneration remains variable in both the models.

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