Post-treatment with an ultra-low dose of NADPH oxidase inhibitor diphenyleneiodonium attenuates disease progression in multiple Parkinson’s disease models

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Nicotinamide adenine dinucleotide phosphate oxidase, a key superoxide-producing enzyme, plays a critical role in microglia-mediated chronic neuroinflammation and subsequent progressive dopaminergic neurodegeneration in Parkinson’s disease. Although nicotinamide adenine dinucleotide phosphate oxidase-targeting anti-inflammatory therapy for Parkinson’s disease has been proposed, its application in translational research remains limited. The aim of this study was to obtain preclinical evidence supporting this therapeutic strategy by testing the efficacy of an ultra-low dose of the nicotinamide adenine dinucleotide phosphate oxidase inhibitor diphenyleneiodonium in both endotoxin (lipopolysaccharide)- and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using post-treatment regimens. Our data revealed that post-treatment with diphenyleneiodonium significantly attenuated progressive dopaminergic degeneration and improved rotarod activity. Remarkably, post-treatment with diphenyleneiodonium 10 months after lipopolysaccharide injection when mice had 30% loss of nigral dopaminergic neurons, showed high efficacy in protecting the remaining neuronal population and restoring motor function. Diphenyleneiodonium-elicited neuroprotection was associated with the inhibition of microglial activation, a reduction in the expression of proinflammatory factors and an attenuation of α-synuclein aggregation. A pathophysiological evaluation of diphenyleneiodonium-treated mice, including assessment of body weight, organs health, and neuronal counts, revealed no overt signs of toxicity. In summary, infusion of ultra-low dose diphenyleneiodonium potently reduced microglia-mediated chronic neuroinflammation by selectively inhibiting nicotinamide adenine dinucleotide phosphate oxidase and halted the progression of neurodegeneration in mouse models of Parkinson’s disease. The robust neuroprotective effects and lack of apparent toxic side effects suggest that diphenyleneiodonium at ultra-low dose may be a promising candidate for future clinical trials in Parkinson’s disease patients.

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Introduction

Parkinson’s disease is an age-associated movement disorder that progresses over decades in afflicted individuals. The pathological hallmark of Parkinson’s disease is the progressive nigrostriatal dopaminergic neurodegeneration coupled with intracellular inclusions known as Lewy bodies (Olanow and Tatton, 1999). Current clinical dopamine replacement interventions for patients with Parkinson’s disease provide temporary symptomatic relief but fail to halt disease progression (Salawu et al., 2010). Thus, alternative strategies must be developed to target Parkinson’s disease progression to modify the course of the disease.

Microglia-mediated neuroinflammation has been linked to multiple neurodegenerative diseases, including Parkinson’s disease (Gao et al., 2002, 2003; Gao and Hong, 2008; Perry et al., 2010; Czirr and Wyss-Coray, 2012; Phani et al., 2012). These findings prompted pharmaceutical companies to investigate the use of anti-inflammatory drugs as potential treatments for Parkinson’s disease. Early epidemiological and animal studies supported that non-steroidal anti-inflammatory drugs have been shown to reduce the risk of acquiring Parkinson’s disease (Teismann and Ferger, 2001; Chen et al., 2005). However, recent meta-analyses and case-control studies failed to support these findings (Samii et al., 2009; Becker et al., 2011). The development of novel anti-inflammatory strategies to treat neurodegenerative diseases has been further hampered by the failure of several clinical trials (McGeer and McGeer, 2007). The inability of translating successful strategies from animal studies to human therapy highlighted the need for better therapeutic strategies and more suitable animal models in Parkinson’s disease therapy development.

One recent strategy for Parkinson’s disease therapy has been to deviate from conventional anti-inflammatory targets and inhibit upstream mediators, such as microglial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Gao et al., 2012), a key superoxide-producing enzyme. Once activated, NADPH oxidase produces extracellular and intracellular reactive oxygen species (Lambeth, 2004), which are critical in initiating and maintaining chronic neuroinflammatory responses, leading to progressive dopaminergic neurodegeneration (Block et al., 2007; Gao and Hong, 2008; Lambeth et al., 2008). As a proof of concept, we used the NADPH oxidase inhibitor diphenyleneiodonium (DPI) as a therapy for Parkinson’s disease. Although DPI lacks clinical use at its recommended dose (mg/kg) because of non-specificity and high toxicity (Aldieri et al., 2008), we recently reported that DPI at sub-picomolar concentrations ($10^{-14}$ to $10^{-13}$ M) specifically inhibits NADPH oxidase activation and protects dopaminergic neurons in vitro (Wang et al., 2014a). Beyond using a new class of anti-inflammatory drugs, we recognized that the choice of suitable animal models was essential for the successful development of Parkinson’s disease therapies. Widely used parkinsonian animal models, including those generated by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine, acutely lesion dopaminergic neurons within days but fail to recapitulate the progressive feature of Parkinson’s disease (Cannon and Greenamyre, 2010). Moreover, the use of pretreatment regimens for candidate therapies in these acute parkinsonian animal models together with their toxicities after long-term usage have hampered the progress of successful translational therapies. Here, we aimed to overcome these obstacles by post-administering an ultra-low dose of DPI in chronic inflammation-based Parkinson’s disease models, which recapitulate the delayed, progressive features of dopaminergic degeneration. We found that DPI at an extreme low dose (10 ng/kg/day, subcutaneously for 2-week infusion) showed no apparent toxicity and successfully protected dopaminergic neurons in two inflammation-driven mouse models after the loss of ~30% dopaminergic neurons. The therapeutic potential of DPI was further verified in a sub-chronic MPTP Parkinson’s disease mouse model. Parallel experiments in NADPH oxidase-deficient mice validated that NADPH oxidase is the molecular target of DPI-elicited neuroprotection. Our findings suggest a promising strategy for arresting Parkinson’s disease progression by mitigating neuroinflammation through the inhibition of NADPH oxidase.

Materials and methods

Animal treatments

A repeated MPTP regimen (15 mg/kg, subcutaneously for six consecutive days) or a single systemic lipopolysaccharide (LPS) injection (Escherichia coli 0111:B4, Sigma) were administered to C57BL/6J and/or transgenic mice over-expressing human A53T mutant α-synuclein (B6.C3-Tg [Prnp-SNCA*A53T] 83 Vle/J, The Jackson Laboratory) mice. The dosage of MPTP (Zhang et al., 2004; Wang et al., 2014b) or LPS (Qin et al., 2007, 2013) was selected based on our previous studies. Mice used as vehicle controls received an equal volume of 0.9% saline. In both MPTP and LPS regimens, mice were treated subcutaneously with DPI at 10 ng/kg/day for 2 weeks via an Alzet osmotic pump. In LPS-treated C57BL/6J mice, DPI was administered after 3 months (pre-motor group,
n = 8 to 11 each group with total 37 mice) or 10 months (motor group, n = 6 each group with total 24 mice) after LPS injection. LPS-treated transgenic mice over-expressing human A53T mutant α-synuclein (n = 5 to 6 each group with total 16 mice) received DPI infusion after 1 month of LPS challenge. In MPTP-injected C57BL/6J mice (n = 8 to 10 each group with total 27 mice), DPI infusion started 3 days after the first injection of MPTP. All the mice were euthanized at the desired time points. Housing, breeding and experimental use of the animals were performed in strict accordance with the National Institutes of Health guidelines. All procedures were approved by the National Institute of Environmental Health Sciences/National Institutes of Health animal care and use committee.

Immunohistochemistry and double-labelling immunofluorescence

Immunostaining and double-labelling immunofluorescence were performed as described previously (Gao et al., 2011; Wang et al., 2012, 2014b). For details of immunostaining assays and quantitative analysis see the online Supplementary material.

Cell counts

The number of tyrosine hydroxylase-immunoreactive neurons in the substantia nigra pars compacta was estimated using stereological methodology with the optical fractionators method (MBF Science) as described previously (Wang et al., 2014b). For details see Supplementary material.

Rotarod test

The rotarod behaviour test was measured using a Rotamex device (Columbus Instruments). The parameters of the rotarod system include start speed, acceleration and highest speed (1 rpm, accelerate 12 rpm/2 s, 50 rpm). The mice underwent three consecutive trials. The rest period between each trial was 30 min. The mean latency time to fall off the rotating rod for the last two trials was used for the analysis.

In situ visualization of superoxide and superoxide-derived oxidant production

In situ visualization of oxidative stress was assessed by dihydroethidium histochemistry according to previous reports with minor modifications (Quick and Dugan, 2001; Wu et al., 2003). Briefly, LPS-injected mice were administered single injections [intraperitoneally (i.p.)] of dihydroethidium at a dose of 20 mg/kg. Eighteen hours later, mice were perfused transcardially with PBS, and coronal substantia nigra sections were examined for the dihydroethidium product using fluorescence microscopy (excitation 534 nm; emission 580 nm).

Real-time PCR analysis

Total RNA was extracted with the RNeasy® Mini kit and reverse-transcribed with an oligo dT primer. Real-time PCR amplification was performed using SYBR® Green PCR Master Mix (Applied Biosystems) and Applied Biosystems 7900HT Fast Real-Time PCR System according to the manufacturer’s protocols. The primers were designed by Vector NTI Version: Advance 11 software (Invitrogen, Supplementary Table 1). The PCR conditions were 95°C for 10 s, 55°C for 30 s, and 72°C for 30 s for 40 cycles. All of the data were normalized to Gadph.

Catecholamine content analysis

The levels of dopamine and its metabolite dihydroxyphenylacetic acid (DOPAC) were measured using high-performance liquid chromatography and coupled with electrochemical detection as described previously (Zhang et al., 2004). For details see Supplementary material.

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) and were analysed statistically with Graph-Pad Prism (GraphPad Software Inc.). Differences with two groups were analysed using unpaired two-tailed Student’s t-test. For more than two groups, one- or two-way ANOVA was applied. When ANOVA showed significant differences, pair-wise comparisons between means were tested by Tukey’s post hoc testing. In all analyses, P < 0.05 was considered statistically significant.

Results

Post-treatment with an ultra-low dose of DPI prevents dopaminergic neurodegeneration and motor deficits in LPS-treated mice

A neuroinflammation-driven progressive dopaminergic neurodegenerative model generated by a single systemic injection of the endotoxin LPS (Qin et al., 2007) was used to determine whether post-treatment of DPI can halt disease progression. The advantages of using this inflammation-based model are twofold: (i) progressive nigral dopaminergic neuron loss over a period of 10 months after LPS injection coincides with motor deficits that can be reversed by L-DOPA (Qin et al., 2007, Liu et al., 2008); and (ii) mimicking the therapeutic window for Parkinson’s disease patients, treatment can be performed at different stages of disease progression. In the present study, LPS-treated mice were separated into two treatment groups (Fig. 1A). The first group was treated with DPI 3 months after LPS injection, a time point prior to nigral dopaminergic neuron loss and behavioral deficits (premotor stage). The second group was treated with DPI after 10 months of LPS injection,
when significant dopaminergic neurodegeneration (30–35% loss of neurons) and motor deficits were observed (motor stage). An ultra-low dose of DPI (10 ng/kg/day), which is approximately one-millionth of the standard doses used in previous reports (Vlessis et al., 1995; Miesel et al., 1996), was delivered systemically via a subcutaneously implanted mini-pump for 2 weeks. This ultra-low dose was chosen based on exploratory dose-response studies evaluating the effectiveness of doses from 0.1 to 10 ng/kg. We found that 10 ng/kg was the lowest dose that still showed potent neuroprotection. Although DPI is known to readily cross the blood–brain barrier (Gatley and Martin, 1979), the measurement of the DPI concentration in the brain at this dose was beyond the detection sensitivity of the analysis.

By extrapolating the pharmacokinetic data from a previous report (Gatley and Martin, 1979), we estimated the brain concentration of DPI to be in the subpicomolar range, which is comparable to the in vitro dose we used previously (Wang et al., 2014a). The efficacies of this ultra-low DPI on LPS-induced dopaminergic neuron loss and reduction in rotarod activity were determined 7 months after infusion for both the premotor (i.e. total 10 months after LPS injection) and motor groups (i.e. total 17 months after LPS injection) (Fig. 1A). This time point was selected because dopaminergic neurodegeneration in premotor-group mice is much more evident after 10 months of LPS injection (Qin et al., 2007, 2013). For consistency, we also selected the same time point (7 months after DPI infusion) in motor-group mice.

Consistent with our previous report (Qin et al., 2007), LPS injection reduced the number of nigral tyrosine hydroxylase-immunoreactive neurons in premotor stage mice by 32%. An almost 50% loss of tyrosine hydroxylase-immunoreactive neuron was detected in motor stage mice (Fig. 1B and C). These results replicated our previous finding showing progressive dopaminergic neurodegeneration in LPS-injected mice (Qin et al., 2007, 2013). It is important to note that the loss of nigral dopaminergic neurons was previously confirmed to reflect the death of the neurons rather than the loss of tyrosine hydroxylase immunoreactivity (Gao et al., 2011). DPI post-treatment exhibited significant protection against LPS-induced dopaminergic neuron loss in both premotor (P = 0.0002, compared with LPS alone group) and motor (P = 0.0339, compared with LPS alone group) stage groups. These results indicate that the LPS-elicited progressive...
neurodegenerative process was attenuated by DPI. In concurrence with protecting neuronal cell bodies, DPI post-treatment also maintained the integrity of the neurite network of dopaminergic neurons in the substantia nigra pars reticulata, as demonstrated by greater tyrosine hydroxylase-immunoreactive fibre density and a reduction of dendritic beading (fragmented dendrites) in DPI/LPS-treated mice compared with the LPS alone group (Supplementary Fig. 1A). Consistent with these morphological observations, the LPS-induced decrease of striatal dopamine levels was significantly attenuated by DPI post-treatment, which showed no differences compared with the vehicle controls (Supplementary Fig. 1B).

Post-treatment with an ultra-low dose of DPI not only showed significant neuroprotection but, even more impressively, displayed potent efficacy in attenuating LPS-elicited motor deficits in both the pre-motor and motor groups. DPI post-treatment markedly attenuated the LPS-induced reduction of rotarod activity in both the premotor (\(P = 0.0132\) compared with LPS alone group) and motor group (\(P = 0.0072\), compared with LPS alone group) (Fig. 1D). It is interesting to note that DPI post-treatment was able to restore the rotarod activity of LPS-treated mice to the same level as control mice, despite its inability to fully prevent the loss of nigral dopaminergic neurons. These results suggest that ultra-low dose DPI is capable of halting progressive dopaminergic neurodegeneration and motor deficits.

**Post-treatment with an ultra-low dose of DPI is neuroprotective in LPS-treated human A53T \(\alpha\)-synuclein over-expressing mice**

\(\alpha\)-Synuclein is a major constituent of Lewy bodies in patients with Parkinson’s disease. The A53T mutation in the SNCA gene is known to increase the amount of aggregated \(\alpha\)-synuclein and is closely associated with dopaminergic neurodegeneration (Lee and Trojanowski, 2006). Although transgenic mice over-expressing human A53T mutant \(\alpha\)-synuclein have been previously used as rodent models of Parkinson’s disease, reports have shown that this transgenic mouse fails to develop overt nigral dopaminergic neurodegeneration (Giasson et al., 2002; Gao et al., 2011). We recently developed a ‘two-hit’ model by injecting LPS into transgenic mice over-expressing human A53T mutant \(\alpha\)-synuclein, which showed earlier onset and a much more robust progressive loss of nigral dopaminergic neurons than C57BL/6J mice (Gao et al., 2011). Thus, the LPS-injected transgenic mouse can serve as an ideal model with which to investigate the neuroprotective effects of DPI. Because of the early onset (as early as 2.5 months after LPS injection) of dopaminergic neurodegeneration in LPS-treated transgenic mice over-expressing human A53T mutant \(\alpha\)-synuclein (Gao et al., 2011), the protective effects of DPI were determined after 4 months of LPS injection, a time point that showed 40–50% loss of dopaminergic neurons. Consistent with previous report (Gao et al., 2011), 4 months after LPS injection, a 43% loss of nigral dopaminergic neurons was noted in transgenic mice over-expressing human A53T mutant \(\alpha\)-synuclein compared to untreated littermate controls. Post-treatment with DPI 1 month after LPS injection led to a significant protection (\(P = 0.0029\), compared with LPS alone group) of the nigral tyrosine hydroxylase-immunoreactive neurons (Fig. 2A–C). A similar degree of DPI-elicited protection of dopaminergic terminals in the striatum was also observed, as shown by the high striatal tyrosine hydroxylase density in the DPI-treated group (87 ± 4% of control) compared to the non-DPI-treated group (56 ± 7%, \(P = 0.0006\); Fig. 2B and D).

The expression of human \(\alpha\)-synuclein in transgenic mice over-expressing human A53T mutant \(\alpha\)-synuclein is not homogeneous in the brain with high levels in brainstem and cortex and very low levels in the substantia nigra (Giasson et al., 2002). We recently confirmed this finding showing minimal protein expression in the substantia nigra by western blot and immunohistochemistry using an antibody again human \(\alpha\)-synuclein in untreated transgenic mice over-expressing human A53T mutant \(\alpha\)-synuclein (Gao et al., 2011). Interestingly, after LPS injection, increased expression and insolubility of human \(\alpha\)-synuclein were observed in the substantia nigra (Gao et al., 2011). In addition to protecting dopaminergic neurons, DPI post-treatment mitigated LPS-induced human \(\alpha\)-synuclein accumulation in the substantia nigra, as detected by immunostaining using SYN211 antibody (specific for human \(\alpha\)-synuclein, Fig. 3A). Double-label immunofluorescence analysis revealed an accumulation of \(\alpha\)-synuclein in the cytosol and perinuclear locations of nigral tyrosine hydroxylase-immunoreactive neurons after LPS injection. In contrast, nigral \(\alpha\)-synuclein immunoreactivity in DPI/LPS-treated transgenic mice over-expressing human A53T mutant \(\alpha\)-synuclein was diffuse and barely visible (Fig. 3B). Quantitative analysis of the nigral SYN211 density indicated a 223% increase in the LPS alone group, which was reduced to 127% in DPI/LPS-treated mice (\(P < 0.0001\), compared with LPS alone group; Fig. 3C).

**Post-treatment with an ultra-low dose of DPI attenuates LPS-elicited microglia-mediated neuroinflammation**

To determine whether the neuroprotective effects of DPI were related to its anti-inflammatory properties, we examined the inhibitory effects of DPI on microglial activation using post-treatment regimens at different stages of the neurodegenerative process. Activation of microglia in the substantia nigra was morphologically observed by immunostaining with two microglial markers: ionized calcium binding adaptor molecule 1 (AIF1, also known as
Iba-1) and ITGAM (formerly known as CD11b, alpha chain of the β-2 integrin receptor). In both the premotor and motor stage, activated microglia characterized by a hypertrophied morphology and intensified ITGAM and AIF1 staining were observed throughout the nigral reticulata area (Fig. 4A). Analysis of ITGAM and AIF1 density and cell body size supported these morphological observations. Compared with the LPS alone group, post-treatment with DPI markedly attenuated microglial activation, as shown by a reduced density of ITGAM \( (P = 0.0020 \text{ and } 0.0001 \text{ in premotor and motor stage, respectively}) \) and AIF1 \( (P = 0.0198 \text{ and } 0.0001 \text{ in premotor and motor stage, respectively}) \) staining and microglial cell body sizes \( (P < 0.0001 \text{ and } 0.0001 \text{ in premotor and motor stage, respectively}; \text{Fig. 4B–D}) \).

Activated microglia secrete a variety of toxic factors, such as tumor necrosis factor alpha, interleukin-1 beta and other proinflammatory cytokines, which work in concert to cause neuronal damage (Block and Hong, 2005). Exposure to LPS produced a long-lasting 2-fold increase in the expression of the proinflammatory genes tumor necrosis factor alpha, interleukin-1 beta and major histocompatibility complex II, in both the premotor and motor groups. Interestingly, ultra-low dose DPI post-treatment prevented the LPS-induced increase in the gene expression of these immune factors (Fig. 4E–G).

**Post-treatment with an ultra-low dose of DPI inhibits LPS-induced oxidative stress**

NADPH oxidase is essential for maintaining chronic microglia-mediated neuroinflammation and subsequent progressive dopaminergic neurodegeneration (Qin et al., 2013). Although DPI exhibits potent inhibitory effects on NADPH oxidase at mg/kg doses, it is not clear whether DPI administered at ultra-low dose is still capable of inhibiting NADPH oxidase activation in vivo. To address this question, we determined the effects of...
ultra-low dose DPI on reactive oxygen species production in the brain after 10 months of LPS injection (Fig. 5A). Our previous report showed that a single systemic LPS injection causes persistent NADPH oxidase activation and related reactive oxygen species production in the mouse brain (Qin et al., 2013). Electron spin resonance analysis further confirmed that NADPH oxidase, but not other oxidases, such as xanthine oxidase, accounts for the major (> 90%) source of LPS-induced oxidative stress in vivo (Sato et al., 2002). In situ visualization of reactive oxygen species production was performed using dihydroethidium, a reactive oxygen species-sensitive dye. Dihydroethidium can readily cross the blood–brain barrier and exhibits red fluorescence through interactions with superoxide and other free radicals in the brain (Wu et al., 2003). As shown in Fig. 5B, reactive oxygen species production in the substantia nigra was minimal in vehicle controls, as evidenced by the low levels of red fluorescence. In contrast, exposure to LPS resulted in increased levels of red fluorescence in the substantia nigra, indicating elevated reactive oxygen species production. Co-staining with tyrosine hydroxylase antibody revealed a high degree of oxidative stress in the dopaminergic neurons of LPS-injected mice (Fig. 5C). Post-treatment with ultra-low dose DPI markedly reduced LPS-induced oxidative stress in the nigra and nigral dopaminergic neurons (Fig. 5B and C).

Quantitative analysis revealed 2.3-fold increase in fluorescence density in LPS-injected mice compared with vehicle controls, which was reduced to 1.1-fold in DPI/LPS-treated mice (P < 0.0001; Fig. 5D). The inhibitory effects of DPI on LPS-induced reactive oxygen species production suggest that ultra-low dose DPI can inhibit NADPH oxidase activation.

The source of reactive oxygen species production has recently become a subject of debate. Mitochondria have been traditionally considered a major source of intracellular reactive oxygen species production; however, our previous finding indicates that NADPH oxidase is a key reactive oxygen species-generating enzyme in microglia in response to LPS stimulation (Qin et al., 2013). In addition to NADPH oxidase, DPI at regularly used doses (between 1 to 5 mg/kg) inhibits a variety of electron-transferring flavoprotein enzymes, including mitochondrial complex I (Aldieri et al., 2008). To investigate whether ultra-low dose DPI has specificity toward NADPH oxidase in vivo, we evaluated the effects of DPI on complex I activity. Interestingly, DPI at 10 ng/kg failed to suppress the activities of mitochondrial complex I in the brain (Fig. 5E). This result indicated a high specificity of ultra-low dose DPI in inhibiting NADPH oxidase and revealed a critical role of this superoxide-producing enzyme in the generation of reactive oxygen species in the brain.
Ultra-low dose DPI affords long-term dopaminergic neuroprotection against MPTP lesions

In addition to using LPS models, we tested the protective effects of ultra-low dose DPI in a MPTP Parkinson’s disease mouse model, which has been widely used to screen for therapeutic agents. Unlike LPS, MPTP directly damages dopaminergic neurons in an acute fashion. Therefore, we initially evaluated the neuroprotective potential of DPI using a pre-treatment regimen. DPI infusion (10 ng/kg/day, subcutaneously) started 2 days before MPTP administration and lasted for 2 weeks (Supplementary Fig. 2A). The neuroprotective effects of DPI were evaluated at different time points (27, 60 and 120 days after the initial injection of MPTP). The chosen 27-day point was based on our previous report (Hu et al., 2008) showing nearly 50% loss of dopaminergic neurons in the substantia nigra pars compacta. Consistently, MPTP elicited a 45% loss of tyrosine hydroxylase-immunoreactive neurons in the substantia nigra pars compacta compared with vehicle controls at the 27-day point. The loss of nigral tyrosine hydroxylase-immunoreactive neurons remained close to the same degree at the 60- and 120-day time points. MPTP-elicited tyrosine hydroxylase-immunoreactive neuronal loss was significantly attenuated by DPI ($P = 0.0032$, compared with the MPTP alone group) at the 27-day time point. The long-lasting protective effect of DPI was evident based on the results obtained at the 60- and 120-day time points (Supplementary Fig. 2B). To exclude the
possibility that DPI-afforded protection was due to either alterations of MPTP metabolism or entry into the brain, we measured MPP+ levels in the brain after MPTP injection and found no significant difference between MPTP and DPI/MPTP (data not shown).

In addition to evaluating the DPI-afforded protection of nigral tyrosine hydroxylase-immunoreactive neurons, striatal levels of dopamine and its metabolite, DOPAC as well as their turnover rate (DOPAC/dopamine) were measured as markers for the functional recovery of dopaminergic neurons (Zigmond et al., 1990; Zigmond, 1997). Twenty-seven days after the MPTP injections, marked decreases in striatal levels of dopamine (68%) compared with the vehicle controls were observed (Supplementary Fig. 2C); in contrast, the decrease in DOPAC level was attenuated (Supplementary Fig. 2D). The higher ratio of DOPAC/dopamine in the MPTP-treated group further indicated a higher turnover of dopamine, likely due to a compensatory effect of dopaminergic neuron loss (Supplementary Fig. 2E). Interestingly, whereas DPI did not prevent the MPTP-induced loss of striatal dopamine levels, significantly high levels of DOPAC ($P = 0.0207$, compared with the MPTP alone group) and DOPAC/dopamine ratios ($P = 0.0284$ compared with the MPTP alone group) were observed in the DPI-treated group (Supplementary Fig. 2D and E), suggesting enhanced functional activities of the surviving dopaminergic neurons (Zigmond et al., 1990; Zigmond, 1997). A time-dependent recovery of dopamine levels was found at the 60-day time point for both the MPTP/vehicle and the MPTP/DPI groups compared with the levels at the 27-day time point. Although the striatal levels of dopamine were the same between these two groups, the DOPAC level and DOPAC/dopamine ratio remained high in the DPI-treated group, suggesting that DPI preserved some degree of functional activity in remaining dopaminergic neurons. Continuing recovery of striatal dopamine levels was shown at the 120-day time point in the MPTP-treated group. It is interesting to note that the levels of dopamine in DPI/MPTP-treated mice returned to the same level as non-MPTP-injected mice ($P = 0.9960$;
Supplementary Fig. 2C). The rapid recovery of striatal dopamine levels supported the promising neuroprotective effects of DPI.

**Ultra-low dose DPI attenuates MPTP-induced oxidative stress and microglial activation**

Additional studies were performed to elucidate the mechanism by which DPI protected against MPTP-elicted neurotoxicity. Although MPTP is directly toxic to dopaminergic neurons, microglia-mediated neuroinflammation contributes to its overall neurotoxicity (Wu et al., 2003; Hu et al., 2008; Levesque et al., 2010). Microglia are indirectly activated to produce cytotoxic factors (reactive microgliosis) in response to MPTP-induced neuronal damage, causing additional dopaminergic degeneration (Levesque et al., 2010). To provide evidence that microglia play a role in DPI-elicted neuroprotection, immunostaining of microglial markers was performed. DPI treatment significantly attenuated MPTP-induced microglial activation, as shown by the reduced cell body size and densities of ITGAM and AIF1 staining in the substantia nigra (Supplementary Fig. 3A). Quantitative analysis revealed decreased immunoreactivities of ITGAM \( (P=0.0466) \) and AIF1 \( (P=0.0385) \) in the substantia nigra compared with the MPTP alone group (Supplementary Fig. 3B). Moreover, DPI significantly reduced MPTP-elicted oxidative stress in the substantia nigra based on the low nigral density of dihydroethidium oxidation (red fluorescence) in the DPI/MPTP group compared with the MPTP alone group \( (P=0.0039; \) Supplementary Fig. 4A and C). Consistent with the LPS model, co-staining with tyrosine hydroxylase antibody revealed that DPI treatment reduced oxidative stress in dopaminergic neurons (Supplementary Fig. 4B). Overall, our results show that ultra-low dose DPI inhibits MPTP-induced reactive microgliosis and oxidative stress.

**NADPH oxidase deficiency abolishes ultra-low dose DPI-afforded neuroprotection**

To investigate whether NADPH oxidase mediates ultra-low dose DPI-afforded neuroprotection, NADPH oxidase-deficient \( (gp91^{phox−/−}) \) and wild-type control \( (gp91^{phox+/+}) \) mice were treated with DPI followed by lesioning with MPTP. Consistent with the data in Supplementary Fig. 2B, in wild-type mice, MPTP injection caused a 46% loss of nigral tyrosine hydroxylase-immunoreactive neurons and had only 23% loss in DPI/MPTP-treated group \( (P=0.0001; \) Supplementary Fig. 5A and C), compared to the vehicle control. In contrast, mice lacking \( gp91^{phox} \) were more resistant to DPI-treated lesions than wild-type controls \( (27±3\% \text{ versus } 46±3\% \text{ loss of nigral tyrosine hydroxylase-immunoreactive neurons, } P=0.0027) \), supporting the potential involvement of NADPH oxidase in MPTP-induced dopaminergic neuron damage. Under this condition, ultra-low dose DPI failed to protect nigral dopaminergic neurons in \( gp91^{phox−/−} \) mice with MPTP lesions \( (P=0.9997, \text{ compared with MPTP alone group}) \). Consistently, reduced degeneration of terminals of dopaminergic neurons in the striatum were observed in DPI/MPTP-treated wild type \( (gp91^{phox+/+}) \) mice, but no difference was detected in NADPH oxidase-deficient \( (gp91^{phox−/−}) \) mice (Supplementary Fig. 5B and D).

**Post-treatment with DPI attenuates MPTP-induced dopaminergic degeneration and motor deficits**

The positive results from the pretreatment studies (Supplementary Fig. 2 to 5) led us to explore the possibility of a post-treatment regimen to further assess the therapeutic efficacy of ultra-low dose DPI in the MPTP model. DPI (10 ng/kg/day) was post-administered at Day 3 after the first injection of MPTP for 2 weeks (Fig. 6A). Pilot studies showed a ~20–30% loss of nigral tyrosine hydroxylase-immunoreactive neurons when DPI infusion was initiated. Twenty-seven days after MPTP lesion, a significantly greater number of nigral tyrosine hydroxylase-immunoreactive neurons was found in DPI/MPTP-treated mice \( (P=0.0239, \text{ compared with the MPTP alone group; Fig. 6B}) \). Consistent with the pretreatment regimens, DPI post-treatment enhanced the activity of nigral dopaminergic neurons, as shown by an increased dopamine turnover rate compared to the MPTP alone group \( (P=0.0067; \text{ Fig. 6C}) \). The neuroprotective effect of DPI post-treatment was correlated with the attenuation of MPTP-elicted motor deficits \( (P=0.0470) \), as measured by the rotarod test (Fig. 6D). Longer-term studies similar to Supplementary Fig. 2 were not conducted in this post-treatment regimen. However, based on the high turnover rate of dopamine and improved motor behaviour in DPI-treated mice, it is likely that mice post-treated with DPI would display an accelerated recovery from MPTP treatment at later time points.

**Ultra-low dose DPI displays no overt toxicity in mice**

The severe toxicity of DPI at the recommended dose hampers its potential for clinical usage in patients. Thus, we conducted a toxicological screen of DPI when administered at ultra-low dose. All parameters studied in this project, including the quantitative and morphological analysis (neurons and microglia), neurochemical measurements (proinflammatory factors, complex I activity and dihydroethidium oxidation) and behavioural observations (rotarod activities, Fig. 7A), showed no difference in DPI alone group compared to vehicle controls. General
evaluation of major organ systems and organ weights concluded that ultra-low dose DPI treatment had no significant effects on body and organ weights (including liver, kidney and spleen; Fig. 7B–E) nor any observable histopathological changes in haematoxylin and eosin-staining of sections from liver, spleen, kidney, testes, heart and lung (Fig. 7F) when compared to saline treated controls. Additionally, haematoxylin and eosin staining of brain sections based on the modified National Toxicology Program protocol (Rao et al., 2013) also revealed no significant changes in DPI-treated mice compared to vehicle controls (Fig. 7F). We further determined whether low doses of DPI affect the peripheral immune function by examining both high and low doses of DPI on superoxide production in mice neutrophils. Results indicated that high dose DPI (10^{-5} M) completely inhibited superoxide production, but DPI in low concentrations (10^{-13} M and 10^{-14} M) failed to affect the oxidative burst of neutrophils (Supplementary Fig. 6). Our preliminary data also showed no effect of low-dose DPI on the superoxide production of human neutrophils (data not shown). Overall, these findings suggest that ultra-low dose DPI displayed no overt organ toxicity and does not influence peripheral immune cell functions that are critical for hosting normal immune responses.

**Discussion**

The present study demonstrated that DPI at an ultra-low dose provides potent beneficial effects in three models of dopaminergic degeneration. One salient feature was the high efficacy of DPI in neuroprotection, even when administered in a post-treatment regimen after the onset of dopaminergic neuron damage (see summary in Supplementary Table 2). Our results strongly support that the neuroprotective effects of ultra-low dose DPI occur by specifically inhibiting NADPH oxidase and subsequently reducing microglia-mediated chronic neuroinflammation (Fig. 8). Additionally, mice treated with ultra-low dose DPI showed no overt signs of toxicity. The efficacy of a post-treatment regimen in chronic progressive dopaminergic neuron degenerative models together with its low toxicity suggest that ultra-low dose DPI may be a promising drug candidate for future human studies.

Over the past decade, the development of drugs capable of modifying disease progression in Parkinson’s disease has been unsuccessful. Despite the encouraging results reported in numerous animal studies, a small percentage of these compounds have been tested in clinical trials with even fewer reaching the clinic (Hart et al., 2009; Brichta et al., 2013). One particular failure in translating these drugs has
been the lack of suitable Parkinson’s disease models for drug development, which prompted us to create two inflammation-based progressive neurodegenerative models by using a systemic injection of LPS to either C57BL/6J or transgenic mice over-expressing human A53T mutant α-synuclein (Qin et al., 2007; Gao et al., 2011). These new models improve upon existing models by recapitulating the delayed and progressive degeneration of nigrostrial dopaminergic neurons (Qin et al., 2007; Gao et al., 2011), showing temporary recovery of motor deficits by L-DOPA (Qin et al., 2007; Liu et al., 2008) and generating α-synuclein-positive inclusion bodies in the substantia nigra (Liu et al., 2008; Gao et al., 2011). Administering an ultra-low dose of DPI to these mouse models not only halted the progression of neurodegeneration in mice that already exhibited more than a 30% loss of nigral dopaminergic neurons but also attenuated α-synuclein accumulation in dopaminergic neurons in the transgenic mice over-expressing human A53T mutant α-synuclein (Fig. 3). To our knowledge, this is the first report demonstrating a therapy capable of halting progressive dopaminergic neurodegeneration in mice that already exhibited more than a 30% loss of nigral dopaminergic neurons but also attenuated α-synuclein accumulation in dopaminergic neurons in the transgenic mice over-expressing human A53T mutant α-synuclein (Fig. 3). To our knowledge, this is the first report demonstrating a therapy capable of halting progressive dopaminergic neurodegeneration, reducing α-synuclein accumulation, attenuating the depletion of striatal dopamine, and improving motor behaviours even when administered as a post-degenerative intervention.

Mechanistically, the most critical question to address is why such a low dose of DPI post-administered at the motor stage of disease progression still displays neuroprotection and reverses motor deficits. The results of the present study, together with our previous reports (Block et al., 2007; Gao and Hong, 2008; Qin et al., 2013), suggest that DPI achieves these extraordinary protective effects through the inhibition of NADPH oxidase and subsequent interruption of microglia-mediated chronic neuroinflammation. Although microglial activation is essential to restore brain homeostasis after an injury or infection (Streit, 2000), it may become pathological if the initial inflammation is not properly resolved. Release of noxious endogenous ligands generated by injured neurons, such as μ-calpain, α-synuclein and high mobility group box 1, are thought to continually reactivate microglia (reactive microgliosis) resulting in additional neurodegeneration (Block et al., 2007; Levesque et al., 2010; Gao et al., 2011). Consequently, a self-propelling vicious cycle is created through interactions between injured neurons and dysregulated microglia, inevitably resulting in the delayed and progressive collateral neurodegeneration of dopaminergic neurons in Parkinson’s disease (Gao and Hong, 2008). We previously recognized NADPH oxidase as a key mediator in bridging chronic neuroinflammation and progressive dopaminergic neurodegeneration (Qin et al., 2004; Zhang et al., 2004; Block et al., 2007; Gao and Hong, 2008). This finding led us to theorize that inhibiting NADPH oxidase could effectively disrupt this self-propelling vicious cycle, potentially resulting in a new disease-modifying strategy for Parkinson’s disease. As predicted, ultra-low dose DPI effectively reduced LPS-induced oxidative stress, which was mainly derived from NADPH oxidase activation and subsequent superoxide production (Fig. 5 and Supplementary Fig. 4). The inhibition of NADPH oxidase not only reduced...
extracellular superoxide but also decreased intracellular reactive oxygen species levels thought to be important secondary messengers that regulate the expression of many proinflammatory factors by activating several downstream signalling pathways including protein kinase C, mitogen-activated protein kinase and NF-κB (Block and Hong, 2005). Consistent with this mode-of-action, post-treatment with DPI produced long-term inhibition of LPS-elicited microglial activation and suppressed gene expression of proinflammatory factors tumor necrosis factor alpha and interleukin-1 beta (Fig. 4). Unlike conventional anti-inflammatory therapies that directly suppress certain pro-inflammatory factors, selective inhibition of NADPH oxidase by DPI can inactivate microglia and prevent the generation of a spectrum of pro-inflammatory factors. Although this study focused primarily on microglial NADPH oxidase, neurons are also express this superoxide-producing enzyme at much lower quantities and thus, we cannot exclude the possibility that the inhibition of neuronal NADPH oxidase might also contribute to the DPI-elicited neuroprotection.

In addition to using LPS models, the efficacy of DPI was demonstrated in a more conventional MPTP model. Although previous studies have demonstrated protection by post-treatment with rottlerin (protein kinase Cδ inhibitor) (Zhang et al., 2007) or caffeine (Xu et al., 2010) in a MPTP mouse model, their protective effects were only observed when the drugs were administered within a couple of hours after MPTP lesion. By using a subchronic MPTP model, we showed that DPI (10 ng/kg/day) post-treatment after 3 days of initial MPTP injection attenuated dopaminergic neurodegeneration and improved rotarod activity.

It is interesting to compare the LPS and MPTP models in this study. LPS is known to directly activate microglia and trigger neuroinflammation to produce delayed and progressive nigral neurodegeneration (Qin et al., 2007). In contrast, MPTP causes acute dopaminergic neurotoxicity by inhibiting mitochondrial complex I. The different modes-of-action of these models begs the question how DPI can partially prevent dopaminergic neuronal loss, improve dopamine turnover and restore motor function in a model of direct neuronal lesion (Fig. 6). Although MPTP cannot directly activate microglia, stress signals released by dying neurons derive reactive microgliosis to generate superoxide via NADPH oxidase (Block et al., 2007; Levesque et al., 2010). We and others have previously reported that reactive microgliosis generated in response to the acute lesioning of dopaminergic neurons by MPTP trigger a delayed collateral neurotoxicity from neuroinflammation after the acute phase of MPTP (Gao et al., 2003; Wu et al., 2003; Hu et al., 2008; Levesque et al., 2010). Thus, we believe that DPI protected against this delayed toxicity by attenuating reactive microgliosis through the inhibition of NADPH oxidase activity.

It is important to note that although DPI potently inhibits microglial activation in both LPS and MPTP models, DPI alone did not produce microglial cytotoxicity nor did it suppress the basal microglial activity as illustrated by the similar densities of AIF1 and ITGAM-positive microglia and the gene expressions of proinflammatory cytokines
in comparison with the vehicle controls (Fig. 4). This observation is clinically relevant because DPI attenuates only the induced microglial activation without interfering with normal microglial immune surveillance function. Taken together, our findings suggest that the reduction of chronic microglia-mediated neuroinflammation through the inhibition of NADPH oxidase is an effective strategy to halt disease progression in both inflammation (LPS) and neurotoxin (MPTP)-based rodent Parkinson’s disease models.

This study also addressed some critical issues relevant to the potential clinical use of ultra-low dose DPI in future human clinical trials. The first issue was resolving the duration of treatment necessary to achieve an observable effect. To our surprise, we found that the subcutaneous infusion of DPI for two weeks was sufficient to provide potent neuroprotection. Although it is not clear why long-term administration was not necessary, one possible explanation for this effect is that once the self-propelling vicious cycle is interrupted by DPI, chronic neuroinflammation ceases unless additional inflammation is induced in the brain. The second issue was whether DPI could specifically inhibit NADPH oxidase in vivo. In micromolar concentrations, DPI inhibits several essential cytochrome-containing enzymes beyond just NADPH oxidase, attributing to its high cytotoxicity at these concentrations (Gatley and Martin, 1979; Aldieri et al., 2008). We recently reported that subpicomolar concentrations of DPI have great specificity to potently inhibit NADPH oxidase-generated superoxide without affecting the activities of other cytochrome-containing enzymes, such as inducible nitric oxide synthase, xanthine oxidase, cytochrome P450 reductase, thioredoxin reductase, and complex I, in cultured microglia (Wang et al., 2014a). Although the exact brain concentration of DPI in our in vivo studies was too low to be accurately measured, data extrapolation from a previous pharmacokinetic study (Gatley and Martin, 1979) estimate that the brain DPI levels in treated mice were similar to the range (10^{-14} to 10^{-13}M) used in the aforementioned in vitro studies (Wang et al., 2014a). The specificity of this ultra-low dose DPI in vivo was further supported by showing no changes of brain mitochondrial complex I activity in the DPI-treated mice (Fig. 5).

Another critical issue was whether ultra-low dose DPI could generate toxicity in vivo. To examine this, we conducted a standard pathological evaluation based on the modified National Toxicology Program/National Institutes of Health protocol. This method measures the weight of the body and organs, behavioural activity, and performs histological assessment of organs tissues to verify the safety profile of putative toxicants. Although these methods cannot rule out subtle toxicities, no gross toxicological effects were noted. Furthermore, as we know that patients with chronic granulomatous disease (CGD), a rare mutation on NADPH oxidase that render the subunit functional inactive, and NADPH oxidase deficient mice display immunodeficiency, we confirmed that ultra-low dose DPI did not affect the peripheral immune cell functions in both mice (Supplementary Fig. 6) and human (data not shown) that are critical for hosting normal immune responses. Finally, it is important to point out that although we provided strong evidence for the potential clinical usage of ultra-low dose DPI in Parkinson’s disease, there still have several issues to consider before translating our findings. First, in our study, the DPI infusions are given when about 30–35% of dopaminergic neurons are lost. In humans, the degree of nigral dopaminergic neuron loss is much greater (about 50–60% loss) (Hirsch, 2007) than this when the patients first show motor symptoms. It remains to be determined whether DPI treatment is still effective in Parkinson’s disease patients, who already display motor symptoms. Second, our study showed no overt toxicity in low-dose DPI-treated mice; however, a more detailed evaluation for the toxicity of low-dose DPI in monkeys or even human is needed. Third, although neuroinflammation has been recognized as one of the critical factors that contribute to the progression of Parkinson’s disease, the complexity of disease aetiology makes it difficult to predict whether DPI could work as effectively in human Parkinson’s disease patients.

In summary, this study provides convincing evidence that subchronic infusion of an ultra-low dose of DPI potently reduced microglia-mediated chronic neuroinflammation by selectively inhibiting NADPH oxidase and halted progressive neurodegeneration in both LPS and MPTP models. Our findings may provide a novel and efficient therapeutic strategy for future Parkinson’s disease therapy. The ability to halt progressive neurodegeneration, selective specificity in inhibiting NADPH oxidase and initial safety profiles suggest that ultra-low dose DPI could be a promising candidate for future clinical trials in patients with Parkinson’s disease.

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**Supplementary material**

Supplementary material is available at *Brain* online.

**References**


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