Neuroprotective Effects of Glyceryl Nonivamide against Microglia-Like Cells and 6-Hydroxydopamine-Induced Neurotoxicity in SH-SY5Y Human Dopaminergic Neuroblastoma Cells

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ABSTRACT

Glyceryl nonivamide (GLNVA), a vanilloid receptor (VR) agonist, has been reported to have calcitonin gene-related peptide-associated vasodilatation and to prevent subarachnoid hemorrhage-induced cerebral vasospasm. In this study, we investigated the neuroprotective effects of GLNVA on activated microglia-like cell mediated- and proparkinsonian neurotoxin 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in human dopaminergic neuroblastoma SH-SY5Y cells. In coculture conditions, we used lipopolysaccharide (LPS)-stimulated BV-2 cells as a model of activated microglia. LPS-induced neuronal death was significantly inhibited by diphenylene iodonium (DPI), an inhibitor of NADPH oxidase. However, capsazepine, the selective VR1 antagonist, did not block the neuroprotective effects of GLNVA. GLNVA reduced LPS-activated microglia-mediated neuronal death, but it lacked protection in DPI-pre-treated cultures. GLNVA also decreased LPS activated microglia induced overexpression of neuronal nitric-oxide synthase (nNOS) and glycoprotein 91 phagocyte oxidase (gp91phox) on SH-SY5Y cells. Pretreatment of BV-2 cells with GLNVA diminished LPS-induced nitric oxide production, upregulation of inducible nitric-oxide synthase (iNOS), and gp91phox and intracellular reactive oxygen species (iROS). GLNVA also reduced cyclooxygenase (COX)-2 expression, inhibitor of nuclear factor (NF)-κB (IκBα/IκBβ) degradation, NF-κB activation, and the overproduction of tumor necrosis factor-α, interleukin (IL)-1β, and prostaglandin E2 in BV-2 cells. However, GLNVA augmented anti-inflammatory cytokine IL-10 production on LPS-stimulated BV-2 cells. Furthermore, in 6-OHDA-treated SH-SY5Y cells, GLNVA rescued the changes in condensed nuclear and apoptotic bodies, prevented the decrease in mitochondrial membrane potential, and reduced cells death. GLNVA also suppressed accumulation of iROS and up-regulated heme oxygenase-1 expression. 6-OHDA-induced overexpression of nNOS, iNOS, COX-2, and gp91phox was also reduced by GLNVA. In summary, the neuroprotective effects of GLNVA are mediated, at least in part, by decreasing the inflammation- and oxidative stress-associated factors induced by microglia and 6-OHDA.

ABBRVIATIONS: PD, Parkinson’s disease; TNF, tumor necrosis factor; IL, interleukin; ROS, reactive oxygen species; LPS, lipopolysaccharide; 6-OHDA, 6-hydroxydopamine; HO-1, heme oxygenase-1; GLNVA, glyceryl nonivamide; VR, vanilloid receptor; iROS, intracellular reactive oxygen species; PG, prostaglandin; NOS, neuronal nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; gp91, glycoprotein 91; PHOX, phagocyte oxidase; IκB, inhibitor of nuclear factor-κB; COX, cyclooxygenase; NF, nuclear factor; DMSO, dimethyl sulfoxide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DCF-DA, 2',7'-dichlorofluorescein diacetate; DPI, diphenylene iodonium; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; TBST, Tris-buffered saline/Tween 20; ANOVA, analysis of variance.
toxicity has been implicated in numerous diseases, including PD, Alzheimer’s disease, multiple sclerosis, and AIDS dementia complex (Block et al., 2007). Activated microglia exert cytotoxic effects by oxidative stress (Dickson et al., 1993), releasing inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, NO, arachidonic acid metabolites, and quinolinic acid (Chao et al., 1995). This process may be especially important in the central nervous system, which is vulnerable to reactive oxygen species (ROS) and reactive nitrogen species damage as the result of the brain high O2 consumption, high lipid content, and the relatively low antioxidant defenses in brain, compared with other tissues (Metodiewa and Koska, 2000). Lipopolysaccharide (LPS) is one of the most common agents used to investigate the impact of inflammation on neuronal death, and studies indicate that microglia are necessary for LPS-induced neurotoxicity (Qian et al., 2006a, b). It remains unclear whether an immune/inflammatory component is a primary or secondary event in PD. However, the possibility that chronic inflammation could be implicated in the pathogenesis of PD, and anti-inflammatory agents decreased degeneration of the substantia nigra pars compacta in animal model. It is quite reasonable to presume that activated microglial cells propagated the neurodegenerative process. Therefore, drugs targeting specific aspects of the microglia-related cascade may prove successful.

The progressive deterioration of catecholaminergic cells in Parkinson’s patients, at least in part, is due to an ongoing selective oxidative stress damage. The autoxidation of the neurotransmitter dopamine to 6-hydroxydopamine (6-OHDA) generates ROS and reactive quinones and subsequently induces cell death (Blum et al., 2001). 6-OHDA can produce oxidative stress and induce an ROS-related collapse in mitochondrial membrane potential (Lotharius et al., 1999). In experimental PD models, 6-OHDA is commonly used to induce apoptosis in the dopaminergic cells specifically (Beal, 2001; Przedborski and Ischiropoulos, 2005), including human SH-SY5Y cell lines (Jordán et al., 2004; Guo et al., 2005). Moreover, heme oxygenase-1 (HO-1), a stress response protein, plays an important role in cellular antioxidant system, and it has been reported that moderate overexpression of HO-1 attenuates 6-OHDA-induced ROS generation (Salinas et al., 2003).

Previously, we have shown glyceryl nonivamide (GLNVA), a vaniloid receptor (VR) agonist, dose-dependently attenuated hemorrhage-induced cerebral basal arteries vasoconstriction in an experimental animal model of subarachnoid hemorrhage (Lin et al., 2001). GLNVA enhanced renal excretion of water and electrolytes due to an increased filtered load, and it decreased tubular reabsorption (Lo et al., 1997a). GLNVA also displayed calcitonin gene-related peptide releasing, K+ channel opening, and associated vasorelaxant activities (Lo et al., 1997b). In the present study, we characterize the anti-inflammatory and neuroprotective effect of GLNVA on LPS-activated microglial BV-2 cell-induced toxicity by measuring the ratio of dead cells; production of inflammatory cytokines (TNF-α and IL-1β) and anti-inflammatory cytokines (IL-10, NO, iROS, and PGE2); protein expression of nNOS, inducible nitric oxide synthase (iNOS), gp91phox, and iκBα/iκBβ; and cyclooxygenase (COX)-2 and NF-kB activity. The neuroprotective effect of GLNVA on 6-OHDA-induced toxicity is also evaluated by measuring the ratio of dead cells, iROS level, mitochondrial membrane potential, and protein levels of nNOS, iNOS, gp91phox, and HO-1.

**Materials and Methods**

**Reagents.** Dimethyl sulfoxide, LPS (L8274) from *Escherichia coli* (O26:B6), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 2’,7’-dichlorofluorescein diacetate (DCF-DA), diphenylene iodonium (DPI), capsazepine, 6-hydroxydopamine, Hoechst 33258, mouse antibody against iNOS, and β-actin were obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco’s modified Eagle’s medium, Eagle’s minimum essential medium, Ham’s F-12 medium, fetal bovine serum (FBS), penicillin, amphotericin B, and streptomycin were obtained from Invitrogen (Carlsbad, CA). Mouse antibody against gp91phox and all materials for SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad (Hercules, CA). Goat antibody against COX-2, mouse antibodies against IκBα, rabbit antibody against IκBβ, nNOS, HO-1, and all horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Enhanced chemiluminescence agent was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). All cytokine enzyme-linked immunosorbent assay (ELISA) kits were obtained from Pierce Endogen (Rockford, IL).

**Microglial Cell Culture and Stimulation with LPS.** Mouse microglial cell line BV-2 were cultured in Dulbecco’s modified Eagle’s medium containing 10% (v/v) heat-inactivated FBS, 4 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B at 37°C in a humidified incubator under 5% CO2 and 95% air. For the purpose of experiment, BV-2 cells were plated at the density of 1 × 10^4 cells/ml medium in 24- or 96-well sterile plates. Then, cells were stimulated with LPS alone (100 ng/ml), vehicle (0.25% propylene glycol + 0.75% DMSO) alone, LPS + vehicle, or LPS + GLNVA.

**Coculture of SH-SY5Y Cells with MicroglialBV-2 Cells.** Human neuroblastoma cell line SH-SY5Y (ATCC CRL-2266) were cultured in a medium consisting 1:1 mixture of Eagle’s minimum essential medium and Ham’s F-12 medium containing 10% heat-inactivated FBS, 4 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B at 37°C in a humidified incubator under 5% CO2 and 95% air. For the purpose of experiment, BV-2 cells were plated at the density of 1 × 10^4 cells/ml medium. BV-2 cells were seeded onto cell culture inserts (pore size of 0.2 μm; NUNC A/S, Roskilde, Denmark), and they were placed into the wells where SH-SY5Y cells were grown. Under this coculture condition, the BV-2 and SH-SY5Y cells were separated by filters present in the insert. However, the medium freely passes through the inserts. BV-2 cells then were stimulated with LPS alone (100 ng/ml) or LPS + vehicle or LPS + GLNVA.

**MTT Cell Viability Assay.** Cell viability was measured by quantitative colorimetric assay with MTT, showing the mitochondrial activity of living cells. SH-SY5Y cells in 96-well plates were incubated with 6-OHDA alone, 6-OHDA + vehicle, or 6-OHDA + GLNVA for 24 h. In coculture condition, BV-2 cells were treated with or without LPS (100 ng/ml), LPS + vehicle, or LPS + GLNVA for 24 h. After the incubation, the inserts containing BV-2 cells were removed. MTT reagent (0.5 mg/ml) was added to the test cell culture medium and incubated further for 4 h. The medium was removed, and the formazone formed in the well was extracted with isopropanol containing 0.04% HCl (200 μl/well) and read at 540/630 nm in a microplate reader.

**Nitrite Measurement.** The production of NO was determined by measuring the level of accumulated nitrite, a metabolite of NO in the culture supernatant using Griess reagent. Briefly, after 24 h of treatment with LPS (100 ng/ml) alone, LPS + vehicle, or LPS + GLNVA, the culture supernatants were separately collected and mixed with an equal volume of Griess reagent [1% sulfanilamide and...
0.1% N-(1-naphthyl)ethylenediamine in 5% phosphoric acid] and incubated at room temperature for 10 min. The absorbance was measured at 540 nm (optical density 540).

Enzyme-Linked Immunosorbent Assay. The production of IL-1β, IL-10, PGE2, and TNF-α was detected by using ELISA kits from Pierce Endogen according to the manufacturer’s instructions. IL-1β and PGE2 in supernatant were determined after 15 h of LPS treatment. TNF-α was determined after 2 h of LPS treatment. IL-10 was determined after 24 h of LPS treatment.

NF-κB DNA Binding Activity. The DNA binding activities of transcription factors in cells were determined by enzyme-linked DNA-protein interaction assay essentially according to a described method by Benotmane et al. (1997). Nuclear protein extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent as described previously (Baumgarten et al., 2001). DNA-p50 NF-κB binding activity was measured with a BD Mercury TransFactor kit (Clontech, Mountain View, CA), which detects DNA binding by specific transcription factors according to the manufacturer’s instructions.

Western Blotting Analysis. After treatment with LPS, LPS + vehicle, or LPS + GLNVA, BV-2 cells were collected and lysed to determine the expression of iNOS, COX-2, IkBa, IxBβ, and gp91phox expression. SH-SY5Y cells were collected to analyze nNOS, iNOS, COX-2, gp91phox, and HO-1 expression. The lysates were centrifuged at 15,000 g for 30 min at 4°C. Equal amount of protein (20 µg/lane) were separated by a polyacrylamide gel (10%)-separated protein and transferred to polyvinylidene fluoride membranes (PerkinElmer Life and Analytical Sciences). Non-specific binding was blocked with 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20 (TBST) containing 5% nonfat milk for 1 h at room temperature. The membranes were then incubated overnight at 4°C with one of the following specific primary antibodies: mouse anti-iNOS (1:1000), mouse anti-gp91phox (1:1000), mouse anti-IkBα (1:1000), mouse anti-β-actin (1:20,000), rabbit anti-nNOS (1:1000), rabbit anti-IkBβ (1:1000), rabbit anti-HO-1 (1:1000), and goat anti-COX-2 (1:5000). Then, membranes were washed six times for 5 min with TBST. The appropriate dilutions of secondary antibodies (diluted 1:1000) were incubated for 1 h. After six washes with TBST,
the protein bands were detected with the enhanced chemiluminescence reagent (PerkinElmer Life and Analytical Sciences).

**Measurement of Intracellular Reactive Oxygen Species.** The level of intracellular ROS was quantified by fluorescence with DCF-DA. After incubations with the indicated treatments, microglial BV-2 cells or SH-SY5Y cells were loaded with 10 μM DCF-DA for 30 min at 37°C. Then, cells were washed three times with phosphate-buffered saline, pH 7.4, and the relative levels of fluorescence were quantified in a spectrophotofluorimeter (FLUOstar OPTIMA; BMG Labtech, Offenburg, Germany; 495-nm excitation and 520-nm emission). Intracellular ROS-containing cells were identified as those with increased FITC fluorescence of oxidized DCF.

**Morphological Changes.** The changes in nuclear morphology of apoptotic cells were investigated by labeling the cells with the nuclear stain Hoechst 33258 and examining them under fluorescent microscopy. After being treated with 6-OHDA, GLNVA, or both for 24 h, the cells were fixed with 3.7% paraformaldehyde, and incubated with Hoechst 33258 (3 μg/ml) for 30 min at 37°C. Then, the nuclear morphology was observed under a fluorescence microscope (IX 70; Olympus, Tokyo, Japan). Cells that exhibited reduced nuclear size, chromatin condensation, intense fluorescence, and nuclear fragmentation were considered as apoptotic cells.

**Measurement of Mitochondrial Membrane Potential.** Mitochondrial membrane potential was measured by the incorporation of a cationic fluorescent dye rhodamine 123. After 24-h incubation in normal medium with or without treatment, the cells were changed to serum-free medium containing 10 μM rhodamine 123, and they were incubated for 15 min at 37°C. The cells were then collected, and the fluorescence intensity was analyzed within 15 min by a spectrophotofluorimeter (FLUOstar OPTIMA; 490-nm excitation and 515-nm emission).

**Statistical Analysis.** Data were expressed as mean ± S.E.M. Analysis of variance (ANOVA) was used to assess the statistical significance of the differences followed by the Dunnett’s test for comparison of multiple means. A value of p < 0.05 was considered statistically significant. Analysis of the data and plotting of the figures were done with the aid of software (SigmaStat and SigmaPlot, version 8.0 (Systat Software, Inc., Point Richmond, CA) and GraphPad Prism, version 2.0 (GraphPad Software Inc., San Diego, CA)] run on an IBM-compatible computer.

**Results**

**GLNVA Inhibited LPS-Activated BV-2 Cell- and 6-OHDA-Induced Loss of SH-SY5Y Cell Viability.** Cells were incubated in drug-free medium or medium containing different concentrations of GLNVA for 24 h. The MTT assay was used as an index of mitochondrial activity. As shown in Fig. 1, GLNVA and vehicle had no acute cytotoxicity on BV-2 cells (Fig. 1A) and SH-SY5Y cells (Fig. 1B). Therefore, inhibition of GLNVA on activated BV-2- or 6-OHDA-induced inflammatory-related responses was not the results of its possible cytotoxic effects on BV-2 or SH-SY5Y cells.

The effect of GLNVA on activated microglia-mediated neurotoxicity was investigated under BV-2 and SH-SY5Y coculture conditions. During coculture, BV-2 cells were stimulated with LPS, and viability of SH-SY5Y cells was quantitated by MTT methods. As shown in Fig. 1C, LPS (100 ng/ml) caused a significant decrease in SH-SY5Y cell viability. Vehicle had no effect on LPS-activated BV-2-induced neurotoxicity. Pre-treatment of GLNVA concentration-dependently decreased LPS-activated microglia-mediated neurotoxicity.

As shown in Fig. 1D, SH-SY5Y cells incubated with 6-OHDA for 24 h and results indicated that 6-OHDA significantly induced cell death. Vehicle had no effect on LPS-activated 6-OHDA-induced neurotoxicity. However, pretreated with different concentration of GLNVA for 30 min, showed protective effects against cell damage caused by 6-OHDA.

**Fig. 2.** Effect of DPI (1 μM), an inhibitor of NADPH oxidase, on LPS (100 ng/ml)-induced SH-SY5Y cell death. Bars represent the mean ± S.E.M. from at least three independent experiments. *, p < 0.05 versus DPI untreated group. ANOVA followed by Dunnett’s test. Vehicle group: 0.25% propylene glycol + 0.75% DMSO + LPS 100 ng/ml.

**Fig. 3.** Effect of capsazepine (1 μM), a selective vanilloid receptor antagonists, on the protection of GLNVA (100 μM) on LPS-activated (100 ng/ml) BV-2 cell (A) or 6-OHDA (100 μM)-induced SH-SY5Y cell (B) death. Cultures were pretreated with capsazepine for 30 min followed by LPS treatment for 24 h. The vehicle groups had either LPS (A) or 6-OHDA (B), respectively. Bars represent the mean ± S.E.M. from at least three independent experiments. *, p < 0.05 versus capsazepine untreated group. ANOVA followed by Dunnett’s test Vehicle: 0.25% propylene glycol + 0.75% DMSO.
DPI Inhibited LPS-Activated BV-2 Cell-Induced SH-SY5Y Cell Death. To examine whether the NADPH oxidase was involved in the LPS-induced neuronal death, DPI (1 μM), an inhibitor of NADPH oxidase, was added 1 h before the addition of LPS treatment. Results indicated that DPI significantly blocked LPS-induced neuronal toxicity. GLNVA (100 μM) lacked neuroprotection in DPI-treated cultures (Fig. 2).

Capsazepine Did Not Block Protective Effect of GLNVA on LPS-Activated BV-2 Cell- and 6-OHDA-Induced SH-SY5Y Cell Death. GLNVA is a VR agonist. To clarify the role of VR1 receptor on the neuroprotection of GLNVA, the selective VR1 antagonist capsazepine was used. As shown in Fig. 3, capsazepine (1 μM) did not inhibit neuroprotective effects of GLNVA (100 μM) on LPS-activated BV-2 cells- or 6-OHDA-induced SH-SY5Y cell death.

GLNVA Attenuated gp91phox and nNOS in SH-SY5Y Cells under Coculture. As shown in Fig. 4, under coculture conditions, LPS exposure induced up-regulation of gp91phox (Fig. 4A) and nNOS (Fig. 4B) on SH-SY5Y cells in a concentration-dependent manner.

GLNVA Reduced LPS-Induced Oxidative Stress-Related Factors in BV-2 Cells. To investigate whether GLNVA inhibited microglial activation-induced oxidative stress, we looked at the effect of GLNVA on the oxidative stress factors, including overexpression of iNOS and gp91phox, and their consequent overproduction of NO and iROS from BV-2 cells stimulated with LPS, respectively. In mouse microglial BV-2 cells, LPS (100 ng/ml) treatment for 24 h could produce large amounts of NO (Fig. 5A). The increased NO release was accompanied by the induction of iNOS (Fig. 5B). Cotreatment of GLNVA with LPS markedly reduced LPS-induced NO production and iNOS expression.

The iROS level was determined by using DCF-DA. As shown in Fig. 6A, treatment with LPS resulted in an increase of iROS in BV-2 cells. The increased iROS release was accompanied by the induction of gp91phox (Fig. 6B). The in-
creases of iROS level and gp91phox expression were reduced by pretreatment with GLNVA (Fig. 6).

**GLNVA Attenuated COX-2 Expression and PGE2 Production in LPS-Activated BV-2 Cells.** BV-2 cells incubated with LPS (100 ng/ml) for 15 h markedly induced COX-2 expression and increased PGE2 production (Fig. 7). However, GLNVA attenuated LPS-induced COX-2 expression (Fig. 7A) and PGE2 production (Fig. 7B) in a concentration-dependent manner in microglial BV-2 cells.

**GLNVA Attenuated Degradation of IkBa and IkBβ and Translocation of NF-κB on LPS-Treated BV-2 Cells.** As shown in Fig. 8, LPS (100 ng/ml) increased degradation of IkBa (Fig. 8A) and IkBβ (Fig. 8B). GLNVA significantly reduced LPS-induced degradation of IkBa and IkBβ in BV-2 cells. Using NF-κB TransFactor Colorimetric kit to determine the NF-κB translocation, LPS significantly induced p50 translocation. However, pretreatment of GLNVA inhibited LPS-induced p50 translocation (Fig. 8C).

**GLNVA Inhibited LPS-Induced Proinflammatory Cytokines TNF-α and IL-β and Augmented Anti-Inflammatory Cytokine IL-10.** As shown in Table 1, LPS (100 ng/ml) caused a marked up-regulation of TNF-α at 2 h and IL-1β at 15 h in BV-2 cells. GLNVA (1–100 μM) significantly inhibited LPS-induced overproduction of TNF-α and IL-1β in a concentration-dependent manner. Vehicle had no effect on LPS-induced overproduction of TNF-α and IL-1β. Furthermore, the anti-inflammatory cytokine IL-10 is induced by activated BV-2 following LPS treatment for 24 h. In this study, we found GLNVA significantly up-regulated IL-10 production in a concentration-dependent manner. However, we also found the vehicle attenuated LPS-induced IL-10.

**GLNVA Attenuated 6-OHDA-Induced Increase in Intracellular ROS Level and gp91phox Expression in SH-SY5Y Cells.** Exposure of SH-SY5Y cells to 100 μM 6-OHDA for 24 h led to a significant increase in iROS level (Fig. 9A). Results indicated GLNVA significantly inhibited 6-OHDA-induced iROS. Moreover, exposure of SH-SY5Y cells to...
6-OHDA for 24 h induced an increased expression of gp91phox (Fig. 9B). GLNVA also inhibited 6-OHDA-induced gp91phox.

**GLNVA Inhibited Up-Regulation of iNOS, nNOS, and COX-2 and Down-Regulation of HO-1 Induced by 6-OHDA in SH-SY5Y Cells.** As shown in Fig. 10, 6-OHDA (100 μM) induced up-regulation of nNOS (Fig. 10A), iNOS (Fig. 10B), and COX-2 (Fig. 10C) as revealed by Western blot analysis. However, pretreatment with GLNVA for 30 min significantly inhibited 6-OHDA-induced up-regulation of nNOS, iNOS, and COX-2. However, 6-OHDA was also found to down-regulate HO-1 and down-regulation was prevented by GLNVA pretreatment (Fig. 10D).

**GLNVA Attenuated 6-OHDA-Induced Changes in Nuclear Morphology and Decrease in Mitochondrial Membrane Potential.** Apoptotic nuclei indicated by condensed nuclei and nuclear fragmentation were apparent after exposure to 100 μM 6-OHDA (Fig. 11A, b). These changes in nuclear characteristics of apoptosis were rescued significantly in the cell pretreated with 1 to 100 μM GLNVA (Fig. 11A, d–f).

Exposure of SH-SY5Y cells to 6-OHDA for 24 h decreased the fluorescent intensity of rhodamine 123 staining, representing a fall in the mitochondrial membrane potential. GLNVA pretreatment inhibited the fall of mitochondrial membrane potential caused by 6-OHDA (Fig. 11B).
TABLE 1  
Effects of GLNVA (1, 10, and 100 µM) on LPS (100 ng/ml)-induced TNF-α, IL-1β, and IL-10 formations in microglia BV-2 cells 

Data are shown for cells (10⁶ cells/ml) with or without preincubated with GLNVA for 30 min before the addition of LPS. Supernatants were collected at 2 h for measurement of TNF-α, at 15 h for measurement of IL-1β, and at 24 h for measurement of IL-10. The results are expressed as mean ± S.E.M. for three independent experiments.

<table>
<thead>
<tr>
<th>Formation in Microglia</th>
<th>PG/ml</th>
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<tr>
<td>TNF-α</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>93.8 ± 6.8</td>
</tr>
<tr>
<td>LPS only</td>
<td>2014.6 ± 182.0*</td>
</tr>
<tr>
<td>LPS + vehicle</td>
<td>1928.3 ± 146.2</td>
</tr>
<tr>
<td>LPS + GLNVA 1 µM</td>
<td>1374.2 ± 101.8**</td>
</tr>
<tr>
<td>LPS + GLNVA 10 µM</td>
<td>1235.2 ± 118.2***</td>
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<tr>
<td>LPS + GLNVA 100 µM</td>
<td>928.3 ± 102.4**</td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12.1 ± 2.3</td>
</tr>
<tr>
<td>LPS only</td>
<td>70.6 ± 6.8*</td>
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<td>LPS + vehicle</td>
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<tr>
<td>LPS + GLNVA 1 µM</td>
<td>54.9 ± 4.3*</td>
</tr>
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<td>LPS + GLNVA 10 µM</td>
<td>48.6 ± 1.5**</td>
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<tr>
<td>LPS + GLNVA 100 µM</td>
<td>38.1 ± 3.3**</td>
</tr>
<tr>
<td>IL-10</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>66.0 ± 10.6</td>
</tr>
<tr>
<td>LPS only</td>
<td>1149.6 ± 117.4*</td>
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<td>LPS + GLNVA 10 µM</td>
<td>1505.4 ± 121.6*</td>
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<tr>
<td>LPS + GLNVA 100 µM</td>
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*p < 0.05, **p < 0.01 vs. LPS only (ANOVA followed by Dunnett’s test).  
*p < 0.01 vs. control (basal level) (ANOVA followed by Dunnett’s test).  
*Vehicle is 0.25% propylene glycol + 0.75% DMSO.

Discussion

The roles of microglial activation and neurotoxin in the pathogenesis of neurodegenerative disorders have been noticed, and it is important to develop a novel class of neuroprotective agents that can protect neuron against microglia and neurotoxin-induced neuronal cell death. Previously, we have shown GLNVA, a vanilloid receptor agonist, attenuated hemorrhage-induced cerebral basilar arteries vasospasm in an experimental animal model of subarachnoid hemorrhage (Lin et al., 2001). The present study demonstrates that GLNVA protects against activated microglia- and 6-OHDA-induced neuronal death. The major findings provided in this study are as follows. 1) GLNVA significantly reduced LPS-activated microglia-like cell and 6-OHDA-induced neuronal cell death, and these effects were not mediated by the vanilloid receptor. 2) In LPS-activated microglia-like cell-mediated toxicity, the neuroprotection of GLNVA was mediated by attenuating inflammatory cytokine (TNF-α and IL-1β); enhancing anti-inflammatory cytokine (IL-10); decreasing the production of iROS, NO, and PGE₂; down-regulating iNOS, nNOS, COX-2, and gp91phox; decreasing degradation of IkBα and IkBβ; and decreasing the translocation of NF-κB. 3) In 6-OHDA-induced neurotoxicity, the protective effect of GLNVA was mediated by down-regulation of nNOS, iNOS, COX-2, and gp91phox; up-regulation of HO-1; decreased accumulation of iROS; and increased mitochondria potential.

Increasing evidence suggests that microglial-induced neurotoxicity plays a crucial role in the majority of neurodegenerative diseases. Increasing intracellular ROS levels in microglia will amplify proinflammatory gene expression, and it might contribute to overactivation and neurotoxic consequences. It also represents an ideal therapeutic target (Block et al., 2007). ROS have been implicated in cellular damage during neurodegenerative disorders. ROS can react with cellular macromolecules through oxidation and cause cells to undergo necrosis or apoptosis. During brain injury, oxidative state plays a key role in the regulation and control of the cell survival and cell death through its interaction with cellular macromolecules and signal transduction pathways (Loh et al., 2006). In the microglia-neuron coculture, we found that treatment with GLNVA significantly protected dopaminergic neuron SH-SY5Y cells from LPS-stimulated microglial BV-2-mediated damage. GLNVA also showed significantly inhibitory effect on LPS-induced iROS level in microglial BV-2 cells.

It is noteworthy that the general term “oxidative stress” is commonly and intimately associated with nigral cell death in Parkinson’s disease (Abou-Sleiman et al., 2006). ROS-in-
duced 6-OHDA reacts with biological target molecules, induces lipid peroxidation, and damages the mitochondrial membrane, resulting eventually in the collapse of mitochondrial membrane potential, leading to cell death (Guo et al., 2005). In this study, GLNVA decreased ROS levels in SH-SY5Y cells exposed to 6-OHDA. GLNVA also reduced the decrease of mitochondrial membrane potential and cell death induced by 6-OHDA. Therefore, GLNVA prevention of neuronal cell death might be partly mediated via decreasing iROS and increasing mitochondrial membrane potential.

The HO enzyme, which is a rate-limiting enzyme in heme catabolism, has a number of potential protective effects against oxidative stress (Salinas et al., 2003). The present results showed that GLNVA not only decreased 6-OHDA-induced iROS level but also decreased the down-regulation of HO-1 induced by 6-OHDA.

The phagocyte NADPH oxidase, dormant in resting cells, becomes activated during phagocytosis to deliberately produce superoxide. The catalytic center of this oxidase is the membrane-integrated protein gp91phox, tightly complexed with p22phox, and its activation requires the association with p47phox, p67phox, and the small GTPase Rac, which normally reside in the cytoplasm. Since the recent discovery of non-phagocytic gp91phox-related enzymes of the NADPH oxidase family, seven homologues identified in humans, deliberate ROS production has been increasingly recognized as important components of various cellular events (Sumimoto et al., 2005; Takeya et al., 2006). The present study showed GLNVA inhibited LPS-induced gp91phox expression in microglial BV-2 cells. In addition, the current results showed that GLNVA inhibited LPS-induced gp91phox expression not only in BV-2 but also in SH-SY5Y under coculture conditions. Moreover, GLNVA down-regulated 6-OHDA-induced gp91phox overexpression. Therefore, GLNVA protects neuron against activated microglia-like cells- and 6-OHDA-induced neurotoxicity partly through inhibition of the expression of gp91phox and the production of ROS.

![Fig. 10](https://example.com/figure.png)

**Fig. 10.** Inhibitory effects of GLNVA on 6-OHDA (100 μM)-induced overexpression of nNOS (A), iNOS (B), and COX-2 (C) and down-regulation of HO-1 (D) in SH-SY5Y cells. Cultures were pretreated with GLNVA for 30 min followed by 6-OHDA treatment for 24 h. Densitometry analyses are presented as the relative ratio of protein/β-actin protein, and they are represented as percentages of the 6-OHDA response in the absence of GLNVA. Bars represent the mean ± S.E.M. from at six independent experiments. The vehicle group was treated with vehicle (0.25% propylene glycol + 0.75% DMSO) and 6-OHDA. #, p < 0.01 versus control; *, p < 0.05; and **, p < 0.01 versus 6-OHDA only. ANOVA followed by Dunnett’s test.
and increase gene expression, such as of PGE2 and TNF-α. In the present study, the induction of iNOS, thereby amplifying NO-mediated neuronal death (Arimoto and Bing, 2003). In the present study, the induction of iNOS, thereby amplifying NO-mediated neuronal death (Arimoto and Bing, 2003).

Hoechst 33258. Scale bar, 50 μm. (f) GLNVA. The cells were stained with the DNA-binding fluorochrome preincubated 6-OHDA with vehicle (c), 1 μM (d), 10 μM (e), and 100 μM (f) GLNVA. The cells were stained with the DNA-binding fluorochrome Hoechst 333258. Scale bar, 50 μm. B, GLNVA attenuated 6-OHDA-induced decrease of mitochondrial membrane potential. Cells exposed to 6-OHDA with or without GLNVA were incubated with rhodamine 123, and then the fluorescence intensity was measured. Bars represent the mean ± S.E.M. from at three independent experiments. #, p < 0.01 versus control; *, p < 0.05; **, p < 0.01 versus 6-OHDA only. ANOVA followed by Dunnett’s test.

Neurotoxic amounts of reactive nitrogen species are formed by the activity of iNOS (Floyd, 1999). NO, together with nNOS, is known to be involved in the pathogenesis of PD (Przedborski et al., 1996; Klivenyi et al., 2000). The current results indicated that GLNVA not only reduced LPS-activated microglia-induced NO/iNOS and decreased the overexpression of iNOS and nNOS in microglia-stimulated SH-SY5Y cells but also attenuated the overexpression of iNOS and nNOS induced by 6-OHDA. ROS also enhances proinflammatory signaling in phagocytes. An increase in intracellular ROS is known to increase kinase phosphorylation, increase activation of transcription factors, such as NF-κB, and increase gene expression, such as of PGE2 and TNF-α (Block et al., 2007). The proinflammatory cytokines TNF-α and IL-β are thought to play a major role in inducing neuronal death (Aloisi, 2001). Both TNF-α and IL-1β increase the permeability of the blood-brain barrier (Brabers and Nottet, 2006). Proinflammatory cytokines may also be involved in the induction of iNOS, thereby amplifying NO-mediated neuronal death (Arimoto and Bing, 2003). In the present study, we found that GLNVA significantly inhibited the release of TNF-α and IL-1β in a dose-dependent manner in LPS-stimulated BV-2 cells. Moreover, activation of TNF-α receptor is associated with nuclear translocation of NF-κB and with the development of apoptosis in cultured dopaminergic neurons (Hunot et al., 1996, 1997). GLNVA prevented the degradation of IkBα and IkBβ and the translocation of NF-κB. Therefore, it is likely that suppressing the production of proinflammatory cytokine through the NF-κB signaling pathway is at least partially responsible for the observed neuroprotective effect of GLNVA.

IL-10 is a pleiotropic cytokine that plays a critical role in the regulation of inflammatory responses and immune reactions, acting on both hematopoietic and nonhematopoietic cells. Increasing evidence indicates that IL-10 has the ability to improve neurological outcome after central nervous system injury, and this ability relies on its anti-inflammatory effects. The neuroprotective effect of IL-10 was attributed to inhibition of LPS-stimulated microglial activation. IL-10 significantly inhibited the microglial production of TNF-α, NO, ROS, and superoxide free radicals after LPS stimulation. Moreover, IL-10 inhibits LPS-induced dopaminergic neurotoxicity through the inhibition of PHOX activity in a Janus tyrosine kinase 1-dependent mechanism (Qian et al., 2006a,b). The anti-inflammatory cytokine IL-10 is also induced by activated microglia following LPS treatment (Mi-zuno et al., 1994). The present results indicated GLNVA significantly augmented IL-10 production, and this might result in decreasing NADPH oxidase activity. The augmentation of IL-10 production by GLNVA might be a benefit in the defense of microglia-induced neurotoxicity.

Among many inflammatory factors found in the PD brain, COX, specifically the inducible isofrom, COX-2, is thought to be a critical enzyme in the inflammatory response (Vijith et al., 2006). COX-2 has been implicated in neuronal survival and death. COX-2 functions as a cellular factor that induces superoxide-mediated cell death in primary cortical neurons (Im et al., 2006). Cytokines (particularly TNF-α) also activate COX-1 and COX-2 enzymes that catalyze the first step in the conversion of arachidonic acid to prostaglandins and thromboxanes. Previous studies have shown inhibition of COX-2 provides neuroprotection (Teismann and Ferger, 2001), and COX-2 inhibitors can lower the risk of PD (Chen et al., 2003). We showed here that GLNVA not only decreased TNF-α production but also dose-dependently inhibited COX-2 expression and PGE2 synthesis in microglial BV-2 cells.

Previously, we have shown GLNVA is a capsaicin nonpungent analog (Liu et al., 1997). Capsaicin, an antioxidant and vanilloid receptor agonist, produced a protective effect against N-methyl-D-aspartate-induced neurotoxicity. The protective effect of capsaicin was not blocked by the specific vanilloid receptor antagonist capsazepine (Chen et al., 2005). However, capsazepine also protects against neuronal injury caused by oxygen glucose deprivation by inhibiting I(h) (Ray et al., 2003). Their results demonstrate that the neuroprotection of capsaicin or capsazepine is not mediated by vanilloid receptor. In this study, the protective effect of GLNVA was also not blocked by capsazepine. Therefore, the protective effect of GLNVA was not mediated by the vanilloid receptor.

In conclusion, findings from our research indicate that GLNVA can effectively attenuate LPS-activated microglial-like cell- and 6-OHDA-induced neurotoxicity. Considering the importance of inflammation, apoptosis, and ROS in neurodegeneration, GLNVA might have a protective potential in
the microglia-mediated or oxidative stress-related neurodegenerative disorders such as Parkinson’s disease.

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References


