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Downregulation of Inducible Nitric Oxide Synthetase by Neurotrophin-3 in Microglia

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Abstract Microglia activated after many neurological degeneration of the central nervous system (CNS) act as important regulators for neuropathogenesis in the injured CNS via producing proinflammatory mediators, such as nitric oxide (NO), TNF-α, and IL-1β. Neurotrophin-3 (NT-3) is a well-known trophic factor for neural survival, development, and plasticity. Activated microglia are NT-3-producing cells in the injured CNS, and express its receptor-TrkC. However, little is known about the effect of NT-3 on activated microglia. In this study, pre-treatment of a mouse microglial cell line, BV2, with NT-3 for 24 h indicated that NT-3 reduced the inducible form of NO synthase (iNOS), NO, and TNF-α in BV2 stimulated with lipopolysaccharide (LPS). NT-3 exerted less effect on the reduction of these proinflammatory mediators when it was added to BV2 cultures either simultaneously with LPS or post LPS treatment. These findings indicate that NT-3 may serve as an anti-inflammatory factor to suppress microglial activation. J. Cell. Biochem. 90: 227–233, 2003.
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Key words: iNOS; neurotrophin-3; microglia; lipopolysaccharide; nitric oxide

Microglia, central nervous system (CNS) resident macrophages, are quiescent in the normal CNS. They are activated by cytokines that are produced by infiltrating immune effectors after CNS injury [Gonzalez-Scarano and Baltuch, 1999; Stoll and Jander, 1999]. Activated microglial cells are observed in various neuropathological conditions, such as brain/spinal cord trauma and cerebral ischemia. They are also believed to have the regulatory role in CNS pathophysiologies, including Alzheimer's disease and AIDS [Kreutzberg, 1996; Stoll and Jander, 1999]. In addition to functionally acting as debris scavengers around the lesion site, activated microglia can secrete neurotrophic factors and growth factors, suggesting that they have the beneficial role in CNS repair. Yet, the activated microglia primarily produce proinflammatory cytokines including IL-1β and TNF-α, and generate nitric oxide (NO), reactive oxygen species (ROS) such as superoxide anion (O_2^-), fatty acid metabolites such as eicosanoids, and quinolinic acid and hydrogen peroxide [Woodroofe et al., 1991; Halliwell, 1992; Matsuo et al., 1995; Dugan et al., 1996; Espey et al., 1997]. A growing body of evidence indicates that excessive expression of these activated microglia secreted factors is deleterious to neurons [Boje and Arora, 1992; Chao et al., 1992; McGuire et al., 2001]. NO secreted by activated microglia can rapidly react with O_2^- to produce peroxynitrite anion [Banati et al., 1993; Brosnan et al., 1994]. Moreover, the synthesis of the inducible form of NO synthase (iNOS) along with the release of NO by microglia is correlated with the progression of neurodegeneration, both in vivo and in vitro [Boje and Arora, 1992; Chao et al., 1992; Espey et al., 1997].

Neurotrophin-3 (NT-3) is a target-derived trophic factor, which was originally found to be produced by target neurons or tissues in the nervous system [Bandtlow et al., 1990; Maisonpierre et al., 1990]. NT-3 can be produced by astrocytes [Rudge et al., 1992] and microglia [Elkabes et al., 1996]. NT-3 function is focused on neuronal survival, differentiation, and function. Evidence indicates that NT-3 deficiency causes deficits in specific cutaneous

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innervation [Airaksinen et al., 1996], and partially loses trigeminal mesencephalic neurons [Matsuo et al., 2000]. In addition, NT-3 acts as a survival factor for vestibular ganglion neurons [Ernfors et al., 1994; Farinas et al., 1994], early sympathetic neuroblasts [Birren et al., 1993; Verdi et al., 1996], and spinal motor neurons [Oorschot and McLennan, 1998; Liu and Jaenisch, 2000]. NT-3 is also mitogenic for oligodendrocyte progenitors [Marmur et al., 1998], and enhances neural differentiation of stem cells or neural progenitors [Romero-Ramos et al., 2002; Tzeng, 2002]. Additionally, NT-3 promotes the differentiation of transplanted neural stem cells in the injured spinal cord [Castellanos et al., 2002]. On the other hand, Elkabes et al. [1996] have indicated that microglia proliferate in response to NT-3, suggesting that NT-3 acts on microglia via autocrine and/or paracrine fashion [Nakajima et al., 1998]. Nevertheless, the biological effect of NT-3 on microglia is not fully understood.

We show here that pre-treatment with NT-3 reduced the production of iNOS, NO, and proinflammatory cytokines in a mouse microglial cell line (BV2) stimulated with inflammagen lipopolysaccharide (LPS), indicating the diverse functions of NT-3 for neural cells in response to CNS injury.

MATERIALS AND METHODS

Cell Culture

The immortalized murine microglial cell line, BV2, was the kind gift provided by Dr. J.S. Hong (NIEHS, NIH, Bethesda, MD). The BV2 cell line was grown in Dulbecco's modified Eagle medium (DMEM)/F-12 (1:1) containing 5% heat inactivated fetal bovine serum (FBS), 50 U/ml penicillin, and 50 mg/ml streptomycin. Culture medium and antibiotics were purchased from Gibco-BRL (Grand Island, NY). FBS was the product of Hyclone (Logan, UT).

WST-1 Cell Viability Assay

WST-1 was used to determine the effect of NT-3 on microglial growth. WST-1 reacts with mitochondrial dehydrogenase in viable cells to generate a formazan product. Thus, measurement of the level of formazan product by a colorimetric method is correlated with the relative number of cells. To study the growth effect of NT-3 on microglia, we replated BV2 cells onto 96-well microtiter plates at a density of 1×10^4 cells/well. Twenty-four hours after 10 ng/ml LPS treatment with or without NT-3 (R&D, Minneapolis, MN) at the distinct concentrations, cultures were subjected to WST-1 assay. WST-1 solution (Roche, Germany) was added to each well 2 h later, WST-1 metabolite was measured using an ELISA reader at 450 nm.

Nitrite/Nitrate, TNF-α, and IL-1β Assay

BV2 cells at a density of 1×10^4 cells/well or 1×10^5 cells/dish were replated onto 96-well plates or 35-mm petri dishes, respectively. Twenty-four hours after 10 ng/ml LPS treatment with or without NT-3 at the distinct concentrations, culture media were collected for NO production assay and cytokine ELISA assay. The production of NO was assessed as the accumulation of nitrate and nitrite in the culture medium using a colorimetric reaction kit from R&D (Minneapolis, MN). TNF-α and IL-1β were measured using an enzyme immunoassay kit from R&D (Minneapolis, MN), following the procedure provided by the vendor.

Western Blot Analysis

BV2 cells at a density of 1×10^5 cells/dish were replated onto 35-mm tissue petri dishes. Protein samples were extracted from BV2 cell cultures at 24 h after 10 ng/ml LPS treatment with or without NT-3 at the distinct concentrations. Cells were harvested and gently homogenized on ice using PBS containing 1% SDS, 1 mM phenylmethyl-sulfonylfluoride (PMSF), 1 mM EDTA, 1.5 mM pepstatin, 2 mM leupeptin, and 0.7 mM aprotinin. Protein concentration was determined using a Bio-Rad DC kit. Subsequently, 10–20 μg of total protein was loaded onto 7.5–12.5% SDS–PAGE, and transferred to a nitrocellulous membrane. The protein was identified by incubating the membrane with anti-iNOS antibodies (Calbiochem, Cambridge, MA) or anti-α-tubulin antibodies (Sigma, St. Louis, MO) overnight at 4°C, followed by horseradish peroxidase conjugated secondary antibodies and ECL solution (NEN LifeScience, Boston, MA).

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Each experiment was repeated at least twice. Statistical significance of differences between groups of data (P-value < 0.01) was evaluated using one-

way analysis of variance (ANOVA) followed by Tukey's pairwise comparison (Release 8.01, SAS Institute, Cary, NC). Statistical significance was determined when P-value < 0.01 was obtained.

RESULTS

Effect of NT-3 on BV2 Cell Growth

NT-3 has been reported to stimulate primary microglial proliferation [Elkabes et al., 1996]. Here we show that treatment with NT-3 at the distinct concentrations for 24 h caused no change in WST-1 absorbance, which was correlated with the relative number of BV2 cells in the presence or absence of 10 ng/ml LPS stimulation (Fig. 1). Treatment of BV2 cells with LPS showed no effect on BV2 cell growth when compared to that observed in control.

Effect of NT-3 on iNOS and NO Production

We measured the amount of NO in the culture supernatant of BV2 cells. As shown in Figure 2A, 24 h pre-treatment with NT-3 significantly reduced NO production of LPS-stimulated BV2 in a dose-dependent manner. However, NO production in LPS-stimulated BV2 remained unchanged after simultaneous treatment with NT-3 and LPS (Fig. 2B). Similarly, treatment with NT-3 for 18 h in the presence of 10 ng/ml LPS failed to reduce NO production in BV2 cells that were pre-treated with 10 ng/ml LPS for 6 h (Fig. 2C).

To determine whether NT-3 reduced the production of NO in LPS-treated BV2 cells via

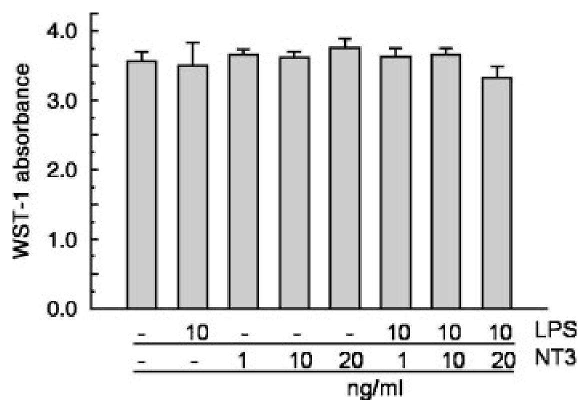

Fig. 1. Effect of NT-3 on BV2 cell viability. BV2 cells were treated with NT-3 at concentrations of 1, 10, or 20 ng/ml in the presence or absence of LPS for 24 h. Data are the means $\pm$ SEM values (n = 8). The experiments were repeated at least twice with similar results.

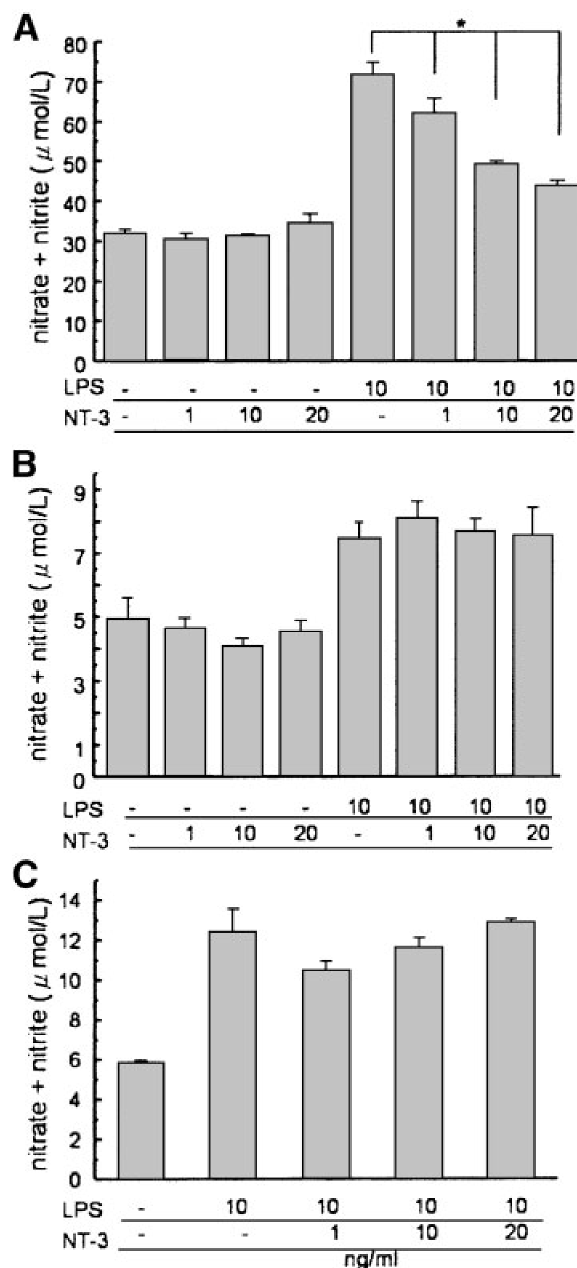

Fig. 2. Effect of NT-3 inhibition on NO production in LPS-treated BV2 cells. **A**: BV2 cells were treated with NT-3 at concentrations of 10 or 20 ng/ml for 24 h, and then followed by 10 ng/ml LPS stimulation. Twenty-four hours later, the levels of NO in the supernatants of BV2 cultures were measured. **B**: BV2 cells were treated for 24 h with 10 ng/ml LPS and NT-3 at concentrations of 10 or 20 ng/ml. **C**: BV2 cells were stimulated by 10 ng/ml LPS for 6 h, and then treated with 10 or 20 ng/ml NT-3 in the presence of 10 ng/ml LPS for 18 h. Levels of NO in the culture supernatants of BV2 treated with NT-3 was comparable to that observed in control.The graphs are representative of two experiments with assays in triplicate. Data are the mean $\pm$ SEM values. *$P < 0.01$ calculated according to one-way ANOVA.

suppressing iNOS expression, Western blot analysis was performed. The results indicated that treatment of BV2 simultaneously with 10 ng/ml LPS and NT-3 at 10 or 20 ng/ml slightly reduced levels of iNOS in BV2 (Fig. 3B). Six hours after LPS stimulation, treatment of BV2 with NT-3 did not cause any significant change on iNOS production when compared to that observed in LPS-stimulated BV2 cells without NT-3 treatment (Fig. 3C). Pre-treatment with

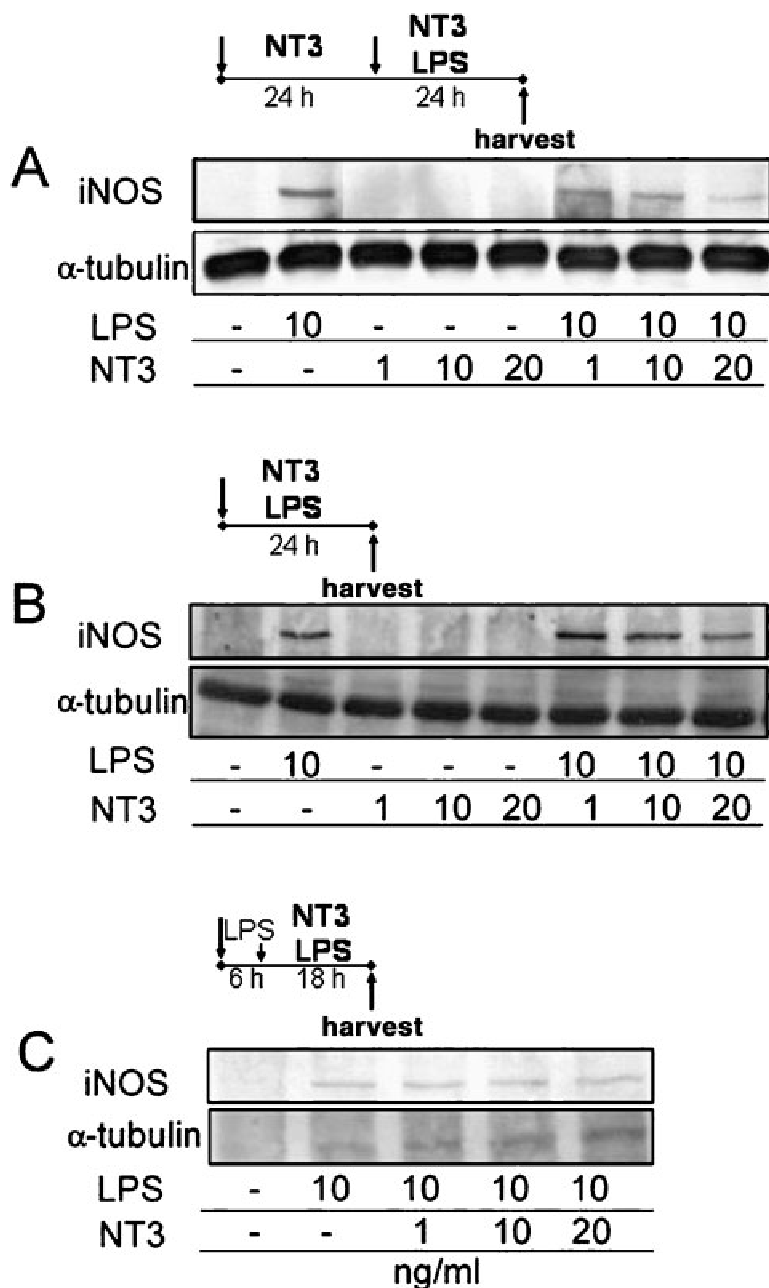

Fig. 3. Effect of NT-3 on the production of LPS-induced iNOS in BV2. **A**: BV2 cells were treated with NT-3 at concentrations of 1, 10, or 20 ng/ml for 24 h, and then followed by 10 ng/ml LPS stimulation for 24 h. iNOS production in BV2 was analyzed by Western Blot assay. **B**: BV2 cells were treated for 24 h with 10 ng/ml LPS and NT-3 at concentrations of 1, 10, or 20 ng/ml. **C**: BV2 cells were stimulated by 10 ng/ml LPS for 6 h, and then treated with NT-3 at concentrations of 1, 10, or 20 ng/ml in the presence of 10 ng/ml LPS for 18 h. Noted that iNOS production was undetected in BV2 cultures treated with NT-3 at the different concentrations. The experiments were repeated at least twice with similar observations. Each experiment was probed with alpha-tubulin antibodies as internal loading protein.

NT-3 for 24 h, however, reduced LPS-induced iNOS production in BV2 cells (Fig. 3A). This decrease showed in a dose-dependent manner of NT-3. It was noted that NT-3 itself induced no or rare production of iNOS in BV2.

Effect of NT-3 on TNF-α Production

To further explore the effect of NT-3 on the production of proinflammatory cytokines, we examined the level of TNF-α in the culture supernatant of LPS-stimulated BV2. IL-1β was not detectable in the supernatant of BV2 cells treated with 10 ng/ml LPS (data not shown). We found that the level of TNF-α dramatically decreased when BV2 cells were pre-treated with NT-3 for 24 h followed by 10 ng/ml LPS stimulation (Fig. 4A). However, the levels of TNF-α in LPS-stimulated BV2 cells were not changed by simultaneous treatment with NT-3 and LPS (Fig. 4B). Moreover, there was no significant reduction in the levels of TNF-α in BV2 cells that were stimulated with 10 ng/ml LPS for 6 h followed by LPS plus treatment with NT-3 at 10 or 20 ng/ml for 18 h (Fig. 4C).

DISCUSSION

Activated microglia generate various NTs and growth factors and contribute to protecting neurons from CNS injuries. They also express the receptors for NTs. NT-3 has recently been shown to inhibit the release of LPS-stimulated NO from microglia through its receptor Trk and/ or LNGFRp75 [Nakajima et al., 1998]. The precise biological activity of the NTs, however, on microglia remains to be defined. Using a mouse microglial cell line, BV2, we are first to present here that prior to LPS stimulation treatment with NT-3 for 24 h decreases the production of iNOS, NO, and TNF-α in BV2. However, neither its co-treatment nor post-treatment with LPS reduces the levels of iNOS protein, NO, and TNF-α in BV2 cells. These findings indicate that NT-3 may act as an anti-inflammatory factor to inhibit microglial activation.

Similar to other NTs, NT-3 exerts its effect on neuroprotection, neurite outgrowth, and growth

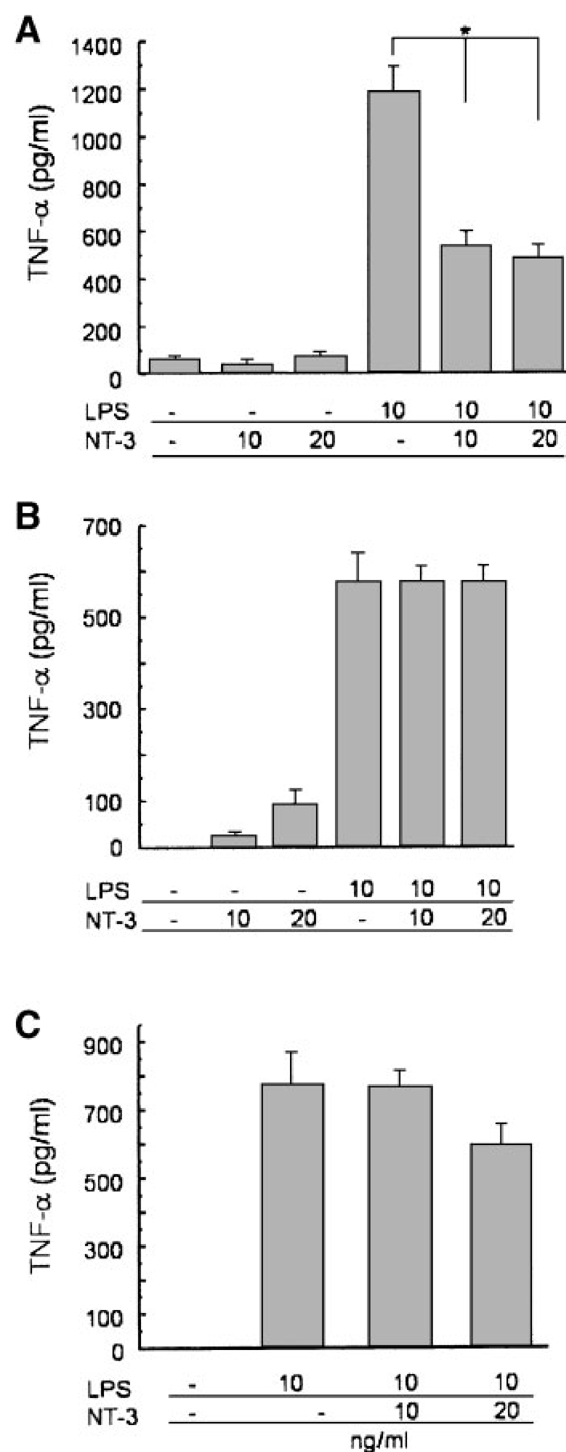

Fig. 4. Effect of NT-3 inhibition on TNF-α production in LPS-treated BV2 cells. **A**: BV2 cells were treated with NT-3 at concentrations of 10 or 20 ng/ml for 24 h, and then followed by 10 ng/ml LPS stimulation. Twenty-four hours later, the levels of TNF-α in the supernatants of BV2 cultures were measured. **B**: BV2 cells were treated 24 h with 10 ng/ml LPS and NT-3 at concentrations of 10 or 20 ng/ml. **C**: BV2 cells were stimulated by 10 ng/ml LPS for 6 h, and then treated with NT-3 at concentrations of 10 or 20 ng/ml in the presence of 10 ng/ml LPS for 18 h. Noted that there was undetectable levels of TNF-α in BV2 cells treated with NT-3 alone. The graphs are representative of two experiments with assay in triplicate. Data are the mean ± SEM values. $^{*}P < 0.01$ calculated according to one-way ANOVA.

cone formation via activating MAP kinase pathway, through PI3 kinase activation, or by triggering PLC-γ signaling [Kaplan and Miller, 1997; Huang and Reichardt, 2001; Yang et al., 2001]. Blockage of the MAP kinase pathway by a MAP kinase inhibitor, U0126, has indicated that a reduction of iNOS production was observed in BV2 treated with LPS alone and LPS plus NT-3 (data not shown). Yet, LPS increases iNOS production via MAP kinase signaling to mediate activator protein-1(AP-1)-dependent iNOS promoter activation [Kristof et al., 2001]. Thus, our observation that U0126-induced iNOS reduction was found in LPS-stimulated microglia pretreated with NT3 can not support our hypothesis concerning the involvement of MAP kinase signaling in NT-3-induced iNOS downregulation. The role of MAP kinase induced by NT-3 in the inhibition of iNOS expression, therefore, remains to be clarified. In addition, the other signaling pathways induced by NT-3 may also play regulatory role in inhibiting iNOS production of LPS-treated BV2 cells. On the other hands, other molecules, such as transcription factors that are upregulated or activated after early treatment with NT-3 may be also involved in the regulatory route of iNOS inhibition.

In conclusion, pre-treatment with NT-3 suppressed LPS-induced proinflammatory agents, iNOS, NO, and TNF-α in BV2 microglial cell line, suggesting that NT-3 may not only have the protective effect on neurons against various insults, but also suppress microglia to produce proinflammatory agents.

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