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Glial cell line-derived neurotrophic factor protects midbrain dopaminergic neurons against lipopolysaccharide neurotoxicity

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Abstract

Aberrant microglia activation causes dopaminergic neuronal loss and nitric oxide produced by microglia plays a critical role in dopaminergic neuronal degeneration. However, no study has determined if GDNF protects dopaminergic neurons via inhibiting nitric oxide generation in Parkinson's disease animal model. We report that GDNF not only reduces lipopolysaccharide-induced degeneration of dopaminergic neurons, suppresses microglia activation and nitric oxide generation, but also reverses the inhibition of phosphoinositide 3-kinase (PI3K) in dopaminergic neurons and microglia. It suggests that the neuroprotective effect of GDNF on dopaminergic neurons may be related to its suppression of microglia activation-mediated nitric oxide via releasing the inhibition of PI3K in both neurons and microglia.

Keywords

Parkinson's disease; GDNF; lipopolysaccharide; dopaminergic neurons; microglia; nitric oxide; PI3K

1. Introduction

Parkinson's disease (PD) is a common neurodegenerative movement disorder. The progressively selective dopaminergic neuronal degeneration in the substantia nigra of the midbrain is a hallmark of PD pathology. Increasing evidence has suggested that neuroinflammation (KimCunningham et al., 2005; Herrera et al., 2005; Hong, 2005; Jenner, 2003; McGeer et al., 2001) and oxidative stress (Elkon et al., 2004; Jenner, 2003) are involved in the process of dopaminergic neuronal loss. Inflammation and oxidative stress mediated by activated microglia has been known to be a significant pathological feature of PD (Dexter et al., 1994; Jenner and Olanow, 1998), and that suppression of inflammation and oxidative stress from activated microglia is neuroprotective in various animal models of

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PD (Kotake et al., 2005; Li et al., 2005a; Liu et al., 2003; Sherer et al., 2003; Testa et al., 2005; Wu et al., 2002).

Glial cell line-derived neurotrophic factor (GDNF) is one of the most potent neurotrophic factors for survival of dopaminergic neurons (Lin et al., 1993). Evidence has shown that GDNF has a neuroprotective and restorative effect on the nigral dopamine system in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Cheng et al., 1998) and 6-hydroxydopamine (6-OHDA)-induced (Aoi et al., 2000; Ding et al., 2004; Lapchak et al., 1997; Rosenblad et al., 1999; Rosenblad et al., 1998; Yasuhara et al., 2005) animal PD models, in non-human primates (Eslamboli et al., 2003; Gash et al., 1996; Grondin et al., 2002; Kordower et al., 2000; Maswood et al., 2002; Reilly, 2001), and by direct intraputamenal infusion in PD patients (Gill et al., 2003; Patel et al., 2005). In contrast, clinical trials of intracerebroventricular infusion of GDNF showed different outcomes, suggesting not only the necessity of alternative delivery systems to enhance the efficacy on the target reach (Kordower et al., 1999; Lindvall and Wahlberg, 2008), but also of further study on the signaling mechanisms of its neuroprotective effect so that the side effects can be minimized.

A series of *in vitro* studies have explored the signal transduction mechanisms for the neuroprotective effects of GDNF. A recent study showed the ability of GDNF to prevent microglia activation induced by β -amyloid in rabbit hippocampus (Ghribi et al., 2004). On the other hand, the inhibition of phosphoinositide 3-kinase (PI3K) blocked GDNF-mediated neuronal protection in the 6-OHDA-induced PD model (Ugarte et al., 2003). In contrast, PI3K activation enhances the neuroprotective effects of GDNF against H_2O_2 -induced neuronal death in PD cybrids (Onyango et al., 2005). Furthermore, GDNF protect mesencephalic neurons by suppression of oxygen radical accumulation and caspase-dependent apoptosis in the 1-methyl-4-phenylpyridinium (MPP⁺) model, which are mediated by the PI3K/Akt pathway (Ding et al., 2004; King et al., 2001; Sawada et al., 2000). More importantly, immunoblotting and immunocytochemistry assay of PD midbrains in a very recent postmortem study demonstrated that the activity of PI3K/Akt pathway is defective compared to control brains (Malagelada et al., 2008; Timmons et al., 2009), and an increase in its activity was found in the patients with Alzheimer's diseases (Griffin et al., 2005), suggesting its convergent and crucial role in the neurodegenerative diseases. Our previous *in vitro* study on the signaling mechanisms of LPS-induced neurotoxicity suggested that PI3K can be a very critical signaling molecule negatively regulating activated microglia-mediated iNOS function (Xing et al., 2008). However, whether GDNF inhibits lipopolysaccharide (LPS)-induced microglia activation and the subsequent generation of nitric oxide, one of the main reactive nitrogen species released from activated microglia, and whether GDNF influences its expression remained an interesting question to answer. In the present communication the effect of GDNF on the survival of dopaminergic neurons, microglia activation-mediated nitric oxide, and the alteration of PI3K expression in LPS-induced PD models were explored.

2. Materials and Methods

2.1 Animals

Timed-pregnant Sprague Dawley rats were obtained from Harlan (Indianapolis, IN, USA) and were maintained in a strict pathogen free environment. Animal use was performed in strict accordance with the National Institutes of Health guidelines and was approved by the Institute's Animal Care and Use Committee at the University of Kentucky.

2.2 Cortex-striatum-midbrain organotypic cultures

Cortex-striatum-midbrain organotypic cultures were prepared from post-natal day 2–3 Sprague Dawley rats (Harlan, Houston, TX). Briefly, the rat brains were removed and fixed on the mount in the Vibroslice bath chamber with coronal direction, and the chamber was filled with cold oxygenated chopping solution (110mM sucrose, 60mM NaCl, 3mM KCl, 1.25mM NaH₂PO₄, 28mM NaHCO₃, 5mM D-glucose, 0.5mM CaCl₂, 7mM MgCl₂, and 0.6mM ascorbate) until the whole brain was immersed. In order to get comparable slices from individual brains, the start cutting point was set when the cutting blade touched the anterior tip of the brains. With certain times of trimming, cortex-striatum slices (500µm thick) and midbrain slices (350µm thick) were prepared using the vibroslice (Campden Instruments Ltd. Lafayette, IN), then the slices were trimmed in the chopping solution and transferred onto pre-moistened microporous membranes (Millicell-CM, Millipore, Bedford, MA) in 6-well plates. Adding 100µl phosphate buffered saline (PBS) onto the insert membrane, followed by arranging the medial part of striatum adjacent to midbrain with a distance of 500µm apart, and the PBS was gently removed. Culture medium, consisting of 50% minimal essential medium / HEPES, 25% Hank's balanced salt solution and 25% heat-inactivated horse serum (Invitrogen, Carlsbad, CA) supplemented with 6.5mg/ml glucose, 2mM L-glutamine and 10units/ml penicillin-G / 10µg/ml streptomycin, was supplied at a volume of 1ml per well. The culture medium was replaced with fresh medium on the next day, and thereafter, every two days. Organotypic cultures were maintained for two weeks before GDNF and LPS / IFN-γ treatment in a 36°C and 5% CO₂ humidified atmosphere. After 12 days in culture, 50ng/ml IFN-γ (BD, San Jose, CA) was added into culture medium, and 24hr later LPS at a dose of 10, 20, 40, or 80µg/ml (*Salmonella minnesota*, Sigma, St. Louis, MO) was administered. Alternatively, organotypic cultures were treated with GDNF (20ng/ml) 1hr before IFN-γ and LPS treatment.

2.3 Microglia-enriched cultures

Cultures of the rat primary glial cell cultures were performed according to methods described previously (Xing et al., 2008). Briefly, cerebral cortices of 2–3 day-old neonatal SD rats were minced and gently dissociated by repeated pipetting in HBSS supplemented with newborn calf serum (3.5:1 v/v). Cells were collected by centrifugation at 1000×g for 6 min, resuspended in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, and were cultured in 175 cm² cell culture flasks in 5% CO₂ at 37 °C. Floating microglia were harvested between 2 and 8 weeks by shaking off at 200 rpm and were re-seeded back into 24-well plates (4×10⁵) for conditioned medium and into 6-well plates (1×10⁶) for western blot assay. After 30 min, cultures were washed to remove non-adherent cells and fresh medium was added. The purity of the microglia culture was >98% as determined by immunocytochemistry. After 24 hr, the cultures were treated with LPS (1µg/ml) dissolved in DMSO for three days, the conditioned medium was fed into primary neuronal culture and microglia culture, respectively. Alternatively, GDNF (10ng/ml) was administered 1hr before conditioned medium was added.

2.4 Primary mesencephalic neuron-enriched cultures

Neuron-glia cultures were prepared from the ventral mesencephalic tissues of day 13–14 rat embryos. Briefly, midbrain tissues from SD rat embryos were dissected in Ca⁺⁺/Mg⁺⁺ free medium (CMF). Cells were dissociated via gentle mechanical trituration in Hanks' Balanced Salt Solution (HBSS) containing newborn calf serum (3.5:1 v/v), and the cells were seeded in poly D-lysine (50 µg/ml) pre-coated 6-well plates for western blot (1×10⁶ cells/well). Cells were fed with Dulbecco's Modified Eagle Medium (DMEM/F12) containing 10% horse serum and 10% fetal bovine serum. Twenty four hours after seeding, 5µM Ara-C was added into the culture medium for 48hr, followed by replacement with 500µl DMEM/F12

medium with 5% horse serum and fetal bovine serum. After seven days, cultures were fed with the conditioned medium or GDNF (10ng/ml) 1hr before.

2.5 Nitrite oxide assay

The production of NO was assessed by the accumulation of nitrite in culture supernatants by using the colorimetric reaction of the Griess reagent. Culture supernatants were collected 72hr after LPS stimulation and were mixed with Griess reagent (0.1% N-[1-naphthyl] ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H₃PO₄). The absorbance at 548nm was measured with a spectraMAX microplate reader from Molecular Devices (Sunnyvale, CA, USA).

2.6 Immunocytochemistry

Following three days of incubation, organotypic cultures were fixed and immunohistochemistry was processed. Briefly, cultures were fixed with 0.1M phosphate buffer (pH 7.4) containing 4% paraformaldehyde and 4% sucrose for 2hr, and were processed for tyrosine hydroxylase (TH) immunohistochemistry after rinsing with PBS. Fixed cultures were exposed to 2% H₂O₂ in 100% methanol for 30min, then to 0.2% Triton X-100 in PBS for 30min. Cultures were subsequently treated with 10% goat serum for 30min, incubated overnight at 4°C with mouse anti-TH antibody (1:4000) (Santa Cruz, CA, USA). After incubation for 1hr at room temperature with biotinylated anti-mouse IgG, cultures were rinsed with PBS several times, then treated with Vectastain Elite ABC kit (Vector Laboratories, Au Verney) for 1hr at room temperature. After a further wash with 50mM Tris-buffered saline, peroxidase was visualized with 0.07% diaminobenzidine and 0.018% H₂O₂. Specimens were dehydrated using a graded ethanol series and mounted on slide glasses. In several experiments, immunohistochemistry of OX-6 was performed to demonstrate microglia activation. Primary antibody is mouse anti-OX-6 (1:1000, Serotec Ltd, Oxford, UK). Biotinylated anti-mouse IgG from horse (1:1000, Vector Laboratories) was used as a secondary antibody. Other procedures were the same as for TH immunohistochemistry. TH-immunostained neurons were considered healthy, if the length of all the neurites was at least two times longer than the diameter of its cell body and if the cell had at least two neurites. The numbers of TH-positive cells and OX-6 activated microglia were determined using the optical fractionator method of the Bioquant system (West, 1993).

2.7 Western blot

For organotypic cultures, cells were collected after 1hr and 72hr after LPS treatment, and homogenized on ice. The lysates were used for western blot. For primary neuronal and microglia culture, the samples were collected and processed after 10min and 1hr treatment. Protein concentrations were determined with the bicinchoninic acid assay following the manufacturer's guide. Equal amounts of protein were loaded, separated by PAGE gel electrophoresis, and were transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk and were incubated overnight at 4°C with polyclonal anti-PI3K p110 antibody (1:250) (Santa Cruz, CA, USA), or monoclonal anti-β-actin (1:4000). Peroxidase-linked anti-rabbit IgG (1:4000) was used as the secondary antibody and the ECL Plus kit from Amersham Biosciences Inc (Piscataway, NJ, USA) was used for chemiluminescent detection. The optical density was measured using the scion image™ software (Frederick, MD, USA).

2.8 Statistical analysis

The data are expressed as the means $\pm$ SEM and statistical significance was assessed by ANOVA followed by a Tukey comparisons test using the SYSTAT 10 software (SPSS Inc., Chicago, Illinois). A value of $p < 0.05$ was considered statistically significant.

3. Results

After seventy-two hours exposure to different doses of LPS (10 μ g/ml – 80 μ g/ml), the immunostaining with anti-TH antibody was processed. The results showed that LPS markedly induced the loss of TH positive neurons in a dose-dependent manner (Figure 1. A–c, d, e, and f). In contrast, pretreatment with GDNF (20ng/ml) 1hr before LPS treatment significantly protected neurons ($p < 0.01$ or 0.05) except for the 40 μ g/ml LPS group.

The effect of various doses of LPS and GDNF on microglia activation in the substantia nigra area was also observed. The immunostaining of OX-6 was used to show the activated microglia. Similar to control group, very few OX-6 positive microglia was found in GDNF group. In contrast, LPS treatment significantly activated microglia in a dose dependent manner (Figure 2A), and pretreatment with GDNF had a strong inhibitory effect on microglia activation (Figure 2B).

The NO assay of the whole slices showed that nitric oxide is induced by LPS, and pretreatment with GDNF significantly inhibited microglia activation-mediated generation of nitric oxide (Figure 3).

Slices were processed and PI3K expression was immunoblotted at 1hr and 72hr after LPS treatment to test if its expression was altered by LPS and GDNF. No significant difference was observed between the GDNF alone, control group, and LPS group at 1hr. However, about a 40% decrease in PI3K expression was observed after 72hr treatment (Figure 4) and GDNF reversed this effect ($p < 0.05$).

In order to investigate if GDNF acts on PI3K in neurons and / or microglia, the primary mesencephalic neuronal culture and primary microglia culture were treated with the conditioned medium from LPS-treated microglia culture for 1hr, and PI3K expression was immunoblotted. In primary microglia culture, the expression of PI3K was decreased to 35% compared to control group ($p < 0.01$), and its expression was kept up to 71% with GDNF pretreatment ($p < 0.05$).

Similarly, the decrease in its expression was found in the primary neuronal cultures stimulated with LPS-treated conditioned medium, and pretreatment with GDNF 1hr before significantly abolished the inhibition of PI3K by LPS (Figure 5).

4. Discussion

Although *in vivo* animal models provide a very valuable way to study PD, they are usually limited to a “static picture,” especially when studying altered signaling transduction. In contrast, organotypic slice cultures are not only characteristic of relatively intact anatomical structure and cellular connections analogous to that seen *in vivo*, but also provide an effective way to observe and manipulate the tissues biochemically and pharmacologically in a “dynamic and timely” manner. Single ventral mesencephalon slice cultures (Nakanishi et al., 1997) and its coculture with striatum or cerebral cortex slices (Becq et al., 1999; Franke et al., 2003; Gramsbergen et al., 2002) have been utilized to study the normal physiological function of the nigrostriatal dopaminergic system. Very recently, triple slice culture systems with more naïve properties in anatomy and physiology demonstrated the essential role of N-

methyl-d-aspartate in the neurotoxicity of rotenone, 6-OHDA, and MPP⁺ on dopaminergic neurons (Kress and Reynolds, 2005). However, the role of LPS in the cerebral cortex-striatum-midbrain slice cultures had not yet been clarified, and we for the first time reported the neuroprotection of GDNF against LPS insult to the dopaminergic neurons and explored the possible signaling molecules regulated by GDNF on the neuroprotection of dopaminergic neurons in our culture systems.

Microglia activation has been suggested to play an important role in initiating and / or amplifying neuronal injury since not only the substantia nigra has an extremely high density of resting microglia but also reactive microglia are found in close proximity to the damaged nigral neurons in the brain of PD patients (Hirsch et al., 1998; Iravani et al., 2002; Lawson et al., 1990; Le et al., 2001; McGeer et al., 1988; Ouchi et al., 2005). It is believed that activated microglia-mediated inducible nitric oxide synthase (iNOS) plays a deleterious role in the dopaminergic neurodegeneration. Postmortem studies showed that nitric oxide synthase-containing microglia were up-regulated in substantia nigra and striatum of human parkinsonian but not in control patients (Hunot et al., 1996; Knott et al., 2000). A line of the parallel animal studies have demonstrated that iNOS activation precedes or accompanies neurotoxin-induced dopaminergic degeneration, and its inhibition is neuroprotective upon LPS- (Arimoto and Bing, 2003; Gayle et al., 2002; Hunter et al., 2007; Xing et al., 2008), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Dehmer et al., 2000; Liberatore et al., 1999), and 6-OHDA-induced insult (Barthwal et al., 2001). Importantly, both postmortem and animal studies suggested iNOS expression is mainly in the activated microglia but not in neurons or astrocytes (Arimoto and Bing, 2003; Knott et al., 2000; Wu et al., 2002). As the free radical product of iNOS, nitric oxide can freely diffuse into cells and form highly-reactive peroxynitrite, which attacks functional molecules such as nitration of tyrosine residue and organelles leading to neuronal death (Beal, 1998; Schulz et al., 1995).

As an environmental inflammation-inducer, LPS has been shown as a potent microglia activator and neurotoxin in *in vitro* and *in vivo* studies (Arimoto and Bing, 2003; Choi et al., 2009; Hunter et al., 2009; Hunter et al., 2007; Xing et al., 2007). The animal PD models induced by LPS demonstrated microglia activation followed by gradual loss of dopaminergic neurons (Gao et al., 2002b; Shibata et al., 2003), and pharmacologically anti-inflammatory drugs or anti-oxidant reagents rescued dopaminergic neurons (Li et al., 2004; Li et al., 2005b; Wang et al., 2002; Wang et al., 2004; Zhou et al., 2005).

A recent study demonstrated that LPS induced dopaminergic neuronal loss in the single midbrain slice culture (Shibata et al., 2003). Similarly, the study on our triple culture showed that various doses of LPS (10 μ g/ml, 20 μ g/ml) / IFN γ markedly induced dopaminergic neuronal loss (Figure 1), accompanied with increased microglia activation and nitric oxide production (Figure 2 and Figure 3). In contrast, GDNF protected dopaminergic neurons and inhibited microglia activation. Since Toll-like receptor (TLR4) is required for LPS signaling transduction of its neurotoxicity (Hoshino et al., 1999; Poltorak et al., 1998), and TLR4 is solely expressed on the microglia but not on the neurons and other CNS glial cells (Lehnardt et al., 2002; Lehnardt et al., 2003), the neuronal degeneration observed in our culture system must be initially secondary to the LPS-induced microglia activation, which is consistent with our previous demonstration that LPS induced dopaminergic neurodegeneration only in the presence of microglia (Xing et al., 2007). A variety of deleterious proinflammatory factors and cytokines released from activated microglia trigger dopaminergic neurodegeneration (Gao et al., 2002a; Le et al., 2001), and in turn the dying neurons can release microglia-activating molecules such as neuromelanin and aggregated α -synuclein (Zecca et al., 2008; Zhang et al., 2009; Zhang et al., 2005), forming a vicious degenerating cycle. On the opposite side, pretreatment with GDNF may directly inhibit

microglia activation since both GDNF receptor Ret and GDNF family receptor alpha1 (GFR α -1) are expressed in rat primary cultured microglia (Honda et al., 1999).

It is interesting to find that LPS 40 μ g/ml induced dopaminergic neuronal loss and GDNF 20ng/ml failed to protect neurons. In addition, a parallel pattern of response to LPS and GDNF was observed when microglia activation was measured as, LPS 40 μ g/ml induced microglia activation and GDNF failed to inhibit it. Furthermore, our result for measured nitric oxide showed that GDNF can inhibit its production when induced by LPS at 40 μ g/ml, suggesting that nitric oxide alone is not sufficient to induce the neuronal loss. Indeed, the other proinflammatory mediator cyclooxygenase-2 and its product prostaglandin E₂ are implicated in this process presumably via its EP1 receptor on the dopaminergic neurons (Carrasco et al., 2007; Teismann and Ferger, 2001; Teismann et al., 2003; Vijitruth et al., 2006). Furthermore, the production of nitric oxide induced between 40 μ g/ml and 80 μ g/ml LPS failed to show any significant difference, suggesting that 40 μ g/ml LPS could be sufficient for maximal activation of microglia, releasing not only nitric oxide and prostaglandin E₂ but also other reactive species and cytokines, leading to the failure of GDNF to rescue dopaminergic neurons.

When 80 μ g/ml LPS was administered, it was surprising to find that GDNF “recovered” its ability to rescue neurons, accompanied with the inhibition of microglia activation and nitric oxide production. Although it is generally accepted that aberrantly activated microglia can produce various proinflammatory factors ultimately inducing neuronal death, recent studies on the function of microglia subsets in the brain demonstrated two basic phenotypes of microglia physiologically in primary cultures (Floden and Combs, 2007), and they showed quite a distinct ability to produce reactive species in the rodent brain (Kawahara et al., 2009; Nagatsu and Sawada, 2006). We speculate that non-neurotoxic microglia have higher initial response threshold to LPS stimuli and produce neuroprotective molecules instead of deleterious ones. Indeed, locomotor function in spinal cord injury model was improved to a greater degree with higher doses of LPS, which is correlated with GDNF level in microglia (Hashimoto et al., 2005). To further reveal the profile of the neurotoxic microglia and neuroprotective microglia could be very important for finding their clinical biomarker(s) and facilitating pharmaceutical design to inhibit or enhance the activity from certain group of microglia. It is less likely to activate astrocytes to produce antioxidants and neurotrophic factors (Damier et al., 1999; Saavedra et al., 2006) in our culture system since they are resistant to LPS insult even at the 10,000 times higher than the concentration needed to activate microglia (Liu et al., 2001).

The doses of LPS administered in the experimental scheme is much higher than that used in the primary mesencephalic / glia cultures (Kim et al., 2000; Li et al., 2005b; Xing et al., 2007). In our preliminary study a series of lower doses of LPS from 10ng/ml to 1 μ g/ml were utilized to induce dopaminergic neuronal loss, however, no significant difference on the survival of dopaminergic neurons compared to the control group was found (data not shown). In contrast, LPS is able to induce neuronal loss at higher concentrations used in our triple culture system. Although we can not exclude the possibility that the response of dopaminergic neurons to LPS stimuli in primary culture and slice culture follows the different mechanisms, it is very likely that the culture insert, as a physical barrier, slowed down the diffusion rate of LPS, on the other hand, tissue slices entailed high doses of LPS needed to sufficiently induce neuronal death in a relatively short time, compared to the condition in primary cultures.

The activation of PI3K/Akt pathway has been generally accepted playing a critical role in neuronal survival (Brunet et al., 2001; Frebel and Wiese, 2006), and the dysfunction of this pathway was observed in the PD patient midbrains (Malagelada et al., 2008; Timmons et al.,

2009). According to our previous study, PI3K negatively mediated LPS-induced increased activity of p38 MAP kinase, which was found activated at 72hr treated with thrombin in rat midbrain slice culture (Granziera et al., 2007). Based on the above observation and possibility that PI3K could be activated 1hr after LPS treatment in slice cultures, these two time points were chosen to determine the PI3K expression in the immunoblot assay. The current study showed for the first time that suppression of PI3K expression by LPS is inhibited with GDNF treatment in triple slice cultures (Figure 4). Further study in the primary culture demonstrated that GDNF abolished the inhibition of PI3K by LPS in microglia cultures (Figure 5). Together with the previous observation that PI3K negatively regulates LPS-induced activated microglia-mediated nitric oxide generation (Xing et al., 2008), the results suggests that GDNF rescued dopaminergic neurons via activating PI3K pathway and accordingly inhibiting aberrant nitric oxide generation in microglia. Although it is not clear how PI3K regulates nitric oxide expression in activated microglia, a series of evidence suggested that LPS induces NF-kappa-B nuclear translocation, leading to its binding onto CD40 promoter and causing its gene expression (Qin et al., 2005), which is highly associated with enhanced iNOS activity and nitric oxide production (Kawahara et al., 2009). On the opposite side, PI3K inhibits NF-kappa-B activation via up-regulation of Ikappa-B α in the activated microglia (Jana et al., 2007). It is very interesting to answer if GDNF inhibits CD40 expression and function in the activated microglia because transforming growth factor (TGF)- β 1, sharing common structural feature with GDNF, inhibits CD40 expression in microglia (Nguyen et al., 1998).

Parallel to our finding in the primary microglia culture, the results showed that GDNF also abolished the inhibition of PI3K expression induced by LPS in the primary neuronal culture, which is consistent with recent findings that GDNF increased PI3K activity and protected mesencephalic neurons against apoptotic insult (Sawada et al., 2000) and that PI3K activity is required for the neuroprotection of GDNF against 6-OHDA insult in the dopaminergic cell line (Ugarte et al., 2003). Several studies have shown that both GDNF receptor Ret and GFR α -1 are expressed on the neurons in the substantia nigra (Glazner et al., 1998; Sarabi et al., 2001). More importantly, phosphorylation of Ret by GDNF (Creedon et al., 1997) induced PI3K activity in human neuroepithelioma cell line (van Weering and Bos, 1997). Although the neuroprotective signaling mechanisms downstream of PI3K/Akt in dopaminergic neurons is not clear, increased activity of Akt (Protein Kinase B) promoted granule cell survival via phosphorylation of BCL-2 family member BAD on Ser-136 (Datta et al., 1997). Whether same type of correlation between Ret and PI3K activity occur in dopaminergic neurons is interesting to be further studied. Interestingly, a recent study demonstrated that nitric oxide S-nitrosylates Parkin (Chung et al., 2004), a ubiquitin E3 ligase responsible for removal of aberrant proteins, leading to neuronal death. Furthermore, loss of Parkin function increases LPS-induced dopaminergic neuronal death (Frank-Cannon et al., 2008). It is very interesting to know if GDNF increase Parkin expression and/or function via abolishing its S-nitrosylation and further work should be done in this area. As the other important signaling pathway regulating neuronal survival, the immunostaining analysis demonstrated strong immunoreactivity of c-Jun in dopaminergic neurons from PD patient midbrains (Hunot et al., 2004), and our previous study suggested that inhibition of LPS-activated c-Jun N-terminal kinase (JNK) rescued dopaminergic neurons via suppression of cyclooxygenase-2 activity, another presumably accepted proinflammatory factor following different signaling mechanism (Xing et al., 2007). Whether GDNF inhibits JNK activation in LPS-induced PD model is an interesting question to address in the future since it appears to suppress JNK activity induced by proteasome inhibitor lactacystin (Du et al., 2008).

The present study showed that pretreatment of triple cortex-striatum-midbrain organotypic cultures with GDNF protected dopaminergic neurons from LPS-induced neurotoxicity,

which was paralleled by the suppression of microglia activation and nitric oxide production, and GDNF abolished the inhibition of PI3K induced by LPS in both neurons and microglia. The results suggested that GDNF blocked some but not all of the cytotoxic activity of LPS in the triple organotypic culture and that GDNF protected dopaminergic neurons partially by suppressing microglia activation and excessive nitric oxide production probably via increasing the activity of PI3K in both dopaminergic neurons and microglia.

Acknowledgments

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Figure 1.

Pretreatment with GDNF protected dopaminergic neurons in the slice cultures. **A.** Representative pictures of TH-positive neurons in the substantia nigra area upon LPS stimuli and pretreatment with GDNF (DIV16). As shown in A-a and A-b, the neurite length in the GDNF group (A-b) appears longer than control group (A-a). LPS at 10, 20, 40, and 80 μg/ml (A-c, d, e, f) reduced the number of TH-positive neurons and pretreatment with GDNF (A-g, h, j) prevented neuronal loss. **B.** Graph representing LPS neurotoxicity and the neuroprotection of GDNF. LPS induced loss of TH-positive neurons in a dose-dependent manner; GDNF treatment significantly reduced the loss of TH-positive neurons ($**p < 0.01$ or $***p < 0.001$ control versus LPS; $\#p < 0.05$ and $\#\#p < 0.01$ LPS versus GDNF plus LPS, where $n = 5/\text{group}$).

Figure 2. Pretreatment with GDNF inhibited microglia activation in the slice culture. **A.** Representative pictures of OX-6-positive microglia in the substantia nigra area upon LPS stimuli and pretreatment with GDNF (DIV16). LPS at 10, 20, 40, and 80 μ g/ml (A–c, d, e, f) increased the number of OX-6 positive microglia and pretreatment with GDNF (A–g, h, j) inhibited microglia activation. **B.** Graph representing LPS-induced microglia activation and inhibition by GDNF. LPS induced microglia activation in a dose-dependent manner and GDNF pretreatment inhibited microglia activation except at LPS at 40 μ g/ml (** $p < 0.01$ or *** $p < 0.001$ control versus LPS; # $p < 0.05$ or ### $p < 0.01$ LPS versus GDNF plus LPS, where $n = 6$ /group).

Figure 3.

GDNF suppressed LPS-induced microglia-mediated nitric oxide production in the triple cortex-striatum-midbrain organotypic cultures (DIV16). LPS significantly increased the production of nitric oxide in a dose-dependent way. In contrast, pretreatment with GDNF attenuated nitric oxide generation (** $p < 0.01$ or *** $p < 0.001$ control versus LPS; ## $p < 0.01$ LPS versus GDNF plus LPS, where $n = 5/\text{group}$).

Figure 4. GDNF reduced LPS-induced suppression of PI3K expression. The slice cultures were treated with GDNF and/or LPS (20μg/ml) at DIV13, and protein samples were immunoblotted with polyclonal PI3K antibody after 1hr and 72hr treatment, respectively. **A.** Representative bands of PI3K expression at 1hr and 72hr time-points. There was no significant difference between control and the other groups at 1hr. However, at 72hr the suppression of PI3K expression by LPS was observed, and pretreatment with GDNF reversed its inhibition. **B.** Graph showing optical density of PI3K bands demonstrated GDNF prevented LPS-induced decrease in PI3K expression. (* $p < 0.05$ control versus LPS; # $p < 0.05$ LPS versus GDNF plus LPS, where $n = 3$).

Figure 5. GDNF abolished the inhibition of PI3K by LPS in both primary neuronal culture and microglia. Primary microglia culture was treated with LPS (1μg/ml) for 72hr, the conditioned medium was fed into primary neuronal-enriched culture and primary microglia culture for 10min and 1hr, followed by the immunoblotting of PI3K. **A.** Immunoblotting bands of PI3K from microglia and neuronal culture. No significant difference was found after 10min treatment. LPS-treated conditioned medium suppressed the expression of PI3K after 1hr treatment in both different cultures, and pretreatment with GDNF (10ng/ml) 1hr before markedly inhibited its inhibition. **B.** Optical density of PI3K bands showed GDNF prevented the LPS-induced decrease in PI3K expression in microglia. **C.** GDNF prevents the LPS-induced inhibition of PI3K expression in neurons. (** $p < 0.01$ control versus LPS; # $p < 0.05$ LPS versus GDNF plus LPS, $n = 3$).