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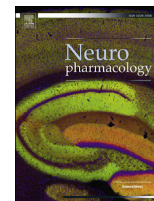

Trans-cinnamaldehyde improves memory impairment by blocking microglial activation through the destabilization of iNOS mRNA in mice challenged with lipopolysaccharide

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A B S T R A C T

Microglia activation and neuroinflammation are critically involved in pathogenesis of neurodegenerative disorders. Patients with neurodegenerative disorders often suffer memory impairment and currently there is no effective treatment for inflammation-led memory impairment. *Trans*-cinnamaldehyde (TCA) isolated from medicinal herb *Cinnamomum cassia* has been shown to exhibit anti-inflammatory capability. However, the potential of TCA to be used to improve memory impairment under neuroinflammation has not been explored. Primary microglia stimulated by lipopolysaccharide (LPS) were used to evaluate the potential anti-neuroinflammatory effects of TCA by examining the production of nitric oxide (NO), expression of inducible nitric oxide synthase (iNOS), pro-inflammatory cytokines, and activation of MAPKs. A mouse model of LPS-induced memory impairment was established to assess the neuroprotective effects of TCA against memory deficit and synaptic plasticity inhibition by both behavioral tests and electrophysiological recordings. TCA pretreatment decreased LPS-induced morphological changes, NO production and IL-1β release in primary microglia. Decreased NO production was due to the accelerated degradation of iNOS mRNA in LPS-stimulated microglia through TCA's inhibitory effect on MEK1/2-ERK1/2 signaling pathway. TCA was able to reduce the levels of iNOS and phosphorylated ERK1/2 in hippocampus of mice challenged with LPS. Most importantly, TCA significantly lessened memory deficit and improved synaptic plasticity in LPS-challenged mice. This study demonstrates that TCA suppressed microglial activation by destabilizing iNOS mRNA, which leads to improved memory impairment in mice suffering neuroinflammation.

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1. Introduction

Neuroinflammation and microglial activation are crucial components of the pathogenesis in neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and Multiple sclerosis (Glass et al., 2010; Gonzalez-Scarano and Baltuch, 1999; Minagar et al., 2002). Microglia, the innate immune cells of central nervous system (CNS), play an important role in actively monitoring their microenvironment by turning into activated state in response to toxic invasion, infection, brain injury and activation of the peripheral immune system (Davalos et al., 2005; Nguyen et al., 2002; Nimmerjahn et al., 2005). Once activated, microglia secrete a number of pro-inflammatory and neurotoxic mediators including interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), nitric oxide (NO), complements, excitatory amino acids and reactive oxygen species (ROS), that not only control neuronal function and synaptic transmission (Block et al., 2007; Hanisch and Kettenmann, 2007; Pascual et al., 2012; Yirmiya and Goshen, 2011) but also contribute to cognitive deficit and sick behavior (Pannu and Singh, 2006). Pharmacological intervention to prevent the activation of microglia and the subsequent release of pro-inflammatory mediators is currently considered as a potential

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therapeutic approach against neuroinflammatory and neurodegenerative diseases.

NO, one of bioactive products of mammalian cells, exhibits important physiological functions in vasodilatation, neurotransmission and host defense (Tripathi, 2007). NO is synthesized by three distinct isoforms of NO synthase (NOS): neuronal NOS (nNOS) and endothelial NOS (eNOS), and inducible NOS (iNOS) (Pautz et al., 2010). In CNS, iNOS is not expressed in microglia or astroglia of the healthy brain; however, its expression is induced in microglia or astroglia in response to immunological and inflammatory stimuli such as endotoxin, pre-inflammatory cytokine and amyloid beta peptide (Aβ) (Nathan and Xie, 1994; Wang et al., 2004). Excessive iNOS induction leads to higher concentration of NO, which is neurotoxic and contributes to the pathophysiology of various neuroinflammatory and neurodegenerative diseases (Abramson et al., 2001; Broom et al., 2011; Glass et al., 2010). Evidences generated from *in vitro* and *in vivo* experiments strongly indicate that inhibition of iNOS expression has suppressive effects on microglial activation and neuroinflammatory processes (Huang et al., 2011; Terazawa et al., 2013; Wen et al., 2011). For instance, administration of iNOS inhibitor and silencing iNOS expression were shown to have neuroprotective effects on dopaminergic neurons damage in Parkinson's Disease (Broom et al., 2011; Li et al., 2012). iNOS inhibitors, aminoguanidine and 1400 W, were found to have the ability to prevent the Aβ-mediated inhibition of long-term potentiation (LTP) induction in murine model. However, Aβ also have inability to inhibit LTP induction in iNOS knockout mice (Wang et al., 2004). These studies all pinpoint that elevated iNOS expression and NO production are the major causes of memory deficit and synaptic plasticity depression in neurodegenerative diseases.

The expression of iNOS can be transcriptionally regulated by nuclear factor κB (NF-κB), activator protein-1 (AP-1), signal transducers and activators of transcription-1α (STAT-1α) and CCAAT enhancer binding protein (C/EBP) in microglia (Pautz et al., 2010). Recent studies suggest that iNOS expression may also be regulated post-transcriptionally through iNOS mRNA stability (Hubbard et al., 2010; Lisi et al., 2011; Murphey et al., 2011). Especially, all three members of mitogen-activated protein kinase (MAPK) families, signal-regulated kinases (ERK1/2), c-Jun N-terminal kinase (JNK1/2), and p38 MAPK (Martindale and Holbrook, 2002) can regulate iNOS expression transcriptionally or post-transcriptionally (Bhat et al., 1998, 2003; Jeohn et al., 2000). It is thus of great interest to investigate whether MAPKs are involved in iNOS expression in microglia.

Cinnamomumassia (*C. cassia*) is a medicinal herb that has been used to treat dyspepsia, diabetes, anxiety, ischemia, cancers and inflammatory disease worldwide (Liao et al., 2012; Shimada et al., 2000; Yu et al., 2007; Zaidi et al., 2015). *Trans*-cinnamaldehyde (TCA), a major bioactive component isolated from the stem bark of *C. cassia*, has been reported to exhibit antitumor, antipyretic, antimicrobial, antidiabetic and anti-mutagenic properties (Chew et al., 2010; Lin et al., 2013; Nikzamir et al., 2014; Trinh et al., 2015). Recent studies have also revealed that TCA possesses potent anti-inflammatory activity in aging rats, endothelial cells and monocytes/macrophages (Kim et al., 2007, 2010; Liao et al., 2008). TCA's anti-inflammatory activity appears to be associated with its suppressive role in diverse signaling axes including toll-like receptor 4 (TLR4) (Youn et al., 2008) and NF-κB and MAPKs (Kim et al., 2007). The observation that TCA displays anti-neuroinflammatory effect in LPS-stimulated BV2 microglia and neuroprotective role in dopaminergic degeneration of mice (Ho et al., 2013; Pyo et al., 2013) strongly suggests that TCA may represent as an effective anti-inflammatory agent to deter neurodegenerative processes. The objective of this study is to investigate the potential and mechanism associated with TCA's role in improving memory impairment under neuroinflammation.

Lipopolysaccharide (LPS) is widely used as an inflammagen to activate microglia. In fact, mouse challenged with LPS suffers neuroinflammation and displays memory deficit and behavioral disorder, thereby used as an experimental model of neurodegeneration (Lee et al., 2008; Qin et al., 2007). Here, we show that TCA decreased NO production in LPS-induced primary microglia by abolishing the expression of iNOS mRNA through acceleration of mRNA decay. In LPS-induced neurodegeneration mouse model, TCA diminishes microglial activation and exhibits potent neuroprotective effects evidenced by lessening microglia-mediate memory impairment and synaptic plasticity inhibition. This study suggests that TCA should be seriously considered as a potential therapeutic agent for neuroinflammatory and neurodegenerative disorders.

2. Materials and methods

2.1. Reagents

Bacterial lipopolysaccharide (LPS) from *E. coli* serotype 0111:B4 and *trans*-cinnamaldehyde (TCA) were purchased from Sigma-Aldrich (St.Louis, MO, USA). LPS was dissolved in sterile saline. TCA for cell-based studies was reconstituted in dimethyl sulfoxide (DMSO), whereas TCA for animal studies was solubilized in oil with the aid of sonication.

2.2. Primary microglial preparation

Primary microglia were prepared from cerebral cortices of neonatal Sprague-Dawley rats at postnatal day P0 to P2 and cultured as previously described with minor modifications (Mecha et al., 2011). Briefly, rat brains were harvested followed by completely removing meninges and blood vessels away. The cerebral cortex were dissociated from brain in ice-cold Earle's balanced salt solution (EBSS; KCl 0.4 g/L, NaCl 6.8 g/L, NaHCO 32.2 g/L, NaHPO4·2H2O 0.16 g/L, pH 7.4) and then digested with 0.025% trypsin (Invitrogen, Carlsbad, CA, USA) for 15 min in a 37 °C with gentle agitation. Trypsinization was stopped by adding an equal volume of Dulbecco's modified Eagle's medium (DMEM; HyClone, south Logan, Utah, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, Hyclone), 10% horse serum (Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen), and mixtures were centrifugated for 5 min at 1500 r. p.m. After discarding supernatants, the remaining brain tissues were treated with EBSS supplemented with deoxyribonuclease (DNase) 4 ml/L, 3.82% $MgSO_4$ 30 ml/L, Glucose 2.5 g/L, BSA 3 g/L. Cells were collected by centrifugation for 5 min at 1000 r. p.m, and then seeded in Poly-D-Lysine-covered T-75 flasks. Culture medium was changed every four to five days after seeding, and microglia were isolated and purified from mixed (astrocyte-microglia) primary cultures by mild shaking of the culture dish between days 7–11. The purity of primary microglia was >98% as determined by immunofluorescence with anti-CD11b monoclonal antibody (OX42, microglial marker, Abcam, Cambridge, UK).

2.3. Cell viability assay

Cell viability was analyzed by 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Briefly, primary microglia (1×10^4 cells/well) were seeded in 96-well plates and then subjected to TCA (1.25–20 μM) and/or LPS (100 ng/ml) treatment for varying times followed by addition of 20 μl MTT solution (5 mg/ml in PBS). After a 4-h incubation, supernatant was

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