Antineuroinflammatory effects of lycopene via activation of adenosine monophosphate-activated protein kinase-α1/heme oxygenase-1 pathways

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ABSTRACT

Microglia play an important role in the immune defense in the central nervous system. Activation of microglia leads to the production of excessive inflammatory molecules and deleterious consequences, including neuronal death. Lycopene, 1 of the major carotenoids present in tomatoes, has been shown to exert antioxidant properties and to inhibit cancer cell proliferation. However, the effects of lycopene on neuroinflammatory responses in microglia remain unknown. In this study, we investigated the signaling pathways involved in lycopene-inhibited expression of cyclooxygenase (COX)-2 and inflammation mediators in BV-2 microglia, mouse primary cultured microglia, and rat primary cultured microglia. Lycopene inhibited the enhancement of lipopolysaccharide (LPS)-induced nuclear factor-kappaB (NF-κB) and activator protein 1 (AP-1) DNA binding activity. In the present study, we demonstrated that lycopene inhibits LPS-induced COX-2 expression through heme oxygenase-1 (HO-1) activation. Our results also demonstrate that stimulation with lycopene increases the phosphorylation of liver kinase B1 (LKB1), calmodulin-dependent protein kinase II (CaMKII), and adenosine monophosphate-activated protein kinase (AMPK)-α1. Treatment with AMPK inhibitors effectively antagonized lycopene-stimulated HO-1 expression. Interestingly, we also found that lycopene increased phospho-AMPKα1 accumulation in the nucleus in microglia. Preincubation of cells with HO-1 and AMPK selective pharmacological inhibitors dramatically reversed the inhibitory effect of lycopene on LPS-induced COX-2 and prostaglandin E2 production. Transfection of microglia with HO-1 and AMPKα1 small interfering RNA (siRNA) also effectively reversed the inhibitory effect of lycopene on LPS-induced COX-2 expression. In a mouse model, lycopene showed significant antineuroinflammatory effects on microglial activation and motor behavior deficits. These findings suggest that lycopene-inhibited LPS-induced COX-2 expression is mediated by HO-1 activation through the AMPK pathway. Therefore, lycopene might be useful as a therapeutic agent for the treatment of neuroinflammation-associated disorders.

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1. Introduction

Microglia are glial cells that comprise approximately 15% of the total cells of the central nervous system (CNS) and play important roles in immune defense and tissue repair. Microglial activation contributes to neuronal toxicity through secretion of proinflammatory mediators that increase oxidative stress, and directly trigger neuronal cell death mechanisms (Gao et al., 2011; Hirsch et al., 2005; Kaneko et al., 2012; Qin et al., 2007). The characteristics of neuroinflammation include upregulation of proinflammatory cytokines, an increase in the blood–brain barrier permeability, and glial activation. Neuroinflammation by microglial activation has beneficial and detrimental consequences in the CNS (Czeh et al., 2011; Wee Yong, 2010). It has been reported that when neuroinflammation begins, microglial activation is followed by clearance of debris or invading pathogens and release of neurotrophic and anti-inflammatory molecules that regulate the...
microenvironment (Ziv et al., 2006). However, overactivated microglia can cause a cytokine storm, leading to neurotoxicity by releasing several cytotoxic mediators. Therefore, microglial activation and neuroinflammation need to be better regulated or properly directed. Under pathological conditions, microglial activation has been regarded as a regulator of inflammation-mediated neuronal damage, which has been thought to contribute to the pathogenesis of neurodegenerative diseases (Polazzi and Monti, 2010; Tian et al., 2012). Numerous reports have shown that activated microglia were present in the substantia nigra pars compacta in postmortem brains of patients who had Parkinson’s disease (PD) (Imamura et al., 2003; McGeer et al., 1988; Ros-Bernal et al., 2011). It has also been found that the expression of inflammation-related enzymes, inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, are increased in the striatum of PD patients (Knott et al., 2000). Expression of proinflammatory molecules such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 have also been found to be elevated in the brain tissue of Alzheimer’s disease patients (Rasmussen et al., 2011; Uslu et al., 2012). COX-2, an in20ble enzyme, is primarily responsible for the production of prostaglandins (PGFs), and it is associated with inflammatory diseases. It is believed that COX-2 expression and PGE2 production are implicated in the initiation and progression of various neurodegenerative diseases (Teismann et al., 2003a, 2003b). It has been reported that microglia-mediated COX-2 expression contributes to neuronal cell death (Consilvio et al., 2004; Manabe et al., 2004). Importantly, COX-2-mediated inflammatory responses play important roles in the pathogenesis of many neurodegenerative diseases, such as Alzheimer’s disease (Giovannini et al., 2003; O’Banion, 1999) and PD (Levin et al., 2012; Teismann, 2012). Moreover, inhibition of COX-2 appears to prevent or slow the progression of neurodegenerative diseases (Giovannini et al., 2003; O’Banion, 1999; Sanchez-Pernaute et al., 2004). Importantly, PGE2 synthesis and COX-2 upregulation relative to microglial activation in chronic cerebral ischemia (Tomimoto et al., 2000) and prion diseases (Minghetti and Pocchiari, 2007) have been reported.

Heme oxygenase (HO) is a cytoprotective and a rate-limiting enzyme, and it degrades heme to bilirubin, carbon monoxide, and iron (Jazwa and Cuadrado, 2010; Jeong et al., 2010). There are 3 types of heme oxygenases, namely, HO-1 (heat shock protein 32; HSP32), HO-2, and HO-3 (Sypain, 2008). HO-1 is a phase II enzyme upregulated in conditions of oxidative stress, cellular injury, and disease (Boehning et al., 2004). HO-2 and HO-3 are constitutively expressed in various tissues (Farombi and Surh, 2006; Wu and Wang, 2005). It has been reported that induction of HO-1 expression and related signal pathways exert anti-inflammatory effects in macrophages (Choi et al., 2012; Choy et al., 2008; Tsou et al., 2011a). Our recent reports also showed that neuroinflammation is repressed by the induction of HO-1 expression in microglia (Lu et al., 2010b) and astrocytes (Chen et al., 2012), and that increased HO-1 expression protects neurons against neurotoxin-induced cell death (Lin et al., 2012; Lu et al., 2013). In the present study, we investigated whether HO-1 expression contributes to lycopene-regulated anti-inflammatory responses.

2. Methods

2.1. Experimental animals

ICR mice weighing 30–35 g were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Male ICR mice were used at 7 weeks of age. The animals were housed in conventional conditions of adequate temperature (23 ± 5 °C), 12-hour light/dark cycle, and provided with free access to food and water. All mice were manipulated in accordance with the Animal Care and Use Guidelines of the China Medical University (Taichung, Taiwan).

2.2. Experimental protocols

Lycopene was dissolved in saline containing 0.1% tetrahydrofuran. Mice received an intraperitoneal injection of vehicle or lycopene (5 or 10 mg/kg) for 3 consecutive days. On the third day, after injection of vehicle or lycopene (5 or 10 mg/kg) for 2 hours, mice were intraperitoneally injected with saline or Escherichia coli lipopolysaccharide (LPS) (20 mg/kg; serotype 0127:B8, Sigma-Aldrich, St Louis, MO, USA) for another 24 hours.

2.3. Materials

Lycopene was purchased from Extrasynthese (Genay Cedex, France). Primary antibody against β-actin, control small interfering RNA (siRNA), AMPKx1 siRNA, and HO-1 siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against AMPKα phosphorylated at Thr172, calmodulin-dependent protein kinase II (CaMKII) phosphorylated at Thr286, and liver kinase B1 (LKB1) phosphorylated at Ser428 were purchased from Cell Signaling and Neuroscience (Danvers, MA, USA), Rabbit anti-iNOS antibody was purchased from BD Transduction Laboratories (Lexington, KY, USA). Rabbit anti-COX-2 antibody was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Compound C and adenosine regulated by various stresses, including heat shock, energy depletion, and oxidants (Kodilha et al., 2007). Importantly, a recent study has shown that nuclear translocation of AMPKx1 potentiates striatal neurodegeneration (Ju et al., 2011). Accumulating evidence has shown that AMPK regulates a variety of biological functions such as cell migration, synaptic plasticity, neuronal survival, brain tumor development, and inflammatory responses (Hardie et al., 2012). An increasing number of studies suggest that AMPK-inhibited COX-2 expression exerts a variety of biological functions (Kim et al., 2012; Yi et al., 2011). However, the actual mechanism of AMPK in antineuroinflammatory remains unclear.

Lycopene is a natural carotenoid that is present in tomatoes and tomato-based products. It has been reported that dietary intake of tomatoes containing lycopene can reduce the risk of chronic diseases and various cancers (Agarwal and Rao, 2000). Lycopene has been found to inhibit tumor progression in several types of cancerous cells, including prostate, breast, and colon cancers (van Breezem and Pajkovic, 2008). It has been shown that lycopene exerts several biological functions, such as acting as an antioxidant and in low-density lipoprotein cholesterol reduction (Karppi et al., 2010; Turk et al., 2010). Moreover, it has been reported that lycopene reduces proinflammatory cytokine and chemokine expression in macrophages (Lee et al., 2012; Marcotorchino et al., 2012). However, the effects of lycopene in microglia have not yet been described thoroughly. In this study, we investigated the molecular mechanisms underlying the anti-inflammatory properties of lycopene in microglia. The present study showed that lycopene-induced accumulation of AMPKx1 in nuclei is closely associated with HO-1 upregulation, which leads to antineuroinflammatory effects in microglia.
9-β-d-arabinofuranosside (Ara A) were purchased from Merck Calbiochem (San Diego, CA, USA). All other chemicals were obtained from Sigma-Aldrich.

2.4. Cell culture

All protocols for animal experiments were approved by the Animal Care Committee of China Medical University (Taichung, Taiwan). Isolated adult mouse microglial cells were prepared from the whole brains of anesthetized 8-week-old mice. After whole-mouse perfusion with ice-cold phosphate buffered saline (PBS), brains were homogenized in Hank’s balanced salt solution (Gibco BRL) with collagenase/DNase (Sigma-Aldrich). Resulting homogenates were passed through nylon mesh (100 μm) and centrifuged at 1000g for 10 minutes. Supernatants were removed, and cell pellets were resuspended in 37% isotonic Percoll (Amersham Biosciences) at room temperature. A discontinuous Percoll density gradient was set up as follows: 70, 37, 30, and 0% isotonic Percoll. The gradient was centrifuged at 200g (at 18 °C–20 °C), and microglia were collected from the interphase between the 37% and 30% Percoll layers. Microglial cells were washed and then resuspended in sterile Hank’s balanced salt solution.

Sprague-Dawley rats were obtained from the National Laboratory Animal Center of Taiwan. Rat primary microglial cultures were prepared according to our previous reports (Lin et al., 2011; Lu et al., 2009). Briefly, glial cells were cultured in 75-cm² flasks for 12–14 days in Dulbecco’s Modified Eagle Medium (DMEM)/F12 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. To separate microglia, flasks were shaken in a rotary shaker. Detached cells were passed through a nylon mesh filter (BD Falcon, Franklin Lakes, NJ, USA) and then plated into 24-well plates. The purity of microglial cultures was assessed using Iba-1 antibody (Wako Pure Chemicals, Osaka, Japan), and more than 95% of cells stained positively.

The murine microglial cell line, BV-2, was generated by infecting primary microglial cell cultures with a v-raf/v-myc oncogene carrying retrovirus (J2). BV-2 cells tested positive for macrophage antigen complex-1 (MAC-1) and -2 antigens. Because BV-2 cells retain most of the morphological, phenotypical, and functional properties described for freshly isolated microglial cells, they can be considered to be immortalized active microglial cells (Blasi et al., 1990). Cells were cultured in DMEM with 10% FBS at 37 °C in a humidified incubator in an atmosphere of 5% CO₂ and 95% air.

2.5. Western blot analysis

Cells were treated with lycopene for the indicated time periods and then lysed with radioimmunoprecipitation assay buffer (50 mM hydroxyethyl piperazineethanesulfonic acid (HEPES) [pH 7.4], 150 mM NaCl, 4 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Na₃PO₄, 100 mM NaF, 2 mM Na₂VO₄, 1% Triton X-100, 0.25% sodium deoxycholate, 50 mM 4-(2-aminoethyl) benzene sulfonylfluoride, 50 μg/mL leupeptin, and 20 μg/mL aprotinin) on ice. Protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with nonfat milk in PBS and then probed overnight with a primary antibody at 4 °C.

After PBS washes, the membranes were incubated with secondary antibodies. The blots were visualized using enhanced chemiluminescence and Kodak XOMAT LS film (Eastman Kodak, Rochester, NY, USA). The blots were subsequently stripped by incubation in stripping buffer (62.5 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, and 0.1 M β-mercaptoethanol) and reprobed for β-actin as a loading control. Quantitative data were obtained using a computing densitometer and Imagej software.

2.6. Preparation of cytosolic and nuclear extracts

Nuclear extracts were prepared as previously described (Lin et al., 2011) with minor modifications. Cells were rinsed with PBS and resuspended in hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride) on ice. The lysates were separated into cytosolic and nuclear fractions using centrifugation at 12,000g for 10 minutes. The supernatants containing cytosolic proteins were collected. A pellet containing nuclear fraction was resuspended in buffer C (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, and 0.4 M NaCl) on ice. The supernatants containing nuclear proteins were collected using centrifugation at 13,000g for 20 minutes.

2.7. PGE₂ assay

Cells cultured in 24-well plates were stimulated with the indicated agents. Production of PGE₂ in the culture supernatant was measured using a commercial kit (Cayman Chemicals) according to the manufacturer’s instructions. Briefly, PGE₂-acetylcholinesetrase conjugate, PGE₂ monoclonal antibody, and culture supernatant were incubated for 18 hours at 4 °C in a 96-well plate precoated with goat polyclonal anti-mouse immunoglobulin G. The wells were rinsed with wash buffer several times. Ellman’s reagent (200 μL) was added to each well to develop in the dark. The absorbance at 405 nm was determined using a microplate reader.

2.8. Nitric oxide assay

Production of nitric oxide was assayed by measuring the stable nitrite levels of nitric oxide metabolism in the culture medium, which was prepared as described previously (Lin et al., 2010). Briefly, the accumulated nitrite in the medium was determined using a colorimetric assay with Griess reagent. The culture supernatant reacted with an equal volume of Griess reagent (1 part 0.1% naphthylethenediamine and 1 part 1% sulfanilamide in 5% H₃PO₄) for 10 minutes at room temperature in the dark. The absorbance at 550 nm was determined using a microplate reader (Bio-Tek, Winooski, VT, USA).

2.9. Quantitative real time polymerase chain reaction

Total RNA was extracted from cells using TRIzol reagent (MDBio Inc, Taipei, Taiwan). Total RNA was reverse transcribed into cDNA using the oligo (dT) primer. Quantitative real-time polymerase chain reaction (PCR) using SYBR Green Master Mix was performed with a model 7900 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). After preincubation at 50 °C for 2 minutes and 95 °C for 10 minutes, the PCR was performed as 40 cycles of 95 °C for 10 seconds and 60 °C for 1 minute. The threshold was set above the nontemplate control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted as CT).

2.10. Transfection

Cells were grown to confluence and transfected with control siRNA, AMPKα1 siRNA, or HO-1 siRNA using Lipofectamine 2000 (LF2000; Invitrogen Life Technologies, Carlsbad, CA, USA). siRNA and LF2000 were premixed in OPTI-medium for 20 minutes and applied to the cells. After 24-hour transfection, LF2000-containing medium was replaced with DMEM medium containing 2% FBS and pretreated with lycopene followed by stimulation with LPS (100 ng/mL) for another 24 hours.
2.11. Immunocytofluorescent staining

Cells were seeded onto glass coverslips and exposed to lycopene for 2 hours, fixed with 4% paraformaldehyde for 15 minutes, and then permeabilized with Triton X-100 for 30 minutes. Cells were incubated with rabbit anti-phospho-AMPKα antibody for 1 hour at room temperature. After a brief wash, cells were incubated with an Alexa 488-fluor-conjugated secondary antibody (Invitrogen Life Technologies). After removing the antibody solution, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) for another 5 minutes. Finally, cells were mounted and visualized with a Zeiss fluorescence microscope.

2.12. Electrophoretic mobility shift assay

The electrophoretic mobility shift assay (Panomics, Redwood City, CA, USA) was performed according to the manufacturer’s protocol. Nuclear extract (4 μg) of cells was incubated with poly d (I-C) at room temperature for 5 minutes. The nuclear extract was then incubated with biotin-labeled probes at room temperature for 30 minutes. After electrophoresis on an 8% polyacrylamide gel, the samples on the gel were transferred onto a presoaked Immobilon-Nyt membrane (Millipore, Billerica, MA, USA). The membrane was cross-linked in an oven for 1 minute and then developed with the peroxidase conjugate, before being subjected to Western blot analysis.

2.13. Immunohistochemistry

The ICR mice were anesthetized with trichloroacetaldehyde and then perfused with phosphate buffer solution with 10% paraformaldehyde. Brains were removed from the skull, postfixed overnight in 10% paraformaldehyde at 4 °C, and stored in a 30% sucrose solution for 1–2 days. The brain slices were then incubated with Triton X-100. After blocking with bovine serum albumin, the slices were incubated with primary antibody against Iba-1 (Wako Pure Chemicals) for microglia staining. Binding was detected using a biotinylated secondary antibody and an ABC standard kit (Vector Laboratories). Visualization was performed using 0.05% diaminobenzene (Sigma-Aldrich).

2.14. Motor coordination (rotarod test)

Motor balance and coordination function were analyzed using an UgoBasile7650 accelerating rotarod (Linton Instruments, Diss, UK). The treadmill was accelerated from 20 to 60 rpm over a period of 5 minutes, and the time spent on the drum was recorded for each mouse. After injection of the vehicle or lycopene (5 or 10 mg/kg) for 2 hours, mice were intraperitoneally injected with saline or LPS (20 mg/kg) for another 24 hours. Mice were placed on the rotating drums; the counter started simultaneously, and the rod was set to accelerate after 20–30 seconds. Any mouse remaining on the apparatus after 5 minutes was removed, and their time scored as 5 minutes. Latency to fall (in seconds) was calculated and used for data analysis.

2.15. Statistics

Statistical analysis was performed using the software GraphPad Prism 4.01 (Graph Pad Software Inc, San Diego, CA, USA). The values given are mean ± standard error of the mean. The significance of

![Fig. 1](image-url) - Lycopene suppresses COX-2 expression in microglial cells. (A) BV-2 microglia and mouse primary cultured microglia were pretreated with various concentrations of lycopene for 30 minutes followed by stimulation with LPS (100 ng/ml) for 24 hours. (B) Rat primary cultured microglia were pretreated with lycopene (5 μM) for 30 minutes followed by stimulation with LPS (100 ng/ml) for 24 hours. The expression of COX-2 was determined using Western blot analysis. The results expressed are representative of 3 independent experiments. (C) BV-2 microglia and mouse primary cultured microglia were pretreated with lycopene (5 or 10 μM) for 30 minutes followed by stimulation with LPS (100 ng/ml) for 24 hours, and then the media were collected for measuring PGE2 production. The results are expressed as mean ± standard error of the mean from at least 3 independent experiments. *p < 0.05 compared with the control group; # p < 0.05 compared with the LPS treatment group. Compared with the LPS group, PGE2 production was significantly lower in the LPS/lycopene groups (1-way ANOVA with Bonferroni correction). (D) BV-2 microglia were pretreated with lycopene (5 or 10 μM) for 30 minutes followed by stimulation with LPS (100 ng/ml) for 6 hours. The COX-2 mRNA expression was determined using real-time polymerase chain reaction. The results are expressed as means ± standard error of the mean from 3 independent experiments. *p < 0.05 compared with the control group; # p < 0.05 compared with the LPS treatment group. As compared with the LPS group, the expression of COX-2 was significantly lower in the LPS/lycopene groups (1-way ANOVA with Bonferroni correction). Abbreviations: ANOVA, analysis of variance; COX, cyclooxygenase; LPS, lipopolysaccharide; LPS-, LPS treatment group; PGE, prostaglandin. The symbol “-“ means without LPS or lycopene treatment, symbol “#“ means with LPS or lycopene treatment.
difference between the experimental group and control groups was assessed using the Student t test. Statistical comparisons of more than 2 groups were performed using 1-way analysis of variance with Bonferroni post hoc test. The difference was considered significant if the p value was < 0.05.

3. Results

3.1. Lycopene suppresses expression of COX-2 and inflammatory mediators in microglial cells

To examine the effects of lycopene on LPS-induced neuroinflammation in microglia, BV-2 microglia or mouse primary cultured microglia were pretreated with various concentrations of lycopene and then stimulated with LPS (100 ng/mL) for 24 hours. Lycopene effectively inhibited LPS-induced COX-2 expression in a concentration-dependent manner (Fig. 1A). The inhibitory effect of lycopene on LPS-induced COX-2 expression is similar to that in rat primary cultured microglia (Fig. 1B). Moreover, the inhibition of LPS-induced PGE2 production was observed in BV-2 microglia and mouse primary cultured microglia (Fig. 1C). We further analyzed COX-2 messenger RNA (mRNA) expression using real-time PCR. As shown in Fig. 1D, lycopene significantly inhibited LPS-induced COX-2 mRNA upregulation in BV-2 microglia. Furthermore, pretreatment with lycopene also inhibited LPS-induced iNOS expression in a concentration-dependent manner in BV-2 microglia and mouse primary cultured microglia (Fig. 2A). Similarly, pretreatment with lycopene significantly inhibited LPS-induced nitric oxide (NO) production in BV-2 microglia and mouse primary cultured microglia (Fig. 2B). Lycopene also inhibited LPS-induced IL-6 expression in a concentration-dependent manner in BV-2 microglia (Fig. 2D).

We further analyzed the effect of lycopene on mRNA expression in microglial cells. As shown in Fig. 2C and E, lycopene effectively inhibited LPS-induced iNOS and IL-6 mRNA expression in microglial cells. Additionally, the inhibitory effect of lycopene on LPS-induced iNOS, and IL-6 expression was similar in rat primary cultured microglia (Fig. 2F). It has been reported that the expression of COX-2 and inflammatory mediators is mainly regulated by nuclear factor-kappaB (NF-κB) and activator protein 1 (AP-1) activation in microglia (Bianchi et al., 2010). To determine the effect of lycopene on transcriptional levels, the DNA-binding activity of transcription factors was determined using electrophoretic mobility shift assay. As shown in Fig. 3A and C, LPS increased NF-κB and AP-1 DNA binding activities. The enhancement of LPS-induced DNA binding activities was attenuated by lycopene treatment in a concentration-dependent manner (Fig. 3A and C).

**Fig. 2.** Lycopene suppresses expression of inflammatory mediators in microglial cells. BV-2 microglia and mouse primary cultured microglia were pretreated with various concentrations of lycopene for 30 minutes followed by stimulation with LPS (100 ng/mL) for 24 hours. (A) The expression of iNOS was determined using Western blot analysis. (B) The culture media were collected and analyzed using Griess reaction. The results are expressed as means ± standard error of the mean from at least 3 independent experiments. Compared with the LPS/- group, nitric oxide production was significantly lower in the LPS/lycopene groups (1-way ANOVA with Bonferroni correction). BV-2 microglia were pretreated with lycopene for 30 minutes followed by stimulation with LPS (100 ng/mL) for 6 hours. Relative mRNA levels of iNOS (C) and IL-6 (E) were determined using real-time polymerase chain reaction. The results are expressed as mean ± standard error of the mean from at least 3 independent experiments. Compared with the LPS/- group, the mRNA levels of iNOS and IL-6 were significantly lower in the LPS/lycopene groups (1-way ANOVA with Bonferroni correction). (D) BV-2 microglia were pretreated with various concentrations of lycopene for 30 minutes followed by stimulation with LPS (100 ng/mL) for 24 hours. The expression of IL-6 was determined using Western blot analysis. (F) Rat primary cultured microglia were pretreated with lycopene (5 μM) for 30 minutes followed by stimulation with LPS (100 ng/mL) for 24 hours. The expression of iNOS and IL-6 were determined using Western blot analysis. The results expressed are representative of 3 independent experiments. Abbreviations: ANOVA, analysis of variance; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; LPS/-, LPS treatment group; mRNA, messenger RNA. The symbol “*” means without LPS or lycopene treatment, symbol “#” means with LPS or lycopene treatment.
The quantitative data are shown in Fig. 3B and D, respectively. Additionally, DNA binding activity of NF-κB was reduced by pre-mixing nuclear extract from LPS-treated cells with anti-p65 but not anti-p50 antibody (Supplementary Fig. 1). These results indicate that lycopene exerts anti-inflammatory effects in microglial cells.

3.2. Involvement of HO-1 in lycopene inhibits LPS-induced COX-2 expression

HO-1 has been shown to play an important role in oxidative stress, and it exerts cytoprotective properties in response to pathological stimulation (Calo et al., 2010; Carter et al., 2004; Lee et al., 2009). In this study, we further investigated whether induction of HO-1 is involved in the anti-neuroinflammatory properties of lycopene. Stimulation of BV-2 microglia or mouse primary cultured microglia with various concentrations of lycopene for 24 hours increased HO-1 protein expression in a concentration-dependent manner (Fig. 4A). Additionally, lycopene also increased HO-1 protein expression in rat primary cultured microglia (Fig. 4B). To further determine whether induction of HO-1 is involved in the inhibitory effect of lycopene on LPS-induced COX-2 expression, BV-2 microglia and mouse primary cultured microglia were pre-incubated with the HO-1 pharmacological inhibitor zinc protoporphyrin IX (0.1 μM) for 30 minutes followed by treatment with lycopene for another 30 minutes, and then cells were stimulated with LPS. As shown in Fig. 4C, zinc protoporphyrin IX dramatically reversed the inhibitory effect of lycopene on LPS-induced COX-2 expression. Involvement of HO-1 in lycopene-inhibited LPS-induced COX-2 expression was further investigated. After transfection with siRNA against HO-1 for 24 hours, microglia were pretreated with lycopene for 30 minutes before LPS stimulation for another 24 hours. Transfection with HO-1 siRNA effectively reversed the inhibitory effect of lycopene on LPS-induced COX-2 and PGE2 expression (Fig. 4D and E, respectively). These results suggest that lycopene inhibited LPS-induced COX-2 expression via upregulation of HO-1 in microglial cells.

3.3. Involvement of AMPKα in lycopene-inhibited COX-2 expression in microglial cells

AMPK plays an important role in cellular energy homeostasis and metabolic regulation. Our previous study showed that activation of AMPK represses proinflammatory responses in microglia (Lu et al., 2010a). Here, we further investigated the molecular mechanisms of AMPKα on the regulatory effects of lycopene in microglia. In BV-2 microglia and mouse primary cultured microglia, stimulation of cells with lycopene increased AMPKα phosphorylation at Thr172 in a time-dependent manner (Fig. 5A). Moreover, lycopene also increased phosphorylation of AMPK upstream molecular regulators, LKB1 (Ser428) and CaMKII (Thr286), in microglia (Fig. 5A). Additionally, lycopene also increased AMPKα phosphorylation at Thr172 in rat primary cultured microglia (Fig. 5B). Importantly, treatment of microglia with lycopene resulted in an accumulation of AMPKα phosphorylation in the nucleus (Fig. 5C). Additionally, we also checked the fractions in nuclear and cytosolic extracts, as shown in Supplementary Fig. 2. Moreover, immunofluorescence staining examination of AMPKα phosphorylation localization showed that the AMPKα phosphorylation was primarily located in the cytoplasm during the control state. However, AMPKα phosphorylation translocated from the cytoplasm into the nucleus in response to lycopene...
treatment (Fig. 5D). To investigate the role of AMPKa1 in lycopene-regulated HO-1 expression and its anti-inflammatory effects, cells were pretreated with the AMPK inhibitors Ara A or Compound C, for 30 minutes followed by stimulation with lycopene for 24 hours. As shown in Fig. 6A, Compound C and Ara A attenuated lycopene-induced HO-1 expression in microglia. We further confirm that AMPKα activation is involved in lycopene-inhibited COX-2 expression in microglia. After pretreatment with Ara A for 30 minutes, lycopene was added for another 30 minutes before stimulation with LPS for 24 hours in the BV-2 microglia. The expression of COX-2 was determined using Western blot analysis. The results expressed as mean ± standard error of the mean from at least 3 independent experiments. * p < 0.05 compared with the control group; # p < 0.05 compared with the LPS treatment; ** p < 0.05 compared with the lycopene and LPS treatment group. Abbreviations: COX, cyclooxygenase; HO, heme oxygenase; LPS, lipopolysaccharide; PGE, prostaglandin; Znpp IX, zinc protoporphyrin IX. The symbol “-” means without LPS or lycopene treatment, symbol “+” means with LPS or lycopene treatment.

3.4. Lycopene inhibits microglial activation and improves the impairment of motor function in a mouse model

It has been reported that systemic inflammation produced by intraperitoneal administration of LPS results in neuroinflammation-associated motor deficits (Jang et al., 2013). To investigate the effect of lycopene on LPS-induced microglial activation and motor coordination dysfunction, we performed an immunohistochemical analysis of microglia and an accelerating rotarod test, respectively. Mice were continuously administered lycopene (5 or 10 mg/kg) once daily for 3 consecutive days, followed by injection with LPS 2 hours later for another 24 hours (Fig. 7A). LPS-treated mice had shorter latency on the rotarod test, thus demonstrating motor impairments compared with the control group. However, treatment with lycopene significantly ameliorated these motor-impaired effects in LPS-injected mice (Fig. 7B). Furthermore, the activation of microglia was assessed morphologically using immunohistochemical analysis with the classic antibody specific for Iba-1. After intraperitoneal injection of LPS, microglia began to retract their processes and enlarge their cell bodies and had more intensive immunoreactivity.
compared with cells in the control group (Fig. 7C). LPS injection induced a pronounced hypertrophy of microglial cells, and activated microglia (Iba1-positive) were observed to be abundant in the striatum (Fig. 7C). Additionally, LPS injection also induced hypertrophy of microglial cells, and activated microglia (Iba1-positive) in the cortex and hippocampus (Supplementary Fig. 4). Parallel to the immunohistochemical results, the activation of microglia was attenuated by lycopene administration, which correlated with the motor performance test. Lycopene altered microglial cells from an activated form back to a ramified morphology with less cytoplasm (Fig. 7C). The immunohistochemistry results further support the hypothesis that lycopene inhibited neuroinflammation and also improved neuroinflammation-associated motor impairment.

**Fig. 5.** Lycopene increases AMPK activation and translocation to the nucleus in microglia. (A) BV-2 microglia and mouse primary cultured microglia were incubated with lycopene for indicated periods (5, 10, 30, 60, or 120 minutes), and the phosphorylated AMPKαThr172, LKB1Ser428, and CaMKIIThr286 were determined using Western blot analysis. (B) Rat primary cultured microglia were treated with lycopene for 30 or 60 minutes, and phosphorylated AMPKαThr172 was determined using Western blot analysis. (C) BV-2 microglia were incubated with lycopene for the indicated periods (10, 30, 60, or 120 minutes), and the levels of nuclear phosphorylated AMPKαThr172 were determined using Western blot analysis. The results expressed are representative of at least 3 independent experiments. (D) BV-2 microglia were treated with lycopene for 120 minutes, and the cellular localization of phosphorylated AMPKαThr172 (top frames) and DAPI localization of nuclei (middle frames) are shown by immunofluorescence (bottom frames illustrate merged images). The results are representative of 3 independent experiments. Abbreviations: AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; CaMK, calmodulin-dependent protein kinase; DAPI, 4',6-diamidino-2-phenylindole; LKB, liver kinase B1.
4. Discussion

Microglia/macrophages exert different activation phenotypes in response to CNS insults, which has been classified as either classically activated or alternatively activated (M2) (Martinez, 2011; Seledtsov and Seledtsova, 2012; Sica and Mantovani, 2012; Van Ginderachter et al., 2006). Although microglial activation has conventionally been linked to neuroinflammation and neurotoxicity (classically activated macrophage phenotype), recent reports have shown that microglial activation leads to neurodegeneration and microglia can generate neuronal growth factors and anti-inflammatory cytokines contributing to neuroprotection (Czeh et al., 2011; Gordon, 2003; Hanisch and Kettenmann, 2007; Michelucci et al., 2009; Schwartz et al., 2006). Microglial polarization toward the alternatively activated phenotype (M2 macrophage) is induced by IL-4, IL-13, or IL-10. M2 microglia/macrophage activation can reverse impaired spatial learning by suppression of inflammatory molecules in the hippocampus in a mouse model (Guerrero et al., 2012). Induction of M2 phenotype polarization and increased anti-inflammatory cytokine expression might be a promising strategy for antineuroinflammation and neuroprotection. Increasing evidence highly suggests that HO-1 expression is involved in microglia/macrophage polarization toward the M2 phenotype (Louvet et al., 2011; Sierra-Filardi et al., 2010; Weis et al., 2009). In this study, our results indicate that lycopene-induced HO-1 expression in activated M2 microglia exerts reduction of the expression of COX-2 and inflammatory mediators.

Lycopene also increases M2 phenotype macrophage gene expression (IL-4, IL-10, IL-13, arginase 1 (ARG1), and chitinase-like-3 (Ym1)) (Supplementary Fig. 5). It has been reported that activation of AMPK is associated with activated-M2 phenotype (Lovren et al., 2010). Our previous study and other reports have shown suppression of neuroinflammatory responses through AMPK activation in microglia, macrophages, and astrocytes (Giri et al., 2004; Lu et al., 2010a; Tsuji et al., 2011b).

Lycopene has been shown to cross the blood–brain barrier and is present in the CNS (Rao and Rao, 2007). The therapeutic benefit of lycopene is well established in prostate carcinoma in various clinical trials (Magbanua et al., 2011) and has been proposed for the management of high-grade gliomas (DeLorenze et al., 2010; Puri et al., 2010). In addition, it has been reported that lycopene prevents neuronal cell death by inhibiting mitochondrial oxidative stress...
molecular mechanisms underlying the anti-inflammatory responses of lycopene in microglia have not been addressed. In this study, we investigated the effects of lycopene in microglia. We demonstrated that lycopene induces phosphorylated AMPK pathway in microglial cells. In our study, we revealed a better understanding of AMPK-regulated anti-inflammatory actions, which could provide a novel strategy for the treatment of neuroinflammatory responses. The detailed regulation of AMPK pathway in the nucleus needs further investigation.

Intraperitoneal injection of LPS is a useful method for investigating inflammation-caused motor and cognitive deficits in rodent models. Peripheral inflammation exacerbates brain cytokine expression, leading to neurodegeneration (Qin et al., 2007), motor impairment (Jang et al., 2013), and cognitive impairment (Bossu et al., 2012). LPS treatment results in motor deficits in normal animals and exacerbated deficits in transgenic neurodegenerative disease mice. A recent study using the rotarod test has shown that intraperitoneal injection of LPS (5 mg/kg) induced progressive motor impairment from 4 to 24 hours (Jang et al., 2013). Franciosi et al. reported that chronic peripheral LPS injection (1 mg/kg) induced microglial activation, striatal volume loss, and motor impairment starting at 8 months (Franciosi et al., 2012). Furthermore, Cunningham et al. (2009) showed that systemic challenge with low doses of LPS (100–500 µg/kg) increased cytokine expression in the long-term period (15 weeks) of postinoculation in control mice. However, the motor coordination abnormality determined using horizontal bar and inverted screen was not observed (Cunningham et al., 2009). We also tested a single injection of LPS (E. coli 0127:B8; 5 mg/kg) which did not have significant effect on motor impairment and microglial activation in our model. Intraperitoneal injection of LPS (20 mg/kg) evoked microglial activation significantly in striatum, cortex, and hippocampus and subsequently impaired the motor deficits. The potency and motor function impairment after LPS administration might depend on the dosage of LPS, treatment duration, LPS strains, and the method of motor evaluation. In the present study, we performed an intraperitoneal injection of LPS (20 mg/kg), which caused a shorter latency on the rotarod test; however, treatment with lycopene significantly alleviated these motor-impaired effects in LPS-injected mice.

Based on our present results, we suggest that lycopene-inhibited LPS-induced COX-2 expression is mediated by HO-1 activation through the AMPKα pathway in microglial cells. In our study, we also found that intraperitoneal injection of LPS impaired motor function and that lycopene significantly alleviated LPS-induced microglial activation and motor coordination dysfunction. Our results indicate that lycopene might be useful as a therapeutic agent for the treatment of neuroinflammation-associated disorders and activate an anti-inflammatory signaling pathway that could contribute to antineuroinflammatory effects.

**Disclosure statement**

The authors report no biomedical financial interests or potential conflicts of interest.

All protocols for animal experiments were approved by Animal Care Committees of China Medical University (Taichung, Taiwan).

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.neurobiolaging.2013.06.020](http://dx.doi.org/10.1016/j.neurobiolaging.2013.06.020).


