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Anti-Inflammatory Effects of Isopropyl 3-(3, 4-Dihydroxyphenyl)-2-Hydroxypropanoate, a Novel Metabolite from Danshen, on Activated Microglia

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Abstract

Down-regulation of microglial activation represents a practical strategy for combating diverse brain disorders such as stroke and neurodegenerative diseases. In the present study, we showed evidence that isopropyl 3-(3, 4-dihydroxyphenyl)-2-hydroxypropanoate (IDHP), a new bioactive metabolite of Danshen (*Salvia miltiorrhiza Bunge*), exerted an anti-inflammatory effect in lipopolysaccharide (LPS)-induced microglia. Our data showed that IDHP significantly reduced the production of nitric oxide (NO), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in LPS-induced BV-2 cells and rat primary microglia in a dose-dependent manner. IDHP also suppressed mRNA expression of LPS-induced inducible nitric oxide synthase (iNOS), TNF-α and IL-1β. Moreover, IDHP significantly suppressed the production of reactive oxygen species (ROS), nuclear factor κB (NF-κB) translocation and DNA binding activity induced by LPS treatment in BV-2 cells. These findings indicated that IDHP might be of value in the treatment of various microglia-mediated neuroinflammatory diseases.

Key Words: isopropyl 3-(3, 4-dihydroxyphenyl)-2-hydroxypropanoate, microglia, neuroprotection, inflammation

Introduction

Microglia are resident innate immune cells in the central nervous system (CNS). Microglia-mediated inflammation is an important contributor to neuronal damage in diverse brain diseases such as stroke, Parkinson's disease (PD), Alzheimer's disease (AD) and multiple sclerosis (reviewed in 5, 10). Under pathological processes, microglia become readily activated in response to brain injuries and activated

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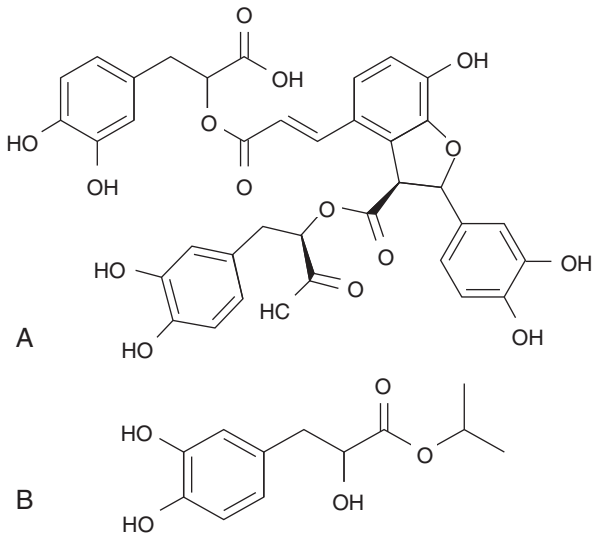

Fig. 1. Chemical structure of Salvianolic acid B (A) and IDHP (B).

microglia are capable of releasing a variety of pro-inflammatory factors, such as nitric oxide (NO), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) which are potentially cytotoxic to neurons. Thus, down-regulation of microglial activation represents a practical strategy for combating these brain disorders (5, 10).

Danshen (*Salvia miltiorrhiza Bunge*), one of the most famous traditional Chinese medicines, is widely used for the treatment of cardiovascular and cerebrovascular diseases in the oriental. A lot of studies have been done to elucidate its chemical constituents and pharmacological activities and dozens of bioactive constituents of danshen have been identified, as reviewed by Zhou *et al*. (17). Our previous study has demonstrated that Salvianolic acid B (Fig. 1A), a major active component of Danshen, exerts an anti-inflammatory effect in lipopolysaccharide (LPS)-induced microglia and provides neuroprotection by regulating microglial activation *in vitro* (12). In the present study, we explored the role of isopropyl 3-(3, 4-dihydroxyphenyl)-2-hydroxypropanoate (IDHP, Fig. 1B), a new bioactive metabolite of Salvianolic acid B in rat brain after oral administration reported in a metabolomics study (16), in modulating microglial activation. The results showed that IDHP also exhibited a strong anti-inflammatory effect in rat primary microglia and in the BV-2 mouse microglial cell line induced by LPS.

Materials and Methods

Reagents

IDHP (purity > 99.0%) was synthesized and verified by elemental analysis, mass spectrometry, infrared spectroscopy and nuclear magnetic resonance spectroscopy as previously described (11). IDHP was dissolved in dimethyl sulfoxide (DMSO). *Escherichia coli* 0111:B4 endotoxin (LPS) and DCFH-DA were obtained from Sigma (St. Louis, MO, USA). All cell culture reagents and Trizol reagent were obtained from Invitrogen (Carlsbad, CA, USA). Cytokine enzyme-linked immunosorbent assay (ELISA) kits were obtained from R&D Systems (Minneapolis, MN, USA). Rabbit anti-NF-κB p65 and goat anti-mouse IgG antibodies were purchased from Cell Signaling (Beverly, MA, USA). TaqMan Reverse Transcription Reagents and SYBR Green PCR Master Mix reagent kit were obtained from Applied Biosystems (Foster City, CA, USA). Nuclear Extraction kit and NF-κB p65 EZ-TFA transcription factor assay kit were the products of Millipore (Billerica, MA, USA).

Cell Culture

Primary microglial cell cultures were prepared from 1-day-old Wistar rats as previously described (12). The purity of microglia was >95% as determined by Iba-1 immunocytochemical staining (data not shown). BV-2 mouse microglial cells were cultured in a Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics.

Cell Viability

BV-2 cells and primary microglial cells were incubated with IDHP at various concentrations for 24 h in serum-free DMEM. Cell viability was measured by the MTT assay.

Determination of Nitrite and Cytokine Levels

To determine nitrite and cytokine levels, BV-2 cells and primary microglial cells were pre-incubated with or without IDHP at various concentrations for 30 min before being stimulated by LPS (BV-2, 0.1 μg/ml; primary microglia, 1 μg/ml) for 24 h in serum-free DMEM. Nitrite levels in the supernatants were determined using the Griess method and the absorbance was read at 550 nm on a fluorescence plate reader (Molecular Device, Flaxstation 3; Sunnyvale, CA, USA). Levels of TNF-α and IL-1β in the supernatants were determined using ELISA kits according to the manufacturer's instructions.

Real-Time Quantitative PCR

For real-time quantitative PCR, BV-2 cells were pretreated with IDHP at 25 μM and were then stim-

ulated by LPS at 0.1 μg/ml for 8 h. Total RNA was extracted from BV-2 cells using Trizol reagent. Reverse transcription (RT) was performed with TaqMan Reverse Transcription Reagents. To quantify inducible nitric oxide synthase (iNOS), TNF-α and IL-1β mRNA expression levels in BV-2 cells, a real-time PCR assay was done using SYBR Green PCR Master Mix reagent kits and the specific primers as follows: GAPDH forward: CTTCACCACCATGGAGA AGGC, reverse: GGCATGGACTGTGGTCATGAG; iNOS forward: GGCAGCCTGTGAGACCTTTG, reverse: GCATTGGAAGTGAAGCGTTTC; TNF-α forward: CGGGGTGATCGGTCCCCAAAG, reverse: GGAGGGCGTTGGCGCGCTGG; IL-1β forward: CGCAGCAGCACATCAACAAGAGC, reverse: TGTCCTCATCCTGGAAGGTCCACG.

Intracellular Reactive Oxygen Species (ROS) Detection

The production of intracellular ROS was measured by DCFH oxidation. BV-2 cells were stimulated by 0.1 μg/ml LPS and/or IDHP at 0.25, 2.5 or 25 μM for 4 h. Following treatment, cells were rinsed with HBSS and 10 μM DCF-DA fluorescent probe was loaded. After 30 min of incubation followed by washing, DCF fluorescence was monitored using a fluorescence plate reader with an excitation wavelength of 492 nm and an emission wavelength of 535 nm.

Immunofluorescence

To determine the effect of IDHP on nuclear translocation of NF-κB from the cytoplasm, immunolabeling of the NF-κB p65 subunit was performed. BV-2 cells were pretreated with 25 μM IDHP and stimulated by 0.1 μg/ml LPS for 45 min. Immunofluorescence was performed with a rabbit anti-NF-κB p65 subunit antibody and goat anti-mouse IgG conjugated with FITC. Samples were also counterstained with the nuclear dye DAPI. The cells were visualized with a laser scanning confocal microscope (Zeiss LSM710; Germany).

NF-κB DNA-Binding Assay

BV-2 cells were treated with LPS in the presence or absence of 25 μM IDHP for 1 h. Nuclear proteins were then extracted and nuclear NF-κB p65 binding to a consensus oligonucleotide was analyzed with a NF-κB p65 EZ-TFA transcription factor assay kit according to the manufacturer's guidelines.

Data Analysis

Data are expressed as means ± SD. Statistical significance was estimated using the non-parametric Mann-Whitney test with SPSS software. In all cases, differences were considered significant at $P < 0.05$.

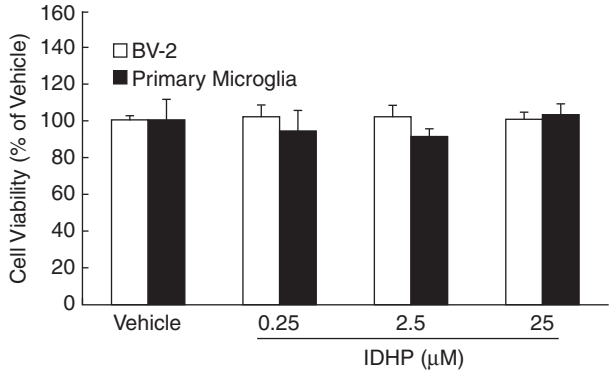

Fig. 2. IDHP does not affect cell viability in both BV-2 cells and primary microglia. BV-2 cells and primary microglial cells were incubated with IDHP at various concentrations for 24 h in serum-free DMEM. Cell viability was measured by MTT assay. The data are expressed as means ± SD, n = 6. Similar results were obtained in at least 3 independent experiments.

Results

IDHP Suppresses LPS-Induced Pro-Inflammatory Factors Production without Cytotoxicity

First, the effects of IDHP on microglial cell viability were measured. The results showed that IDHP alone did not affect cell viability in both BV-2 cells and rat primary microglia as measured by MTT assay (Fig. 2). The effects of IDHP on the production of pro-inflammatory factors (NO, TNF-α and IL-1β) in LPS-stimulated microglia were then examined. LPS dramatically increased NO, TNF-α and IL-1β levels in BV-2 cells compared with the vehicle group with a final concentration of 0.1% DMSO (Fig. 3A). In contrast, IDHP treatment significantly reduced the production of these proinflammatory factors in a concentration-dependent manner (Fig. 3A). In rat primary microglia cultures, a similar pattern of NO and cytokine production and IDHP inhibition was observed (Fig. 3B), confirming the results obtained with the BV-2 cells.

IDHP Suppresses mRNA Expression of LPS-Induced Pro-Inflammatory Factors

To determine whether the decrease in LPS-induced NO, TNF-α and IL-1β measured in supernatants was associated with a decrease in transcription levels, the mRNA expression levels of iNOS, TNF-α and IL-1β were determined. Results showed that pretreatment of BV-2 cells for 30 min with IDHP sig-

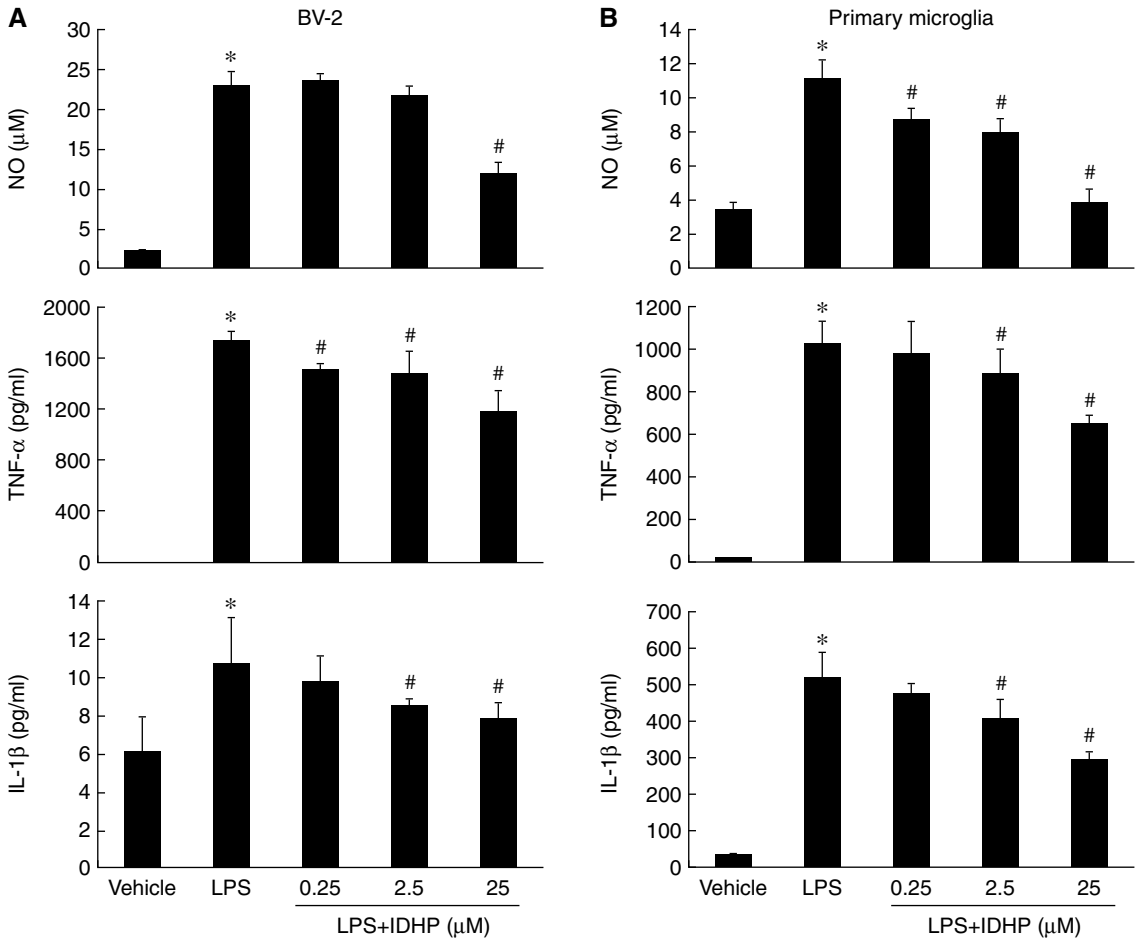

Fig. 3. IDHP suppresses LPS-induced pro-inflammatory factors production in BV-2 cells (A) and primary microglial cells (B). BV-2 cells and primary microglial cells were pre-incubated with or without IDHP at various concentrations for 30 min before being stimulated by LPS (BV-2, 0.1 μg/ml; primary microglia, 1 μg/ml) for 24 h in serum-free DMEM. NO, TNF-α and IL-1β levels in the supernatants were determined using the Griess method or ELISA. The data are expressed as means ± SD, n = 6. $^{*}P < 0.05$, significantly different from the vehicle group. $^{\#}P < 0.05$, significantly different from the LPS-treated alone. Similar results were obtained in at least 3 independent experiments.

nificantly suppressed the LPS-induced increase of TNF-α and IL-1β mRNA (Fig. 4).

IDHP Reduces LPS-Induced ROS Production

Intracellular ROS are critical for LPS-induced neurotoxicity and are components of a signaling pathway regulating proinflammatory gene expression in microglia (7). The results showed that LPS significantly increased ROS in BV-2 cells compared with the vehicle group (Fig. 5). In contrast, pretreatment with IDHP decreased LPS-induced ROS production in a dose-dependent manner.

IDHP Inhibits LPS-Induced NF-κB Translocation and DNA Binding Activity

As the NF-κB is a major regulator of expression of proinflammatory mediators in LPS-stimulated microglia, the effects of IDHP in NF-κB nuclear translocation and DNA binding activity were investigated. Pretreatment of cells with IDHP clearly decreased LPS-induced translocation of NF-κB p65 subunit to the nucleus (Fig. 6A). DNA binding activity of NF-κB was determined in the nuclear extract by an ELISA-based kit. The results showed that LPS-induced DNA binding activity was significantly inhibited by preincubating cells with IDHP prior to LPS stimulation (Fig. 6B).

Discussion

IDHP, a new metabolite from Danshen, has noticeable biological activities. Previous studies conducted by our group have shown that IDHP can exert a vasorelaxant effect by inhibiting both Ca^{2+} release

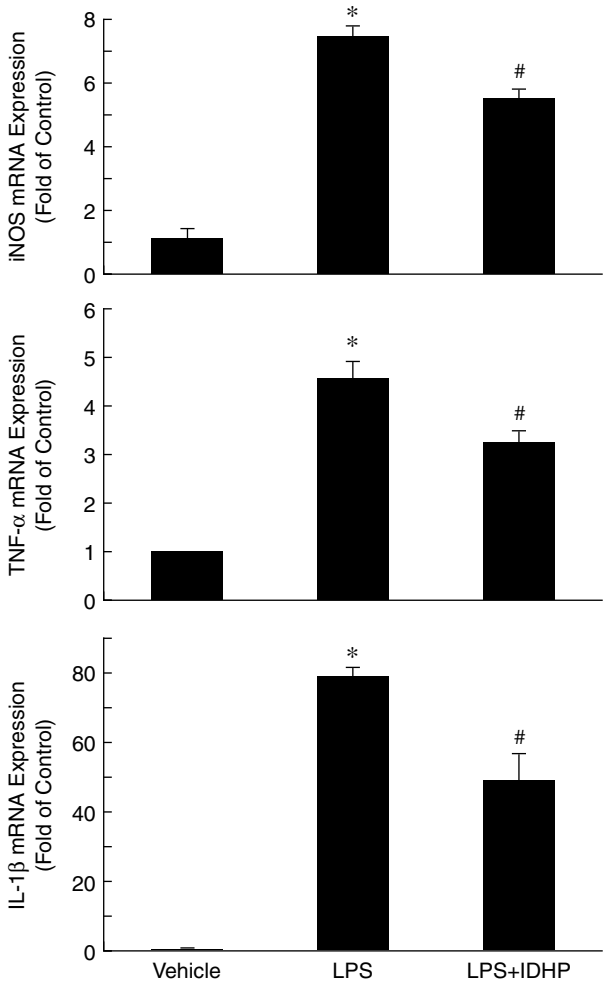

Fig. 4. IDHP decreases mRNA levels of LPS-induced iNOS, TNF-α and IL-1β. BV-2 cells were pretreated with 25 μM IDHP and were then stimulated by 0.1 μg/ml LPS for 8 h. mRNA levels of iNOS, TNF-α, IL-1β were analyzed by RT-PCR. The data are expressed as means ± SD, n = 3. $^{*}P < 0.05$, significantly different from the vehicle group. $^{\#}P < 0.05$, significantly different from the LPS-treated alone. Similar results were obtained in at least 3 independent experiments.

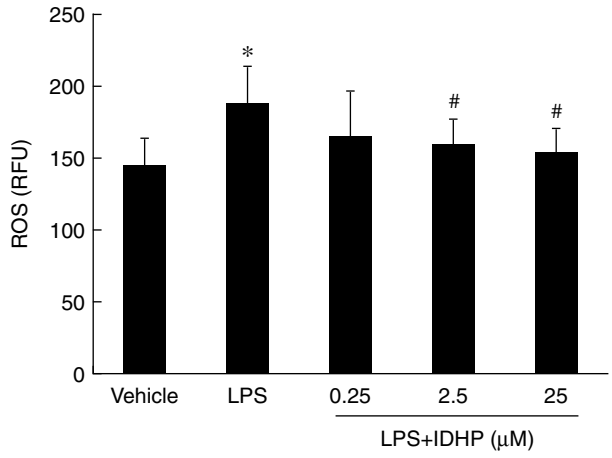

Fig. 5. IDHP reduces LPS-induced ROS production. BV-2 cells were stimulated by 0.1 μg/ml LPS and/or 25 μM IDHP for 4 h. Following treatment, cells were rinsed with HBSS and 10 μM DCF-DA fluorescent probe was loaded. After 30 min incubation followed by washing, DCF fluorescence was monitored using a fluorescence plate reader with an excitation wavelength of 492 nm and an emission wavelength of 535 nm. The data are expressed as means ± SD, n = 6. $^{*}P < 0.05$, significantly different from the vehicle group. $^{\#}P < 0.05$, significantly different from LPS treatment alone. Similar results were obtained in at least 3 independent experiments.

from intracellular stores and Ca^{2+} influx through voltage-dependent calcium channels and receptor-operated calcium channels in vascular smooth muscle cells (13). IDHP also has the protective potential against cerebral ischemia injury due to the amelioration of cerebral energy metabolism and its antioxidant property (11). In the present study, our data further showed that IDHP significantly reduced the production of NO, TNF-α and IL-1β in LPS-induced BV-2 cells and rat primary microglia in a dose-dependent manner. IDHP also suppressed mRNA expression of LPS-induced inducible iNOS, TNF-α and IL-1β. Moreover, IDHP significantly suppressed the production of ROS and NF-κB activation induced by LPS treatment in BV-2 cells.

NO has complicated pathophysiological functions. NO could induce vasorelaxation (14) and increased NO level could inhibit neutrophilendothelium interactions (4). It also could elicit intestinal low vascular reactivity (1). And NO from activated microglia plays an important role in neuroinflammation (5). Other proinflammatory and neurotoxic factors secreted by activated microglia include the cytokines TNF-α and IL-1β, superoxide, Matrix Metallopeptidase 9, prostaglandin E2, chemokines Monocyte Chemotactic Protein 1 and Macrophage Inflammatory Protein 1α. Individual factors often work in concert to induce neurodegeneration, damages to blood-brain barrier (BBB) integrity and leukocytes infiltration (5, 10). There has been some success in clinical studies investigating the effects of anti-inflammatory therapy against cognitive decline in AD patients (6). Researches in several animal models on stroke (3, 15), AD (8) and PD (2) also showed that inhibition of the microglial over-reaction and the inflammatory processes represent a therapeutic target to alleviate these diseases. Inhibition of LPS-activated BV-2 mouse microglial cells as well as rat primary microglia cultures demonstrated in this study suggested that the anti-inflammatory activity of IDHP in microglia, at least in part, contributed to neuroprotection against cerebral ischemia (11), and IDHP may be a new po-

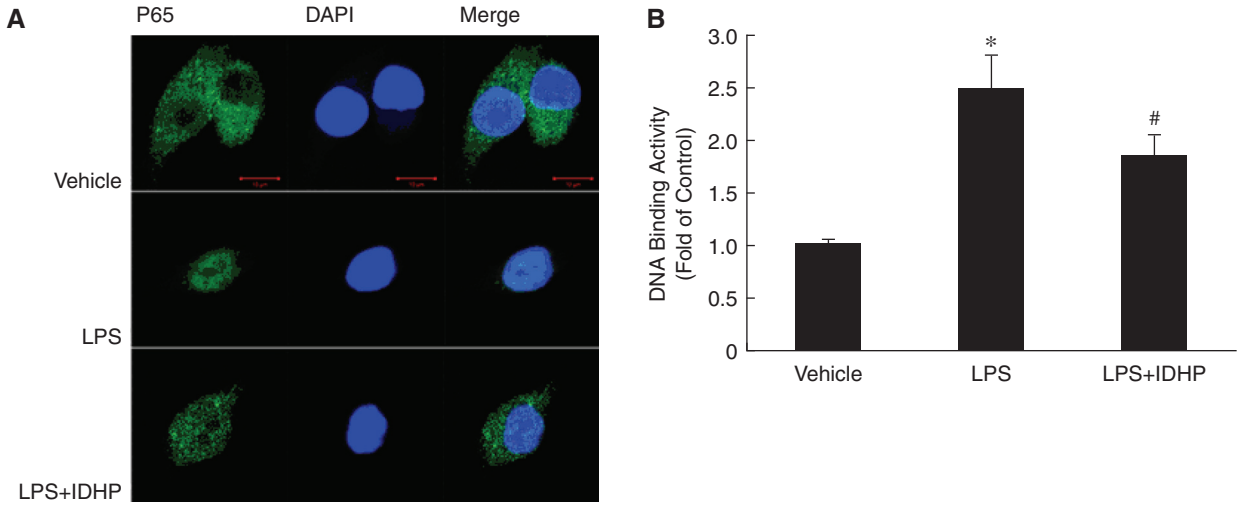

Fig. 6. IDHP inhibits LPS-induced NF-κB translocation (A) and DNA binding activity (B). (A) BV-2 cells were pretreated with 25 μM IDHP and stimulated by 0.1 μg/ml LPS for 45 min. Immunolabeling was performed with a rabbit anti-NF-κB p65 subunit antibody and goat anti-mouse IgG conjugated with FITC. Samples were also counterstained with the nuclear dye DAPI. The cells were visualized with a laser scanning confocal microscope. Bar = 10 μm. (B) BV-2 cells were treated with LPS in the presence or absence of 25 μM IDHP for 60 min, then nuclear proteins were extracted and nuclear NF-κB p65 binding to a consensus oligonucleotide was analyzed with the NF-κB p65 EZ-TFA transcription factor assay kit. The data are expressed as means ± SD, n = 3. $^{*}P < 0.01$, significantly different from the vehicle group. $^{\#}P < 0.01$, significantly different from the LPS-treated alone. Similar results were obtained in at least 3 independent experiments.

tential drug for treatment of other microglia-related neurodegenerative diseases.

The precursors of IDHP include Danshensu, Salvianolic acid A and Salvianolic acid B (16). The results that both IDHP and Salvianolic acid B (12) had anti-inflammatory activities in activated microglia remind us that if Danshensu and Salvianolic acid A have similar activity and the structure-activity relationship of these components should be further studied. Furthermore, it remains obscure whether IDHP could exert the similar anti-inflammatory effects in other CNS cells, for example, the endothelial cells. And the underlying anti-inflammatory mechanisms IDHP in microglial cells, such as NF-κB and its upstream mitogen activated protein kinase signal transduction pathways, are far from clear, and needs further investigation.

When compared with its precursors, IDHP has a smaller molecular mass and less number of hydrogen bonding donors and acceptors, consistent with the criteria of CNS compounds (9). It has also been proved that IDHP could protect against cerebral ischemia injury *in vivo* (11). These observations suggest that IDPH may penetrate the BBB.

In conclusion, we present evidence that IDHP, a new metabolite of Danshen, exerted an anti-inflammatory effect in LPS-induced microglia. These findings indicated that IDHP might be of value in the treatment of various microglia-mediated neuroinflammatory diseases.

Acknowledgments

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References

1. Chen, C.F., Lee, J.F., Wang, G.J., Wang, D. and Chen, H.I. Involvement of nitric oxide in mesenteric vascular reactivity following intraperitoneal pancreatic juice in rats. *Chinese J. Physiol.* 54: 419-426, 2011.
2. Du, Y., Ma, Z., Lin, S., Dodel, R.C., Gao, F., Bales, K.R., Triarhou, L.C., Chernet, E., Perry, K.W., Nelson, D.L.G., Luecke, S., Phebus, L.A., Bymaster, F.P. and Paul, S.M. Minocycline prevents nigrostriatal dopaminergic neurodegeneration in the MPTP model of Parkinson's disease. *Proc. Natl. Acad. Sci. USA* 98: 14669-14674, 2001.
3. Kim, H.J., Rowe, M., Ren, M., Hong, J.S., Chen, P.S. and Chuang, D.M. Histone deacetylase inhibitors exhibit anti-inflammatory and neuroprotective effects in a rat permanent ischemic model of stroke: multiple mechanisms of action. *J. Pharmacol. Exp. Ther.* 321: 892-901, 2007.
4. Krauss, H., Sosnowski, P., Biczysko, M., Biczysko, W., Majewski, P., Jablecka, A., Miskowiak, B., Smolarek, I., Konwerska, A., Ignys, I. and Micker, M. Effects of L-arginine and NG-nitro L-arginine methyl ester (L-NAME) on ischemia/reperfusion injury of skeletal muscle, small and large intestines. *Chinese J. Physiol.* 54: 7-18, 2011.

5. Neumann, H., Kotter, M.R. and Franklin, R.J.M. Debris clearance by microglia: an essential link between degeneration and regeneration. *Brain* 132: 288-295, 2009.
6. Perry, N.S., Bollen, C., Perry, E.K. and Ballard, C. Salvia for dementia therapy: review of pharmacological activity and pilot tolerability clinical trial. *Pharmacol. Biochem. Behav*. 75: 651-659, 2003.
7. Qin, L., Liu, Y., Wang, T., Wei, S.J., Block, M.L., Wilson, B., Liu, B. and Hong, J.S. NADPH oxidase mediates lipopolysaccharide-induced neurotoxicity and proinflammatory gene expression in activated microglia. *J. Biol. Chem*. 279: 1415-1421, 2004.
8. Ranaivo, H.R., Craft, J.M., Hu, W., Guo, L., Wing, L.K., Eldik, L.J.V. and Watterson, D.M. Glia as a therapeutic target: selective suppression of human amyloid-β-induced upregulation of brain proinflammatory cytokine production attenuates neurodegeneration. *J. Neurosci*. 26: 662-670, 2006.
9. Reichel, A. The role of blood-brain barrier studies in the pharmaceutical industry. *Curr. Drug Metab*. 7: 183-203, 2006.
10. Tambuyzer, B.R., Ponsaerts, P. and Nouwen, E.J. Microglia: gatekeepers of central nervous system immunology. *J. Leukoc. Biol*. 85: 352-370, 2009.
11. Tian, J., Li, G., Liu, Z., Zhang, S., Qu, G., Jiang, W. and Fu, F. ND-309, a novel compound, ameliorates cerebral infarction in rats by antioxidant action. *Neurosci. Lett*. 442: 279-283, 2008.
12. Wang, S.X., Hu, L.M., Gao, X.M., Guo, H. and Fan, G.W. Anti-inflammatory activity of Salvianolic acid B in microglia contributes to its neuroprotective effect. *Neurochem. Res*. 35: 1029-1037, 2010.
13. Wang, S.P., Zang, W.J., Kong, S.S., Yu, X.J., Sun, L., Zhao, X.F., Wang, S.X. and Zheng, X.H. Vasorelaxant effect of isopropyl 3-(3, 4-dihydroxyphenyl)-2-hydroxypropanoate, a novel metabolite from *Salvia miltiorrhiza*, on isolated rat mesenteric artery. *Eur. J. Pharmacol*. 579: 283-288, 2008.
14. Yang, A.L., Lo, C.W., Lee, J.T. and Su, C.T. Enhancement of vasorelaxation in hypertension following high-intensity exercise. *Chinese J. Physiol*. 54: 87-95, 2011.
15. Yenari, M.A., Xu, L., Tang, X.N., Qiao, Y. and Giffard, R.G. Microglia potentiate damage to blood-brain barrier constituents improvement by minocycline *in vivo* and *in vitro*. *Stroke* 37: 1087-1093, 2006.
16. Zheng, X.H., Zhao, X.F., Zhao, X., Wang, S.X., Wei, Y.M. and Zheng, J.B. Determination of the main bioactive metabolites of Radix Salvia miltiorrhizae in compound danshen dripping pills and the tissue distribution of danshensu in rabbit by SPE-HPLC-MSn. *J. Sep. Sci*. 30: 851-857, 2007.
17. Zhou, L., Zuo, Z. and Chow, M.S. Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *J. Clin. Pharmacol*. 45: 1345-1359, 2005.