Nicotine protects dopaminergic neurons against lipopolysaccharide-induced damage through a7 nAChRs in microglia

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Abstract: Inflammation plays an important role in the pathogenesis of Parkinson's disease (PD) through over-activation of microglia. Epidemiological studies show that smoking is associated with a lower incidence of PD. Smoking in general and nicotine in particular might be neuroprotective. The a7 nicotinic acetylcholine receptors (a7 nAChRs) signal may be involved in the modification of microglia activation towards a neuroprotective role of nicotine by suppressing the inflammatory state. This study hypothesized that the neuroprotective effect of nicotine is mediated by modulating the activation of microglia via cytokine release through a7 nAChRs. This study found that nicotine pretreatment suppressed the lipopolysaccharide(LPS)-induced inflammatory reaction in rat mesencephalic neuron-glia cultures. Nicotine protected the TH-immunoreactive neurons from inflammation-induced death (P<0.05). Nicotine's neuroprotective effect against LPS-induced dopaminergic neurotoxicity is mediated through modulating activation of microglia and interleukin-6(IL-6) production (P<0.05). Furthermore, methyllycaconitine (MLA), a7 selective nicotinic antagonist, reversed the action of nicotine, namely, the inhibition of LPS-induced IL-6 release and the neuroprotective effect on dopaminergic neurons (P<0.05). These findings suggest that nicotinic receptor agonists that target a7 nAChRs in microglia may have significant therapeutic potential in neuroinflammatory diseases in the brain.

1. Introduction

Parkinson's disease (PD) is a frequent neurological disorder of the substantia nigra pars compacta (SNpc), which is characterized by the progressive loss of dopaminergic (DA) neurons. The rationale for considering nicotine in the present study is based on epidemiological findings that PD is less prevalent in smokers, leading to theories that smoking in general and nicotine in particular might be neuroprotective (Park et al., 2007).

Almost all degenerative diseases of the CNS are associated with chronic inflammation (Shytle et al., 2004). A key role in this process is the activation of brain mononuclear phagocyte cells, called microglia. Activated microglia, as well as to a lesser extent reactive astrocytes, are found in areas associated with cell loss(Le et al.,2004), possibly contributing to the inflammatory process by the release of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 (Sawada et al.,2006; Imamura et al., 2005; Béraud et al.,2013).

In the peripheral nervous system, recent studies suggest that an endogenous 'cholinergic anti-inflammatory pathway' regulates systemic inflammatory responses via a7 nicotinic acetylcholinergic receptors (nAChRs) found on blood-borne macrophages (Olofsson et al., 2012;

Wang et al., 2003; Rosas-Ballina et al., 2009). These data led us to investigate whether a similar cholinergic pathway exists in the brain that could regulate microglial activation. Therefore, the aims of our study were to identify specific microglial activities modulated by nicotine in vitro and this modulation affords neuroprotection against inflammatory stimuli.

2. Material and Methods
Reagents

Embryonic day 14 Sprague–Dawley rats were purchased from Laboratory Animal Center of Medical College of Zhengzhou University, LPS, nicotine and methyllycaconitine (MLA) were obtained from Sigma,the mouse monoclonal anti-ionized calcium binding adaptor molecule 1,(Iba1) antibody and rabbit anti-tyrosine hydroxylase (TH) antibody were purchased from Sigma.

Primary neuronal-glial cultures

Primary rat ventral mesencephalic neuronal-glial cultures were prepared following previously described protocol (Le et al., 2004) with some modifications. Briefly, ventral mesencephalic tissues were dissected from embryonic day 14 Sprague–Dawley rats under pentobarbital anaesthesia and dissociated by a mild mechanical trituration. Dissociated cells were seeded to 24-well (5×

l0^{5}cells/well) culture plates precoated with poly-D-lysine. The cultures were maintained at 37℃ in a humidified atmosphere of 5% CO2. Seven-day-old cultures were used for treatment.

Immunocytochemistry

Dopaminergic (DA) neurons were recognized with the anti-TH antibody and activation of microglia were detected with the anti-Iba1 antibody. The samples were fixed in 4% paraformaldehyde for 1 hour, incubated with 3% H_2O_2 at room temperature for 20 minutes. The samples were treated with Triton X-100 and bovine serum albumin solution (prepared with 0.3% Triton X-100, 5% bovine serum albumin, 0.01 mol/L PBS) at room temperature for 1 hour. The primary antibody (Iba1 or TH)were added at 4 ℃ overnigh. The samples were incubated in biotinylated goat anti-polyclonal antibody at 37 ℃ for 2 hours. They were then incubated in streptavidin-biotin complex solution at 37 ℃ for 2 hours. The samples were colorized in a mixture of 0.05% diaminobenzidine and 0.01% H_2O_2 for 6–8 minutes. The reaction was terminated by addition of 0.01 M PBS. Bovine serum albumin served as the negative control in place of primary antibody.For morphological analysis, image were acquired using an inverted microscope (Leica, Germany) connected to a camera operated with the MetaMorph software. To quantify cell numbers, total TH positive cells in each well were counted by three individuals. The average of these scores was reported.

Assay of proinflammatory cytokines

The amount of IL-6 released into the culture medium was measured using a double-antibody sandwich ELISA method (Lepoutre et al.,2009) following the manufacturer's protocol (R&D Systems Europe, Ltd., Abingdon, UK).The absorbence at 450 nm was measured with a microplate reader. Buffer solution served as the blank control. The resultant IL-6 expression was estimated according to the absorbance reduction in the control wells of unknown samples, by applying a standard curve from the standard concentrations. The detection limit for the IL-6 ELISA kit was 15pg/mL. The antibodies in the kit did not have any detectable cross-reactivity with other antigens.

Statistical Analysis

The SPSS 11.0 software (SPSS, Chisago, IL, UAS) was used for statistical analysis. The data were presented as Mean ± SD. Intergroup comparisons were performed using one-way analysis of variance in conjunction with a Student Newman-Keuls-q post hoc test. The paired-samples t test was used for intragroup comparison at 8, 24 and 72 hours. A value of $p < 0.05$ was considered statistically significant.

3. Results

Nicotine protects the dopaminergic cell against LPS-induced degeneration in primary neuronal-glial cultures

DA neurons were identified in primary mesencephalic cultures by tyrosine hydroxylase (TH) immunocytochemistry. The cultures were divided 4 groups as follows：the control group was incubated in fresh media only ;LPS group was treated with 10 ng/mL LPS only; LPS+nicotine group was pretreated with 100μmol/L nicotine then plus 10 ng/mL LPS; nicotine group was treated with 100μmol/L nicotine only. All groups were treated for 72 h prior to TH immunoreactive staining. The concentration of LPS chosen was based on the results which showed about 50% TH immunoreactive (TH-ir) cells loss. Our previous results (Fan et al.,2011) showed that 10 ng/mL LPS induced about 50% TH-ir cells loss. Based on these experiments, 10 ng/mL LPS was used to test the effect of nicotine's neuroprotection.

As shown in figure 1, the control cultures showed healthy TH-ir cells,which had extensive neurites. Cultures treated with LPS showed a marked reduction in the number of TH-ir cells 72h after treatment. Morphologically, in addition to reductions in the abundance of TH neurons, the dendrites of the remaining TH neurons in LPS-treated cultures were significantly less elaborate than those in the control group. Pretreatment with Nicotine significantly attenuated these changes in TH neurons induced by LPS. Nicotine itself doesn't affect dopaminergic neuron. The protective effect of nicotine was confirmed by counting the cell numbers of TH neurons (Fig. 2). The concentration of nicotine chosen was based on our previous results(Li et al.,2011) that nicotine significantly decreases the production of IL-6 in mixed glia or microglia-enriched cultures and the inhibitory effect of 100 μmol/L nicotine was significantly stronger compared with 1 μmol/L and 10 μmol/L nicotine.
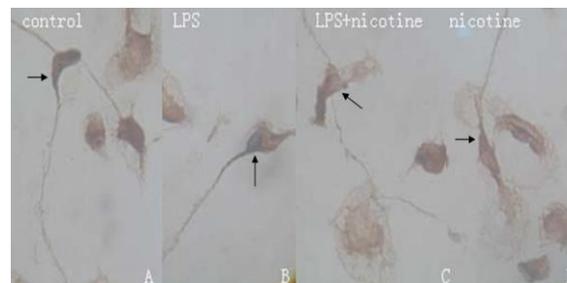

Figure 1 Nicotine protects dopaminergic (DA) neurons from LPS-induced death. Primary mesencephalic cultures were under (A) control conditions or (B) treatment with LPS alone or (C) co-treatment with LPS and nicotine or (D) treatment with nicotine alone. All cells were treated for 72 h

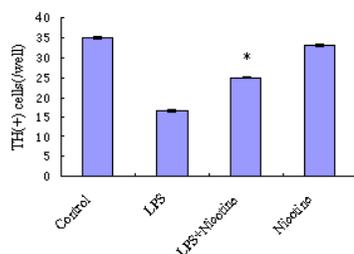prior to TH immunoreactive staining. Arrows show positive staining

Figure 2 The numbers of TH immunoreactive neurons in primary mesencephalic cultures. The cultures were pretreated with or without 100μM nicotine and/or 10 ng/mL LPS for 72h and stained for TH . The control group was incubated in fresh media only. *$p < 0.05$ vs. LPS-treated group

Nicotine prevents activation of microglia and inhibits release of IL-6 in primary neuronal-glial cultures

To examine the effects of nicotine on the LPS induced-activation of microglia and the generation and release of proinflammatory cytokines, mesencephalic cultures were pretreated with nicotine followed by treatment with LPS. The culture supernatants were collected for determining the levels of IL-6 at 8, 24 and 72 hours and cells were fixed and immunostained for specific marker of microglial activation(Iba1)(Zhang et al.,2008) at 72 hours. As shown in figure 3, immunostaining of the cultures revealed predominantly resting microglia in the control group. Treatment with 10ng/mL LPS dramatically altered the morphology of the microglia. Almost all microglia became activated that were characterized by intense Iba1 immunostaining, an increase in size and changes of shape. Pretreatment with 100μM nicotine significantly prevented the LPS-induced activation of microglia, whereas nicotine alone had no effect on their morphology.
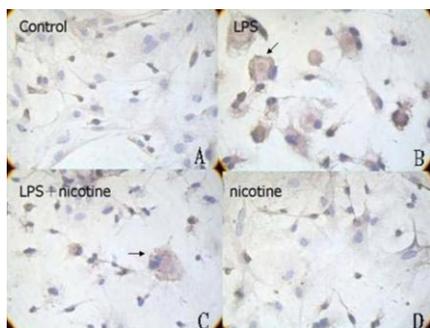

Figure 3 Nicotine suppresses LPS-induced microglia

activation in neuron-glia cultures. Primary mesencephalic cultures were under (A) control conditions or (B) treatment with LPS alone or (C) co-treatment with LPS and nicotine or (D) treatment with nicotine alone. All cells were treated for 72 h prior to Iba1 immunoreactive staining. Arrows show positive staining.

In addition to preventing the activation of microglia, nicotine significantly inhibited the production and release of IL-6. The amount of IL-6 released into the culture supernatant at 8h,24h and 72h after treatment with 10 ng/ml LPS was significantly reduced by nicotine from 3862 ± 83, 4016 ± 112 and 3240 ± 104 pg/5×10^5cells to 3658 ± 85, 3575 ± 138 and 2947 ± 69 pg/5×10^5cells,respectively ($p < 0.05$) (Figure 4).
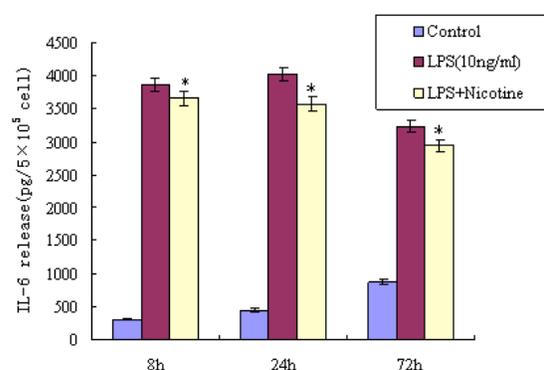

Figure 4 Effect of nicotine on IL-6 production in LPS-stimulated neuron-glia cultures. The amount of IL-6 released into the culture supernatant at 8h, 24h and 72h after treatment with 10 ng/mL LPS was significantly reduced by nicotine(100 μM). Data represent mean $\pm$ SD.*$p < 0.05$ vs. LPS-treated group

Nicotine protects the dopaminergic neurons and modulates proinflammatory mediators release via activation of a7 nAChRs in microglia

The expression of a7 nicotinic acetylcholine receptors (a7 nAChRs) was confirmed in microglia of rat brain by western blot and by RT-PCR(Shytle et al.,2004). To study whether a7 nAChRs could regulate microglial activation and IL-6 release, we examined the effects of methyllycaconitine (MLA), an a7 nAChRs blocker, on nicotine-modulated IL-6 release. The cultures were divided 4 groups as follows：the control group was treated with 10 ng/mL LPS only; LPS+nicotine group was pretreated with 100μM nicotine then plus LPS; LPS+MLA group was pretreated with 10nM MLA then plus LPS; LPS+nicotine+MLA group was pretreated with MLA and nicotine then plus LPS. All groups were treated for 72 h prior to TH immunoreactive staining.

MLA (10 nM) reversed the action of nicotine (P<0.05), namely, the inhibition of LPS-induced IL-6 release (Figure 5a) and the neuroprotective effect on dopaminergic neurons (Figure 5b). MLA did not affect LPS-induced IL-6 release and TH-ir cell loss by itself. These results suggest that nicotine protects the dopaminergic cell and modulates cytokine release, at least partially, through activation of a7 nAChRs.
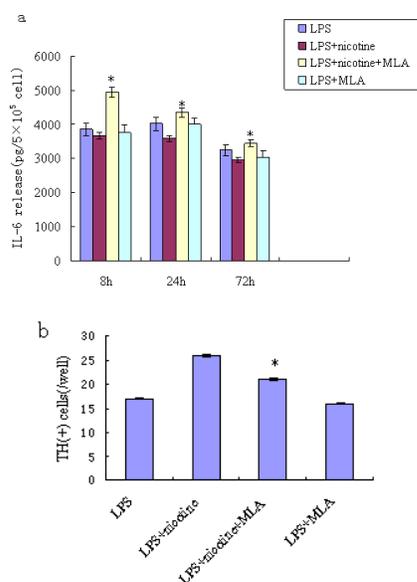

Figure 5 Effects of methyllycaconitine (MLA), an nAChR blocker, on nicotine- modulated IL-6 release and neuroprotective effect on TH neurons. MLA (10 nM) reversed the action of nicotine, namely, the inhibition of LPS-induced IL-6 release (a) . MLA also reversed the neuroprotective effect of nicotine on dopaminergic neurons (b). MLA did not affect LPS-induced IL-6 release and TH-ir cell loss by itself. Data represent mean $\pm$ SD.*$p < 0.05$ vs. LPS + nicotine group

4. Discussions

In this study with rat mesencephalic neuron-glia cultures as a model and LPS as a tool, we demonstrated that nicotine could effectively protect dopaminergic neurons against LPS-induced neurotoxicity by counting of TH-immunoreactive cells. Furthermore, nicotine significantly inhibited the activation of microglia and release of IL-6. Results from the present study emphasize the effects of nicotine's neuroprotection and "anti-inflammatory" treatments in primary cultures that are closer to the in vivo situation.

Microglia is the resident immune cells of the brain and plays a role in immune surveillance under normal condition. However, microglia becomes readily activated in response to infections and neuronal injuries under pathological condition (Lepoutre et al., 2009). Activated microglia produce a wide array of factors, forming the inflammatory cascades leading to tissue injury (Li et al., 2009). This is believed to contribute to microglia-mediated neurodegeneration (Le et al., 2004; Block et al., 2007). Recent studies suggest that the agents which inhibit microglia activation will provide neuroprotective effects (Chen et al., 2007). In the present study, we have shown that nicotine effectively inhibited microglia activation and IL-6 generation and effectively protect dopaminergic neurons in mesencephalic neuron-glia cultures. Our previous results (Li et al., 2011) also showed that nicotine significantly inhibited the activation of microglia and release of IL-6 in mixed glia cultures and in microglia-enriched cultures exposed to LPS treatment, indicating that the mechanism underlying the neuroprotective role of nicotine, at least partially, is attributed to the inhibition of microglia activation.

Microglia perform both neuroprotective and neurotoxic functions in the brain, depending on their state of activation and their release of mediators (Suzuki et al., 2006). Nitric oxide (NO) and proinflammatory cytokines (TNF-α,IL-1) produced by glial cells are known to be involved in the neuropathogenesis of various diseases (Xu et al., 2009; Lee et al., 2010; Wilms et al., 2010). Previously, TNF-α, IL-1β and NO production have been reported (Long-Smith et al., 2010; Liu et al., 2000), but the role of IL-6 remains controversial (Zheng et al., 2008). Therefore, the present study examined the effects of LPS with or without nicotine on the production of IL-6 in primary mesencephalic neuron-glia cultures. By using in vitro primary cultures, this study found that LPS activates inflammatory signaling, which induces the release of IL-6 and causes neurotoxicity. This result implied that LPS-induced IL-6 generation may play a critical neurotoxic role in the induction of dopaminergic neurodegeneration. In this study, we also found that nicotine was significantly potent in inhibiting LPS-induced IL-6 production. It is possible that nicotine with a preferential inhibition toward IL-6 generation may be very effective in providing neuroprotection in the context of LPS-mediated degeneration. The inhibitory and neuroprotective profile of nicotine seems to be similar to that of naloxone stereoisomers. Naloxone is more effective in the inhibition of superoxide generation than in that of proinflammatory cytokines (Liu et al., 2000a). The neuroprotective effect of naloxone has been observed in both in vitro and in vivo models of inflammation-mediated neurodegeneration (Liu et al., 2000a; Liu et al., 2000b).Hence, it is also important to determine

whether the neuroprotective effect of nicotine can be observed in animal models of inflammation-mediated neurodegenerative diseases including PD (Zhang et al., 2011).

Nicotinic acetylcholine receptors are a family of ligand-gated, pentameric ion channels (Wang et al., 2003). Shytle et al. report for the first time that cultured mouse microglial cells express a7 nAChR subunit as determined by RT–PCR, western blot, immunofluorescent and immunochistochemistry analyses (Shytle et al., 2004). Although neuronal a7 nAChRs belong to ligand-gated ion channels, Suzuki et al. demonstrated that rat microglial a7 nAChRs have different properties from conventional neuronal a7 nAChRs, not functioning as ion channels but coupling to phospholipase C (PLC) activation and Ca^{2+} mobilization from IP_3-sensitive Ca^{2+} stores. Such unique receptors should play an important role in neuroprotection, because the activation of these receptors is capable of modulating the stage of microglia from the overactive inflammatory type to a protective type (Suzuki et al., 2006). In this study, we found that in rat primary cultured microglia, the activation of a7 nAChRs by nicotine had a suppressive effect on LPS-induced IL-6 release and activation of microglia. We also investigated methyllycaconitine (MLA), an a7 nAChRs blocker, reversed the action of nicotine, namely, the inhibition of LPS-induced IL-6 release and the neuroprotective effect on dopaminergic neurons. Therefore, we have provided new evidence for the existence of nonconventional a7 nAChRs in rat microglia.

In summary, we demonstrate that nicotine may protect dopaminergic neurons against LPS-induced neurotoxicity and that the neuroprotective effect of nicotine may be associated with inhibition of microglia activation and proflammatory factors generation. However, the mechanisms underlying the neuroprotective effect of nicotine, except inhibition of microglia activation through a7 nAChRs, might not rule out the possibility of other mechanisms. Therefore, further investigation is equired to delineate the precise mechanism of the inhibitory effect of nicotine on microglia activation. Nevertheless, our study may provide insight into the potential application value of nicotine in the treatment of neurodegenerative diseases including PD.

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