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Anti-neuroinflammatory effects of 20C from *Gastrodia elata via* regulating autophagy in LPS-activated BV-2 cells through MAPKs and TLR4/Akt/mTOR signaling pathways

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ABSTRACT

20C, a novel bibenzyl compound, is isolated from *Gastrodia elata.* In our previous study, 20C showed protective effects on tunicamycin-induced endoplasmic reticulum stress, rotenone-induced apoptosis and rotenone-induced oxidative damage. However, the anti-neuroinflammatory effect of 20C is still with limited acquaintance. The objective of this study was to confirm the anti-neuroinflammatory effect of 20C on Lipopolysaccharide (LPS)-activated BV-2 cells and further elucidated the underlying molecular mechanisms. In this study, 20C significantly attenuated the protein levels of nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and interleukin (IL)-1β, and secretion of nitric oxide (NO) and tumor necrosis factor (TNF)-α induced by Lipopolysaccharide (LPS) in BV-2 cells. Moreover, 20C up-regulated the levels of autophagy-related proteins in LPS-activated BV-2 cells. The requirement of mitogen-activated protein kinases (MAPKs) has been well documented for regulating the process of autophagy. Both 20C and rapamycin enhanced autophagy by suppressing the phosphorylation of MAPKs signaling pathway. Furthermore, 20C treatment significantly inhibited the levels of toll like receptor 4 (TLR4), phosphorylated-protein kinase B (Akt) and phosphorylated-mechanistic target of rapamycin (mTOR), indicating blocking TLR4/Akt/mTOR might be an underlying basis for the anti-inflammatory effect of 20C. These findings suggest that 20C has therapeutic potential for treating neurodegenerative diseases in the future.

1. Introduction

Neuroinflammation is recognized as a common disease-related factor influencing neurodegenerative diseases progression (Ransohoff, 2016; Schwartz and Deczkowska, 2016). In neuroinflammation, it is characterized by microglia activation and the presence of neuroinflammatory mediators in the brain microenvironment. Microglia is the major immune cell, act as the resident effector cells in immune defense and maintain central nervous system (CNS) homeostasis through their accurate activation (Colonna and Butovsky, 2017; Yirmiya et al., 2015). The inflammatory response induced by microglia contributes to the development of neurodegenerative diseases and causes neurons death through excessive production of pro-inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), nitric oxide (NO) and enzymes associated with inflammation such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Mendiola and Cardona, 2017; Tiwari and Pal, 2017). Therefore, blocking the activation of microglia-mediated neuroinflammation is considered to be a potential treatment strategy in neurodegenerative diseases.

Autophagy is a ubiquitous intracellular degradation process in eukaryotic cells, which regulates the removal of cellular components or damaged organelles, and ultimately achieves a balance in host homeostasis (Muller et al., 2017). Recent research suggests that autophagy also affects various aspects of immune inflammation and may exhibit protective effect in wide ranges of inflammatory diseases (Deretic et al., 2013; Netea-Maier et al., 2016). The process of autophagy influences

Abbreviations: Akt, protein kinase B; Atg, autophagy related gene; CNS, central nervous system; COX-2, cyclooxygenase-2; CQ, chloroquine; ERK, extracellular signal-regulated kinase; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; JNK, c-jun NH2-terminal kinase; LPS, Lipopolysaccharide; MAPKs, mitogen-activated protein kinases; mTOR, mechanistic target of rapamycin; NO, nitric oxide; PI3K, phosphatidylinositol 3-kinase; PRR, pattern recognition receptor; TLR4, toll like receptor 4; TNF-α, tumor necrosis factor-α

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inflammation *via* regulating immune signaling pathways and equals the beneficial and detrimental influences of inflammatory response (Plaza-Zabala et al., 2017). Autophagy is emerging as a crucial regulator in neurodegenerative disorders and drugs modulating autophagy have been realized in animal models (Levine et al., 2011).

As a significant pattern recognition receptor (PRR), toll like receptor 4 (TLR4) is mainly expressed at microglia. Lipopolysaccharide (LPS) is a main component of the outer membrane of gram-negative bacteria, which can activate TLR4 binding with inflammation and autophagy (Xu et al., 2007). Recent studies have proved that the induction of autophagy involves several signal pathways. Phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (Akt)/ mechanistic target of rapamycin (mTOR) and mitogen-activated protein kinases (MAPKs) signaling pathways exert essential roles. The protein kinase mTOR is one of major downstream regulatory factors and has two different complexes named mTOR complex 1 (mTORC1) and mTORC2. Moreover, mTORC1 is a vital negative regulator of autophagy associated with various signaling pathways including the downstream inflammatory responses (Fu et al., 2017; Hu et al., 2016). Similar to the PI3K/Akt/mTOR signal pathway, extracellular signal-regulated kinase (ERK), c-jun NH2-terminal kinase (JNK) and p38 MAPK are required particularly in the activation of microglia cells to response to autophagy (Zhao et al., 2015).

Gastrodia elata (Tianma, GE) a traditional medicinal herb in China, has been widely used for treating vertigo and hypertension for many years. Recent studies have reported that treatment with this herb was able to reduce memory impairment and enhance cognitive function in neurodegenerative diseases (Duan et al., 2015; Li and Qian, 2016). The bibenzyl compound 20C (Fig. 1) is isolated from *Gastrodia elata.* 20C was able to protect PC12 cells against tunicamycin-induced endoplasmic reticulum stress, rotenone-induced apoptosis and rotenone-induced oxidative damage (Huang et al., 2016; Mou et al., 2016; Zhang et al., 2017). Both oxidative stress and endoplasmic reticulum stress are associated with neuroinflammation, so our study aims at investigating whether 20C exerts anti-neuroinflammatory effect and its potential molecular mechanisms.

2. Materials and methods

2.1. Reagents and antibodies

20C was offered by the Department of Chemosynthesis, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). 20C was dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C. LPS, DMSO, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diph-enyltetrazolium bromide (MTT), Akt1/2 kinase inhibitor Akti, ERK1/2 inhibitor PD98059, JNK inhibitor SP600125, P38 inhibitor SB203580 and rapamycin were bought from Sigma-Aldrich (St Louis, MO, USA). TLR4 inhibitor CLi-095 was purchased from Invivogen (San Diego, California). Chloroquine (CQ) was bought from Selleckchem (Houston, TX, USA). Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12), and fetal bovine serum (FBS) were got from Gibco (Grand Island, NY, USA).

All the primary antibodies including those against NF-κB/p65, COX-2, p-ERK, ERK, p-JNK, JNK, p-p38, p38, Atg12, Atg16 and TLR4 were bought from Santa Cruz Biotechnology (CA, USA). p-mTOR (Ser2448), mTOR, p-Akt (Ser473), Akt, p- NF-κB/p65, iNOS, Atg5 as well as Beclin-1 were ordered from Cell Signaling Technology (Beverly, MA, USA). Anti-p62 and IL-1β antibodies were bought from Abcam (Cambridge, USA). Monoclonal antibodies such as anti-β-actin and anti-LC3II were obtained from Sigma-Aldrich (St. Louis, MO, USA). All secondary antibodies were purchased from KPL (Gaithersburg, MD, USA). Antibodies see Table 1.

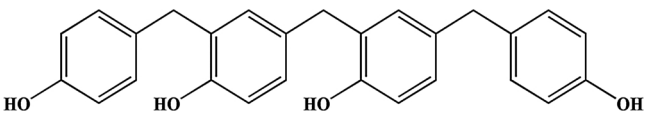

Fig. 1. The chemical structure of 20C.

Table 1
The information of the antibodies used in the present work.

Antibody	Source	Dilutions	Company
NF-κB(p65)	mouse	1: 500	Santa Cruz Biotechnology
COX-2	goat	1:500	Santa Cruz Biotechnology
p-ERK	goat	1: 500	Santa Cruz Biotechnology
ERK	mouse	1:500	Santa Cruz Biotechnology
p-JNK	mouse	1:500	Santa Cruz Biotechnology
JNK	mouse	1:500	Santa Cruz Biotechnology
p-p38	mouse	1: 500	Santa Cruz Biotechnology
p38	rabbit	1:500	Santa Cruz Biotechnology
TLR4	goat	1:500	Santa Cruz Biotechnology
Atg12	mouse	1:500	Santa Cruz Biotechnology
Atg 16	mouse	1:500	Santa Cruz Biotechnology
Atg 5	rabbit	1:1000	Cell Signaling Technology
p-mTOR(Ser2448)	rabbit	1:1000	Cell Signaling Technology
mTOR	rabbit	1:1000	Cell Signaling Technology
p-Akt(Ser473)	rabbit	1:1000	Cell Signaling Technology
Akt	rabbit	1:1000	Cell Signaling Technology
p-p65	rabbit	1:1000	Cell Signaling Technology
iNOS	rabbit	1:1000	Cell Signaling Technology
Beclin-1	rabbit	1:1000	Cell Signaling Technology
p62	mouse	1:1000	Abcam
IL-1β	rabbit	1:1000	Abcam
LC3II	rabbit	1:1000	Sigma
β-actin mA	mouse	1:1000	Sigma

2.2. Microglia cell culture and treatment

The murine BV-2 microglia cells were maintained in our laboratory. BV-2 cells were cultured in DMEM/F12 supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Gibco, USA), 1% penicillin (100 units)/streptomycin (100 mg/mL) at 37 °C in a humidified atmosphere of 5% CO_2. The cells were passaged three times per week.

LPS was dissolved in phosphate buffer saline (PBS) at a concentration of 1 mg/mL stored at −20 °C until it was used. BV-2 cells were pretreated for 1 h with different inhibitors, exposed to LPS (1 μg/mL) in the presence or absence of 0.1, 1, 10 μmol/L 20C. For the MTT assay, the cells were cultured with 20C and LPS for 24 h. For immunofluorescence analysis, the cells were cultured with 20C and LPS for 12 h. Additionally, for western blot analysis and ELISA analysis, the cells were cultured with 20C and LPS for 24 h.

2.3. Assessment of cell viability

BV-2 cells were cultured in Poly-L-lysine (PLL)-covered 96-well plates at a density of 5×10^4 cells/mL per well for 24 h. Then, the attached cells were exposed with LPS (1 μg/mL) in the presence or absence of 0.1, 1, 10 μmol/mL 20C. The MTT assay was used to assess the effect of 20C on the cells viability. The medium was added with 10 μL of 5 mg/mL MTT solution (Sigma-Aldrich) for 4 h at 37 °C, and treatment with 100 μL of MTT-formazan dissolving solution for 10 h could solubilize the formazan crystals. The optical density (OD) was obtained by a Microplate Reader (Thermo, Waltham, MA, USA) at 570 nm.

2.4. RNA isolation and real-time polymerase chain reaction (PCR)

BV-2 cells (2.0×10^6 cells in six-well plate) were incubated with LPS (1 μg/mL) in the presence or absence of 0.1, 1, 10 μmol/L 20C for 6 h. The total RNA of BV-2 cells was extracted with a Trizol reagent (Invitrogen, Carlsbad, CA, USA), and then 1 μg RNA was reverse-transcribed to cDNA. The RNA levels of TNF-α was measured using a real-time PCR system (ABI 7900HT, Foster City, CA, USA). The level of GAPDH gene was used for standardization. All samples were repeated