16α,17α-Epoxypregnenolone-20-oxime Prevent LPS-Induced NO Production and iNOS Expression in BV-2 Microglial Cells by Inhibiting JNK Phosphorylation

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Received September 9, 2013; accepted April 9, 2014

The free radical nitric oxide (NO), a main member of neuroinflammatory cytokines, is a gaseous molecule produced by activated microglia, and has many physiological functions, including neuroinflammation. In the present study, we evaluated the effects of serial 16-dehydropregnenolone-3-acetate derivatives on lipopolysaccharide (LPS)-induced NO production and inducible nitric oxide synthase (iNOS) expression in BV-2 microglial cells. Among the six derivatives tested, the increases in NO production and iNOS expression observed in BV-2 microglial cells after LPS stimulation were significantly inhibited by treatment with 16α,17α-epoxypregnenolone-20-oxime. Moreover, the inhibitory effect of 16α,17α-epoxypregnenolone-20-oxime on NO production was similar to that of S-methylisothiourea sulfate (SMT), an iNOS inhibitor. Further studies showed that 16α,17α-epoxypregnenolone-20-oxime inhibited c-Jun N-terminal kinase (JNK) phosphorylation but not inhibitor kappa B (IκB)-α degradation. Our data in LPS-stimulated microglia cells suggest that 16α,17α-epoxypregnenolone-20-oxime might be a candidate therapeutic for treatment of NO induced neuroinflammation and could be a novel iNOS inhibitor.

Key words: microglia; nitric oxide; neuroinflammation

Microglial cells, the tissue macrophages of the central nervous system (CNS), play a crucial role in the recognition and phagocytic removal of apoptotic neurons. Microglial cell activation is regarded as a marker of brain injury development in many neuronal diseases, such as Alzheimer’s disease (AD) and Parkinson’s disease (PD).

Neuronal inflammation also accompanies neuron degeneration in these diseases. During neuroinflammation, microglia are activated and secrete various cytokines. Microglial cells respond to inflammatory agonists, such as bacterial lipopolysaccharide (LPS), the human immunodeficiency virus (HIV) coat protein gp120, and β-amyloid-related peptides, by producing many inflammatory factors, such as tumor necrosis factor-alpha (TNF-α), interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β) and nitric oxide (NO). These microglial products are thought to be responsible for glia-mediated neurotoxicity.

The free radical NO, a gaseous molecule produced by activated microglia, has many physiological functions, including neuroinflammation. In the brain, NO serves as a neurotransmitter and a second messenger molecule mediating a variety of neuronal functions. Deletion of Osteopontin (OPN) in mice increased thalamic neurodegeneration through regulation inducible nitric oxide synthase (iNOS) expression, an enzyme that produces NO. The interactions of NO are not restricted to a single receptor, it can react with other inorganic molecules (such as oxygen, superoxide or transition metals), structures in DNA (pyrimidine bases), prosthetic groups (such as heme) and proteins (leading to S-nitrosylation of thiol groups, nitration of tyrosine residues or disruption of metal–sulfide clusters such as zinc-finger domains and iron–sulfide complexes). In addition, a recent report demonstrated selective up-regulation of iNOS and cytokines in microglial cells and cytokine stimulation in rat striatum after exposure to LPS. Thus, the molecular pathways controlling the activity of iNOS may be critical in limiting neurotoxicity and its consequences, delaying the progression of neurodegenerative diseases and neuroinflammation in the CNS.

16-Dehydropregnenolone-3-acetate, commonly known as 16-DPA, is the main intermediates for hormone and steroids related drugs. After processing, the 16-DPA can be made into hydrocortisone, prednisone, fluocinolone, hexadecadrol, which affects glycometabolism, and have benefits of anti-inflammation, anti-toxic, anti-shock, anti-allergic, however, the effect of 16-dehydropregnenolone-3-acetate and its derivatives on NO production and iNOS expression in BV-2 microglial cells were determined their mechanism of action. Our findings demonstrate that, 16α,17α-epoxypregnenolone-20-oxime significantly inhibits LPS-induced NO secretion and iNOS expression by inhibiting the JNK signal pathway, without depressing the nuclear factor-kappa B (NF-κB) signaling pathway.

MATERIALS AND METHODS

Chemicals: LPS (from Escherichia coli serotype 0111:B4) from Sigma (St. Louis, MO, U.S.A.), and the iNOS inhibi-
tor S-methylisothioureia sulfate (SMT) were obtained from CALBIOCHEM, 16-dehydropregnenolone-3-acetate derivatives were kindly provided by Professor Wen-Zhong Ge (Heilongjiang Bayi Agricultural University, China).

**Cell Culture**  BV-2 microglial cells, which are immortalized microglial cells, were propagated in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) that contained 10% fetal bovine serum (FBS; HyClone, Logan, UT, U.S.A.) and antibiotics. Exponentially growing BV-2 microglial cells were pre-treated with 10 µg/mL of 16-dehydropregnenolone-3-acetate derivatives, followed by treatment with 1 µg/mL of LPS. The BV-2 microglial cells were kindly provided by Professor Dong-Seok Lee (Kyungpook National University, Korea).

**Biochemical Assay for the Production of NO**  NO production was assessed based on the accumulation of nitrite in the medium using a colorimetric reaction with Griess reagent. Briefly, the culture supernatants were collected and mixed with an equal volume of Griess reagent (0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride, 0.1% sulfanilamide, and 2.5% H₃PO₄). The absorbance at 540 nm was measured with a UV MAX kinetic microplate reader (Molecular Devices, Menlo Park, CA, U.S.A.).

**Western Blotting Analysis**  Protein lysates (30 µg) were separated on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, U.S.A.). The membranes were blotted with antibodies against inhibitor kappa B (IκB)-α (Santa Cruz), iNOS (upstate), phospho-extracellular signal-regulated kinase (pERK), phospho-c-Jun N-terminal kinase (pJNK) (Santa Cruz), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma) at 4°C overnight. The membranes were washed five times with 10 mM Tris–HCl containing 150 mM NaCl (Tris-buffered saline, TBS; pH 7.5) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Sigma) or antimouse IgG (sigma) for 1 h at room temperature (RT). After the removal of excess antibodies by washing with TBS, specific binding was detected using a chemiluminescence detection system (Amersham, Berkshire, U.K.) according to the manufacturer’s instructions.

**Measurement of Phagocytosis and Reactive Oxygen Species (ROS) by Flow Cytometry**  Microglial phagocytosis was analyzed using flow cytometry according to a previously reported method. Briefly, Alexa 488-conjugated Escherichia coli (Ec-A) BioParticles (Invitrogen) were sonicated, added to culture medium without serum at the end of the LPS treatment, and incubated at 37°C in 5% CO₂ for 15 min. After incubation, the cells were washed three times with phosphate buffered saline (PBS) and suspended in 500 µL of PBS. Immediately after suspension, the internalized fluorescence from 10000 cells was determined by FACSscan (BD). To determine the ROS level in microglia, BV-2 cells were incubated with 10 mM CM-H₂DCFDA (Invitrogen), a fluorescence-based ROS indicator, at 37°C for 15 min at the end of the different treatments; DCF fluorescence intensities from 10000 cells were analyzed by FACSscan.

**Statistical Analysis**  Differences between experimental groups were tested for statistical significance using ANOVA. The p values <0.01 were deemed to be statistically significant.

**RESULTS**

**Synthesis of 16-Dehydropregnenolone-3-acetate Derivatives**  Serial derivatives were synthesized from the lead compound 16-dehydropregnenolone-3-acetate (1). Compound 1 was hydrolyzed in KOH to obtain 16-dehydropregnenolone.

Fig. 1.  The Structures of the Compounds Synthesized from 16-Dehydropregnenolone-3-acetate

The structures of the serial derivatives of 16-dehydropregnenolone-3-acetate are shown.
(compound 2), and then oxidized by H₂O₂ in KOH solution yielding 3β-hydroxy-16α,17α-epoxypregn-5-ene (compound 3), flowing oximation of compound 3 resulted in the formation of 16α,17α-epoxypregnenolone-20-oxime (compound 4). We obtained 5,16-pregnadien-3-ol-20-oxime (compound 5) and pregnenolone-20-oxime (compound 7) by oximation from 16-dehydroepiandrosterone (compound 2) and pregnenolone (compound 6), respectively. The serial compounds have similar structures with differences in their side chains (Fig. 1).

**Inhibitory Effects of 16α,17α-Epoxypregnenolone-20-oxime (#4) on LPS-Induced NO Secretion in BV2 Microglia Cells** LPS is a common inflammogen that has been used to activate microglia in several mammalian model systems. As expected, LPS significantly increased NO production, ROS levels and phagocytosis in BV-2 microglial cells (Figs. 2A–C). The inhibitory activity of the six compounds on LPS-induced NO production in BV-2 microglial cells was examined by pre-treating with each compound for 30 min and then treating with LPS (1 µg/mL) for 24 h. Results showed that compounds #1, #3, #4, and #5 significantly inhibited NO production, whereas compounds #2 and #7 had no inhibitory effect (Fig. 2C). Moreover, compound #4 exhibited a greater inhibitory effect on LPS-induced NO production than compounds #1, #3, and #5 (Fig. 2C), with NO secretion almost reduced to basal levels. To investigate whether the compound could affect the cell viability, BV-2 microglial cells were treated with compound #4 without LPS stimulation, and the cell cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Fig. 2D). The results shown compound #4 have not affected on the cell viability.

**16α,17α-Epoxypregnenolone-20-oxime (#4) Dose-Dependently Inhibited LPS-Induced NO Production and iNOS Expression via Inhibition of the JNK Signaling Pathway** Our results showed that compound #4 significantly inhibited NO production in LPS-stimulated BV-2 microglial cells (Fig. 2D). Consequently, the concentration dependence and time course of the inhibitory activity of compound #4 on LPS-induced NO production and iNOS expressions were examined. BV-2 microglial cells were pre-treated with compound #4 at various concentrations with subsequent LPS treatment for 24 h. The results showed that the inhibitory effect of compound #4 on LPS-induced NO production and iNOS expression was dose-dependent (Fig. 3A). To understand the time course of the inhibitory effect of compound #4 on NO secretion, BV-2 microglial cells were treated with LPS (1 µg/mL) for different times (0–24 h). The results showed that the inhibitory activity of compound #4 on LPS-induced NO production increased with the increase in treatment time (Fig. 3B). Furthermore, the concentration dependence and time course of the inhibitory activity of compound #4 on LPS-induced NO production and iNOS expression were examined. BV-2 microglial cells were pre-treated with compound #4 at various concentrations with subsequent LPS treatment for 24 h. The results showed that the inhibitory effect of compound #4 on LPS-induced NO production and iNOS expression was dose-dependent (Fig. 3C). To understand the time course of the inhibitory effect of compound #4 on NO secretion, BV-2 microglial cells were treated with LPS (1 µg/mL) for different times (0–24 h). The results showed that the inhibitory activity of compound #4 on LPS-induced NO production increased with the increase in treatment time (Fig. 3D).
microglial cells were pre-treated with the compound (10µg/mL) followed by LPS stimulation for the times indicated. Treatment with compound #4 significantly inhibited NO production at 12h and 24h (Fig. 3B), and iNOS expression was also decreased (Fig. 3C). Our previous results indicated that LPS could increase microglial ROS production and phagocytosis.15) Therefore, we examined the effect of compound #4 on LPS-induced microglial ROS production and phagocytosis. BV-2 microglial cells were pre-treated with compound #4 for 30min, followed by LPS stimulation for 24h. The results showed that the LPS-induced ROS production and phagocytosis were significantly attenuated by compound #4 treatments (Fig. 3D). To evaluate the inhibitory effect of compound #4 on LPS-induced NO secretion and iNOS expression, we compared its inhibitory activity with SMT, a selective inhibitor of iNOS (Fig. 3E). The result showed that the inhibitory activity of compound #4 on LPS-induced microglial iNOS expression was similar to that of SMT, suggesting that compound #4 may

Fig. 3. Dose-Dependent Inhibition of LPS-Induced NO Production and iNOS Expression by 16α,17α-Epoxyprogrenolone-20-oxime (Compound #4) Is Mediated by Inhibition the JNK Signaling Pathway

(A) Cells were pre-treated with various concentrations of compound #4 (0.1, 1, 10µg/mL) for 30min followed by LPS (1µg/mL) stimulation for 24h, and NO production (lower panel) and iNOS expression (upper panel) were detected using Griess reagent and Western blotting. (B, C) BV-2 microglial cells were pre-treated with compound #4 (10µg/mL) for 30min, followed by LPS stimulation, before the intracellular ROS (upper panel) and phagocytosis (lower panel) were analyzed by FACS. (E) BV-2 microglial cells were pretreated with compound #4 (10µg/mL) and SMT (1mM; a selective inhibitor of iNOS) for 30min, followed by treatment with LPS (1µg/mL). The NO production (upper panel) and iNOS expression (lower panel) were analyzed by Griess reagent and Western blotting. (F) IkB-α degradation and the phosphorylation of JNK and ERK were assessed by Western blot. Three independent replicates were performed for all the experiments. **p≤0.01; ***p≤0.001.
be a candidate of inhibitor for iNOS, but its effect on iNOS enzyme activity should be further studies.

The JNK signaling pathway has been reported to affect LPS-induced NO production and iNOS expression.\textsuperscript{16,17} To understand the mechanism of action for compound #4’s inhibition of LPS-induced NO production and iNOS expression, we examined the inhibitory activity of compound #4 on LPS-stimulated MAPK (JNK, ERK) phosphorylation and IкB-α degradation. As Fig. 3F shows, LPS-induced JNK phosphorylation in the BV-2 microglial cells was significantly reduced by treatment with compound #4, whereas IкB-α degradation and ERK phosphorylation were not. Furthermore, our previous result shown that LPS induced NO production dependent on ROS/JNK cascade in BV-2 microglial cells.\textsuperscript{16,18} So, we also measured the NO production by treatment with NAC (ROS scavenger) and SP600125 (JNK inhibitor) (Fig. 4A). Both of reagents significantly reduced the NO production. In addition, NAC treatment significantly inhibits the JNK phosphorylation (Fig. 4B). These results indicate that the inhibitory effect of compound #4 on NO secretion and iNOS expression in BV-2 microglial cells was through inhibition of the JNK phosphorylation.

**DISCUSSION**

Microglial cells, as the major tissue macrophages of the CNS, play a crucial role in recognition and phagocytic removal of apoptotic neurons. In the present study, we evaluated the inhibitory effect of serial derivatives synthesized from the lead compound 16-dehydropregnenolone-3-acetate on NO production and iNOS expression in LPS-induced BV2 microglial cells. Our results show that compound #4 exhibited the strongest inhibitory effect on LPS-induced NO production in LPS-stimulated microglia among the six derivatives (Fig. 2C). Therefore, we focused on understanding the mechanism of action by which compound #4 regulates NO production and iNOS expression.

LPS is commonly used as an inflammogen to activate microglia in several mammalian model systems. LPS stimulation triggers diverse microglial responses, including inflammatory mediator production, phagocytosis, and ROS production, which entail activation of various signaling cascades through Nox and mitogen-activated protein kinases (MAPKs) in microglial cells.\textsuperscript{18,19} To understand the influence of compound #4 on the kinetics of NO production and iNOS expression in LPS-stimulated microglia, BV-2 microglial cells were treated with different concentrations of compound #4 at different times before LPS stimulation. We found that compound #4 significantly inhibited microglial NO production and iNOS expression in a dose-dependent manner (Figs. 3A–C) and the extent of this inhibition was similar to that of SMT (Fig. 3E). Therefore, we suggesting that compound #4 may be a candidate of inhibitor for iNOS, but it’s effect on iNOS enzyme activity should be further studies.

ROS act as signaling molecules and play a crucial role in microglial activation and in the progression of the neurodegenerative diseases, such as AD and PD.\textsuperscript{20} In our previous report, the microglial phagocytosis was inhibited by the cellular ROS level decrease, through regulation of the Nox/phosphatidyl inositol 3-kinase (PI3K)/P38 signaling pathways.\textsuperscript{15} To investigate whether compound #4 could prevent microglial ROS elevation, result in inhibit the microglial phagocytosis, BV-2 microglial cells were treated with compound #4 prior to LPS stimulation. The results shown that compound #4 significantly reduced LPS-stimulated microglial ROS production, and then inhibited the microglial phagocytosis (Fig. 3D), suggesting that ROS dependent signaling pathways are most likely responsible for the inhibitory effects described above. ROS generated from NADPH oxidase is important in the early activation of microglia.\textsuperscript{19} In the present study, treatment the BV-2 cells with compound #4 inhibit the cellular ROS below basal level (Supplementary 1). It may be correlated with the NADPH oxidase enzyme activity and reduce the superoxide production, consequently, reduced the cellular ROS levels and signaling mechanism studies should be further processed.

Protein kinases in the MAPK family are highly conserved in eukaryotes and play important roles in LPS-induced activation of pro-inflammatory products and neuroinflammation.\textsuperscript{21–23} Our previous study showed that JNK signaling plays a major role in the regulation of LPS-stimulated NO produc-
tion and iNOS expression, and regulatory effect of the JNK signaling pathway on NO production was dependent on cellular ROS. In the present study, compound #4 treatment significantly inhibited the NO production and iNOS expression, as well as ROS. Our results shown, the mechanisms of compound #4 upon these phenomena were through ROS dependent JNK pathways not NF-κB (Fig. 3F). Interestingly, NF-κB dependent cytokines IL-6 was also inhibited by compound #4 treatment but not TNF-α (data not shown). The mechanism of the difference effect of compound #4 on TNF-α and IL-6 productions studies are processing to explain it.

Expression of iNOS is also induced by interferon-beta (IFN-β) via activation of the JAK/signaling transducer and activation of transcription (STAT) signaling pathway. The regulation of cytokine production by TLR4 is complicated by activation of transcription (STAT) signaling pathway. The αβ-oxidation as well as ROS. Our results shown, the mechanisms of compound #4 on Jak/Stat signaling has not been verified in this study.

Taken together, our results showed that compound #4, synthesized from 16-dehydropregnenolone-3-acetate, significantly inhibited NO production and iNOS expression in LPS-stimulated BV-2 microglial cells. Moreover, compound #4 showed great potential as a candidate inhibitor of NO production stimulated by LPS and is a promising candidate for the treatment of neuroinflammatory disease.

Acknowledgments

Financial support for the present study was provided by the Scientific Research Foundation of Heilongjiang Provincial Education Department of China (1251H010) and (12521384).

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