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Anti-inflammatory effects of [6]-shogaol: Potential roles of HDAC inhibition and HSP70 induction

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ABSTRACT

Ginger extracts have been reported to have anti-inflammatory, anti-oxidant, and anti-cancer effects. [6]-shogaol is one of the most bioactive components of ginger rhizomes. This study assessed the [6]-shogaol's ability to protect cultured primary rat astrocytes against lipopolysaccharide (LPS)-induced inflammation. [6]-shogaol was shown to suppress the release of pro-inflammatory cytokines and decreased the level of inducible nitric oxide syntheses (iNOS), cyclooxygenase-2 (COX-2), and phospho-NF-kB in LPS-treated astrocytes. Furthermore, [6]-shogaol treatment markedly up-regulated histone H3 acetylation and suppressed histone deacetylase (HDAC)1 expression. In addition, [6]-shogaol treatment also increased the expression of heat-shock protein (HSP)70. The neuroprotective, neurotrphic, and anti-inflammatory properties of [6]-shogaol may be translated to improvements in neurological performance. [6]-Shogaol's ability to inhibit HDAC was comparable to that of commonly used HDAC inhibitors Trichostatin A and MS275. Taken together, our results suggest that [6]-shogaol can significantly attenuate a variety of neuroinflammatory responses by inducing HSP70, that is associated with HDAC inhibition in cortical astrocytes.

1. Introduction

Neuroinflammation in the central nervous system (CNS) in neurodegenerative diseases is dependent on the production of various inflammatory substances by local neurons and glia (Jung et al., 2009). Until now, most studies in the field of neuroinflammation have focused on the role of microglia. However, recent findings revealed that astrocytes are not only supportive cells for neuronal function, but also actively participate in the initiation and modulation of inflammatory reactions in CNS injury, including Parkinson's disease, Alzheimer's disease, prion diseases, and multiple sclerosis (Salminen et al., 2011; Ridet et al., 1997). The activation of astrocytes may in turn cause neuronal damage in neurodegenerative diseases through the overproduction of pro-inflammatory substances, including pro-inflammatory cytokines, reactive intermediates, proteinases, and complement proteins (Liu et al., 2009). Therefore, attenuation of astrocyte activation may have potential therapeutic options for the treatment of various neurodegenerative conditions.

Ginger is the rhizome of *Zingiber officinale*, which is widely used throughout the world as an important spice and traditional herb (White, 2007). It is known and valued that ginger extract and its biologically active compounds, including gingerols and [6]-shogaol, exhibit pharmacological activities that include anti-inflammation (Grzanna et al., 2005; Lantz et al., 2007), anti-emesis (Sharma et al., 1997), anti-tumor (Katiyar et al., 1996; Surh, 2002), anti-oxidation (Eguchi et al., 2005; Masuda et al., 2004), and analgesic effects (Lantz et al., 2007) in numerous diseases. Ginger extract has been used as an herbal medicinal product that shares pharmacological properties with non-steroidal anti-inflammatory drugs and modulates biochemical pathways that are activated in chronic inflammation (Grzanna et al., 2005).

A recent *in vitro* study has found that [6]-shogaol is an effective anti-inflammatory component of ginger extracts (Dugasani et al., 2010). It has been reported that [6]-shogaol significantly suppresses the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-induced macrophages (Dugasani et al., 2010; Pan et al., 2008) and recent studies have shown that [6]-shogaol also prevents dopamine depression (Kabuto et al., 2005) and apoptotic neuronal cell death (Kyung et al., 2006). However, the specific mechanisms underlying

Abbreviations: CNS, central nervous system; COX-2, cyclooxygenase-2; GFAP, glial fibrillary acidic protein; HSP, heat-shock protein; HDAC, histone deacetylase; iNOS, inducible nitric oxide syntheses; LPS, lipopolysaccharide; MEM, minimal essential media; MS, MS-275; TSA, Trichostatin A; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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its anti-inflammatory effects have yet to be identified. In the present study, we assessed the ability of [6]-shogaol to protect against LPS-induced neuroinflammation in primary astrocyte cultures. We also compared the anti-inflammatory profile and the ability to increase heatshock protein (HSP) 70 of [6]-shogaol and histone deacetylase (HDAC) inhibitors (Trichostatin A and MS-275).

2. Materials and methods

2.1. Cell cultures and treatment

Primary mixed cultures of astrocytes and microglia ("glial cells") were prepared from postnatal day 1 Sprague–Dawley rats. Briefly, cortices were isolated in cold Minimal Essential Media (MEM) without FBS and then mechanically disrupted by pipetting. The cells were then washed and re-suspended in MEM + 10% FBS (Hyclone, Canada). The disrupted cortices were cultured in a 75 cm^2 flask. Enriched astrocyte cultures were obtained after shaking to minimize oligodendrocyte and microglial contamination. After shaking, the astrocytes were grown in Dulbecco's Minimal Essential Medium (DMEM) + 5% FBS (Hyclone, Canada). [6]-shogaol (WAKO chemicals, Japan), Trichostatin A (TSA, Millipore, CA, USA), or MS-275 (MS, Millipore, CA, USA) were added to the culture medium together with LPS (from Escherichia coli 055:B5, Sigma, St. Louis, MO, USA).

2.2. Immunocytochemistry

The enriched astrocyte cultures were identified by evaluating their morphology and by immunocytochemical staining with antibody to glial fibrillary acidic protein (GFAP, an astrocyte marker). The cells were fixed in 100% methanol and permeabilized with 0.1% Triton X-100 in PBS. Non-specific antibody binding was blocked by incubation with 1% BSA in PBS. The cells were then incubated simultaneously with monoclonal mouse anti-CD11b (1:200, CBL1512Z, Millipore, CA, USA) and polyclonal rabbit anti-GFAP (1:5000, AB5804, Millipore, CA, USA). CD11b and GFAP were visualized by successive incubation in goat anti-mouse IgG conjugated to FITC (1:300, ANZ0109, Invitrogen, OR, USA) and goat anti-rabbit IgG conjugated to Cy3 (1:300, A10520, Invitrogen, OR, USA). To determine the proportion of proliferating cells, the nuclei were labeled with Hoechst stain (Gibco, OR, USA). Most of the cells showed GFAP staining (purity > 95%) (Fig. 1).

2.3. Cell survival assay

Cell survival was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit from Sigma. The rat astrocyte cells were grown on 48-well plates (1 × 105/well) in DMEM + 5% FBS for 48 h, then the medium was replaced with serum-free medium and the cells were treated with [6]-shogaol at concentrations up to 20 μM for 24 h. Cell viability was measured as recommended by Sigma and results were expressed as a percentage of viability of the control culture.

2.4. Western blots

Total proteins from rat astrocyte lysates were subjected to SDS–polyacrylamide gel electrophoresis using 10–12% gels, and electrophoretically transferred to polyvinylidene difluoride membranes (Bio-rad, Hercules, CA). Membranes were blocked with 5% non-fat milk in TBS (50 mM Tris base, pH 7.5, 150 mM NaCl) and incubated individually with 1:1000 dilutions of primary antibodies against iNOS (SC-650, Santa Cruz, CA, USA), COX-2 (#4842, Cell Signaling, Beverly, MA), HDAC1 (SC-6298, Santa Cruz, CA, USA), histone H3 and acetyl-histone H3 (#9715, #9675, Cell Signaling, Beverly, MA). The blots were further incubated with peroxidase-conjugated goat anti-rabbit IgG (1:20,000; AP307P, Millipore, CA, USA) and rabbit anti-goat IgG (1:20,000; AP106P, Millipore, CA, USA). The immunoreactions were visualized using a SuperSignal West Dura Extended Duration Substrate (Pierce) and analyzed using a ChemiImager (Alpha Innotech, San Leandro, CA, USA).

2.5. Cytokine release analysis

Concentrations of IL-1β and IL-6 in samples of supernatant from cultured astrocytes were determined with ELISA kits (ALPCO Diagnostics, NH, USA). Plates were read at 450 nm and cytokine concentrations were estimated from the appropriate standard curve and expressed as pg/ml of supernatant.

2.6. Statistical analysis

The results were expressed as the mean ± standard error of the mean (SEM). The data were analyzed using a Student's t test and repeated-measures ANOVA followed by a Bonferroni test. A p value < 0.05 was considered significant.

3. Results

3.1. Inhibition of LPS-induced cell death by [6]-shogaol in primary rat astrocytes

Before examining the protective effects of [6]-shogaol on primary rat astrocytes treated with LPS, we determined the maximum concentration of [6]-shogaol that did not affect cell viability was 10 μM (data not shown). Incubation with 100 ng/ml LPS for 24 h decreased the cell viability to 76.9% with respect to the control. This LPS-induced decrease in viability was abrogated by 10 μM [6]-shogaol treatment that increased the cell viability to 87%) ($p < 0.05$ compared with LPS-treated cells) (Fig. 2B). These data indicated that [6]-shogaol inhibited LPS-induced cell death.

3.2. Inhibition of LPS-induced pro-inflammatory cytokines production by [6]-shogaol in primary rat astrocytes

The anti-inflammatory effects of [6]-shogaol on LPS-induced rat astrocytes were evaluated by measuring the release of pro-inflammatory cytokines. Treatment of primary rat astrocytes with LPS for 24 h induced a marked increase in IL-1β and IL-6 concentrations ($p < 0.001$, compared with no LPS treatment) (Fig. 3). Co-treatment with [6]-shogaol significantly suppressed IL-1β ($p < 0.001$) and IL-6 ($p < 0.05$) expression (Fig. 3). In particular, the robust suppression by [6]-shogaol at 10 μM was noteworthy (Fig. 3A).

3.3. Inhibition of iNOS and COX-2 accumulation by [6]-shogaol in LPS-treated primary rat astrocytes

The activation of astrocytes by LPS leads to the induction of pro-inflammatory gene products such as cytokines. LPS-induced inflammatory cytokine production is required for the activation of signaling pathways involving iNOS and COX-2 (Tanaka et al., 2007). Therefore, we investigated whether [6]-shogaol affected

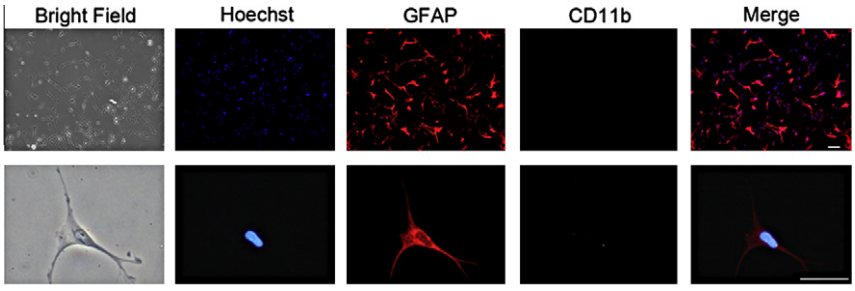

Fig. 1. Immunocytochemistry of primary rat astrocytes. GFAP is a marker for astrocytes and CD11b is a marker for microglia. Most of the cells showed GFAP staining (purity > 95%). Scale bar, 100 μm.