Research article

Effect of apigenin on tryptophan metabolic key enzymes expression in lipopolysaccharide-induced microglial cells and its mechanism

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ABSTRACT

[**Aims**] Flavonoid apigenin (API) has a wide range of biological functions, particularly anti-inflammation. Indoleamine 2,3-dioxygenase (IDO) and 2-Amino-3-carboxymuconate-6-semialde-hyde decarboxylase (ACMSD) are important tryptophan metabolic enzymes that play pivotal roles in the production of toxic metabolite quinolinic acid. However, the relationship between inflammation and ACMSD remains unclear. The present study investigated the relationship between inflammation and tryptophan metabolic key enzymes. Similarly, the anti-inflammatory effect of API on important tryptophan metabolic enzymes was examined in lipopolysaccharide (LPS)-treated microglial cells.

[**Main methods**] MG6 cells were exposed to LPS with or without API treatment for 24–48 h. *IDO* and *ACMSD* mRNA expression and production of inflammatory mediators were analyzed. Activation of inflammatory signaling pathways, such as mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB), was also examined to study the mechanism of API in the inflammatory state.

[**Key findings**] LPS suppressed *ACMSD* expression and enhanced *IDO* expression. However, API elevated *ACMSD* mRNA expression and suppressed *IDO* mRNA expression in LPS-treated MG6 cells. Furthermore, API suppressed interleukin-6 and nitric oxide production, whereas over-production of inflammatory mediators enhanced IDO expression and assisted tryptophan degradation. API also inhibited activation of extracellular signal-regulated kinase (Erk) and jun N-terminal kinase (JNK) MAPK, and degradation of IκBα.

[**Significance**] These results indicate alteration of *ACMSD* expression under inflammatory conditions. Moreover, API recovers expression of tryptophan metabolic key enzymes, which may be mediated by inhibition of proinflammatory mediator production via inactivation of Erk, JNK MAPK, and NF-κB pathways in LPS-stimulated microglial cells.

1. Introduction

Apigenin (API) is a natural flavonoid that has received immense attention because of its biological activities, which include anti-inflammation [1], anti-cancer [2], anti-diabetes [3], its roles in amnesia and Alzheimer's disease [4], and its effects on depression and insomnia [5]. API is abundantly present in various vegetables and fruits. Parsley is a major source of this flavone, which contains 215.46 mg API/100 g [6]. Other rich sources of API are kumquats, celery, peppermint, oregano, and perilla that contain 21.87, 19.1, 5.39, 2.57, and 0.07 mg API/100 g, respectively [6]. Previous studies have reported that API protects neurons and glial cell from Aβ oligomers and inflammatory stimuli [7] and ameliorates cognitive dysfunction and neuronal damage in AD mouse model via apoptosis, amyloidogenesis, and BDNF/TrkB pathways [8]. Nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPK) pathways have been suggested to be involved in API-mediated anti-inflammatory effects [9,10].

The kynurenine pathway (KP) is the main pathway in the catabolism of tryptophan and responsible for ≥90% of tryptophan degradation (Fig. 1) [11]. KP participates in the inflammatory mechanisms of neurogenic and autoimmune diseases through the action of its critical metabolic enzymes. Alterations in KP metabolites and enzyme activities in diseases are accompanied by changes in immune functions that include the neuroimmune system [12].

Indole amine 2,3 dioxygenase (IDO), a rate-limiting enzyme in tryptophan degradation, is expressed in various cell types including macrophages, which is induced by proinflammatory cytokines [13] and bacterial lipopolysaccharide (LPS) [14]. A high level of IDO in the brain is associated with neuronal disorders, such as depression, Alzheimer's disease, Huntington's disease, and Parkinson's diseases, which are partially caused by upregulation of toxic quinolinic acid (QUIN) production in the kynurenine pathway [15–16]. QUIN, a potent excitotoxin, is an agonist of the N-methyl-D-aspartate receptor [17] and is often implicated in the pathogeneses of neuronal diseases [15–18].

2-Amino-3-carboxymuconate-6-semialdehyde decarboxylase (ACMSD) is a critical enzyme in the NAD$^+$ pathway (Fig. 1), which regulates and limits the formation of QUIN by catalyzing the production of another metabolite. An increased level of QUIN due to reduced ACMSD activity may act as a major trigger of excitotoxic neuronal death in Parkinson's disease and other neurodegenerative diseases [19–20]. ACMSD is expressed in the liver, kidney, and central nervous system (CNS) [21]. The expression and activity of ACMSD are influenced by dietary and metabolic conditions [22–23], and have recently begun to be investigated in neurological disorders. However, the relationship between this enzyme and inflammation remains unclear. In the present study, we clarified the direct relationship between ACMSD and inflammation.

Because of the pivotal role of IDO and ACMSD enzymes in the immune system, suppression of IDO and elevation of ACMSD appear to be effective to suppress the pathogeneses of neurodegenerative diseases. Therefore, we investigated the alterations of ACMSD and IDO enzymes in LPS-induced neuroinflammation of microglial cells. Similarly, the potential molecular mechanism that underlies the anti-inflammatory effect of API on tryptophan metabolism and inflammatory processes was examined because the anti-inflammatory mechanism of API in LPS-stimulated microglial cells has not been clarified.
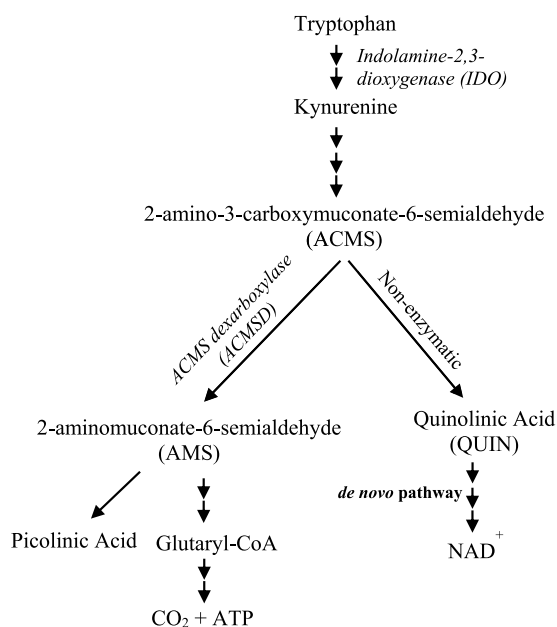

Fig. 1. Simplified diagram of the Tryptophan-kynurenine and -NAD pathway.

2. Materials and methods

2.1. Reagents

API and LPS were purchased from Sigma-Aldrich (St Louis, MO, USA). API and LPS were dissolved in dimethyl sulfoxide (DMSO) and phosphate-buffered saline (PBS), respectively. Antibodies against IκBα (sc-371) was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). Antibodies against p-p38 (#9215), p38 (#9212S), p-Erk (#9101S), Erk (#4695), p-JNK (#9251), JNK (#9252), and β-actin (#4967) were obtained from Cell Signaling Technology (Danvers, MA, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). CellTiter 96® AQueous One Solution was purchased from Promega (Tokyo, Japan). ELISA MAX™ was purchased from BioLegend (San Diego, CA, USA). RNAiso plus reagent was purchased from Takara Biotechnology (Shiga, Japan). ReverTraAce® qPCR RT Master Mix with gDNA Remover and THUNDERBIRD® SYBR qPCR MIX were from TOYOBO CO., Ltd. (Tokyo, Japan). The remaining chemicals and reagents were analytical grade and commercially available.

2.2. Cell culture and treatment

Mouse microglial cell line MG6 cells [24,25] were obtained from RIKEN Cell Bank (Tokyo, Japan). The cells were cultured in DMEM supplemented with 10% FBS and incubated at 37 °C with 5% CO_2 for 2–3 days. Dishes with 80%–90% confluent cells were subcultured and cells from passages 6–7 were used for the treatments. MG6 cells were incubated with 100 ng/mL of LPS concomitant by API at various concentrations for either 30 min, 24 h, 30 h, or 48 h, depending on the experiment. We chose the above-mentioned dose of LPS that was shown remarkable alteration of tryptophan metabolic key enzymes (Fig. 4) and effectively induced the release of proinflammatory mediators in previously reported studies [26,27]. Untreated cells and LPS-treated cells without API co-treatment were cultured in parallel as negative and positive controls.

2.3. Cell viability assay

Cell viability was assessed by a Cell Titer 96® AQueous One Solution Cell Proliferation Assay in accordance with the manufacturer's protocol. Briefly, MG6 cells (0.4×10^5/well) were seeded on 96-well plates and incubated overnight at 37 °C with 5% CO_2. Cells were then treated with or without API (5, 10, 30, and 50 μM) and/or LPS (100 ng/mL). After 24 h of treatment, the cells were incubated with 20 μL Cell Titer reagent for 15 min at 37 °C. Absorbance was then measured at 450 nm using a microplate reader (Infinite® F200 PRO, Tecan Group Ltd., Männedorf, Switzerland).

2.4. Interleukin-6 (IL-6) detection

MG6 cells (0.5×10^6/well) were seeded on 12-well plates and incubated at 37 °C with 5% CO_2 for 24 h. The medium was then replaced with LPS (100 ng/mL)- and/or API (5, 10, and 20 μM)-containing media and the cells were incubated for another 24 h. The culture media were collected at the end of treatments and used to detect IL-6 secretion. IL-6 concentrations in the culture media were measured using an ELISA MAX™ in accordance with the manufacturer's protocol. Absorbance was measured at 450 nm using the microplate reader.

2.5. Nitric oxide (NO) assay

After incubation of MG6 cells for 24 h, NO concentration in the medium was measured by the Griess reaction method [28]. In brief, 100 μL of culture medium was applied to each well of a 96-well plate. Then, 100 μL of an equal mixture of Griess reagent:0.2% N-1-naphthyl ethylenediamine (Wako, Osaka, Japan) and 2% sulfanilamide (Sigma-Aldrich) in 10% H_3PO_4 (Wako) was added to each well. After incubation for 10 min at room temperature, the absorbance was measured at 550 nm using the microplate reader.

2.6. RNA extraction and quantitative real-time PCR analysis

MG6 cells (0.5×10^6/well) were seeded on 12-well plates and incubated at 37 °C with 5% CO_2 for 24 h. The medium was then replaced with LPS (100 ng/mL)- and/or API (5, 10, and 20 μM)-containing media and the cells were incubated for another 30 or 48 h. Subsequently, RNAiso plus reagent was used to extract total RNA from the cells in accordance with the manufacturer's protocol. The total RNA was reverse transcribed to cDNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover in accordance with the manufacturer's protocol. cDNA samples were subjected to PCR using Applied Biosystem StepOnePlus™ Real-time PCR System (Life Technologies, Waltham, MA, USA) and THUNDERBIRD® SYBR qPCR MIX. The following primers were used: *IDO* forward, 5'-AAGGGCTTCTTCCTCGTCTC-3' and reverse, 5'-AAAAACGTGTCTGGGTCCAC-3'; *ACMSD* forward 5'-GCCTCCCACAGTTGGATAGA-3' and reverse 5'-GCCTCAAACACAGACCCATT-3'; *GAPDH* forward 5'-ACCCAGAAGACTGTGGATGG-3' and reverse 5'-CACATTGGGGG-TAGGAACAC-3' and *18S rRNA* forward 5'-CGCGGTTCTATTTTGTTGGT-3' and reverse 5'-AGTCGGCATCGTTTATGGTC-3' as an internal standard for normalization of IDO and ACMDS mRNA expression levels, respectively. The amplification conditions were 95 °C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 31 s, and 95 °C for 15 s, followed by 60 °C for 1 min.

2.7. Protein extraction

MG6 cells (4×10^6/dish) were seeded on 60-mm dishes and incubated at 37 °C with 5% CO_2 for 24 h. Then, the medium was replaced with LPS (100 ng/mL)- and/or API (5, 10, and 20 μM)-containing media. Protein collection of MAPK and NF-κB signaling was performed after 30 min of LPS stimulation when activation of these proteins was clearly observed according to the results of preliminary experiments (data not shown). Subsequently, cells were washed with PBS and collected in protein extraction buffer that contained protease (Calbiochem, San Diego, CA, USA) and phosphatase (Wako) inhibitor cocktails. Aggregates were lysed by an ultrasonicator and centrifuged at 13,000×g at 4 °C for 5 min. The supernatants were collected as whole protein samples.

2.8. Western blot analysis

Protein samples were applied to gels at 20–50 μg/well and then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transferring to a polyvinylidene difluoride membrane (Millipore, Darmstadt, Germany), the blot was blocked with 5% dry skim milk for 1 h and then washed three times with PBS with 0.05% Tween 20 (PBST). Subsequently, membranes were treated overnight with antibodies against p-p38 (1:300), p38 (1:1000), p-Erk (1:2000), Erk (1:1000), p-JNK (1:2000), JNK (1:1000), IκBα (1:300), or β-actin (1:1000). After washing with PBST, the membranes were treated with anti-rabbit IgG horseradish peroxidase conjugate (1:3000) (W4018) (Promega) as secondary antibody. Blots were developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore) and detected by a Light Capture III (ATTO Co., Ltd., Tokyo, Japan). Luminescence intensity was quantified using CS Analyzer 3.0 (ATTO Co., Ltd.).

2.9. Statistical analysis

Data are represented as the mean ± standard error (SE). Statistics were performed using the Bell Curve for Excel ver. 2.10 (Social Survey Research Information Co., Ltd., Tokyo, Japan). Data were analyzed by one-way ANOVA with Dunnett test or Steel's test. Data of Fig. 5 were analyzed by Student's t-test. *$p < 0.05$ and **$p < 0.01$ were considered statistically significant.

3. Results

3.1. Effect of API and LPS on cell viability

The cytotoxicity of API and LPS on the viability of MG6 cells was investigated using an MTT assay. Cells were treated with API (0, 5, 10, 30, and 50 μM) and/or LPS (100 ng/mL) for 24 h. The results revealed that 30 and 50 μM API treatments significantly decreased the viability of MG6 cells compared with the control group (Fig. 2a). However, API treatments up to 50 μM in the presence of LPS had no significant effect on cell viability (Fig. 2b). Therefore, API concentrations of lower than 30 μM were selected for the next stage of study, which are also supported by earlier experiments [1,27].

3.2. Production of inflammatory mediators

The levels of inflammatory mediators, such as NO, IL-6, and TNF, were measured. Many *in vitro* studies reported that the production of inflammatory mediators was sufficiently induced at 24 h of LPS exposure [1,27,29]. Stimulation of MG6 cells with LPS resulted in significant increases in the concentrations of NO (Fig. 3a), IL-6 (Fig. 3b), and TNF (data not shown) in the culture media. API treatment led to significant inhibition of NO and IL-6 releases induced by LPS in a dose-dependent manner. However, API did not affect
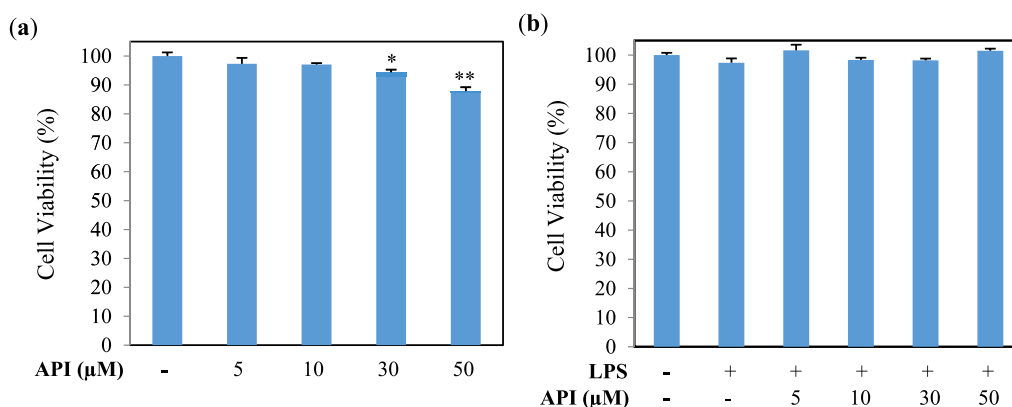

Fig. 2. Viability of MG6 cells treated for 24 h (a) with or without API and (b) with or without LPS (100 ng/mL). Values indicate the mean ± SE (n = 6). *$p < 0.05$ and **$p < 0.01$ vs API (−).
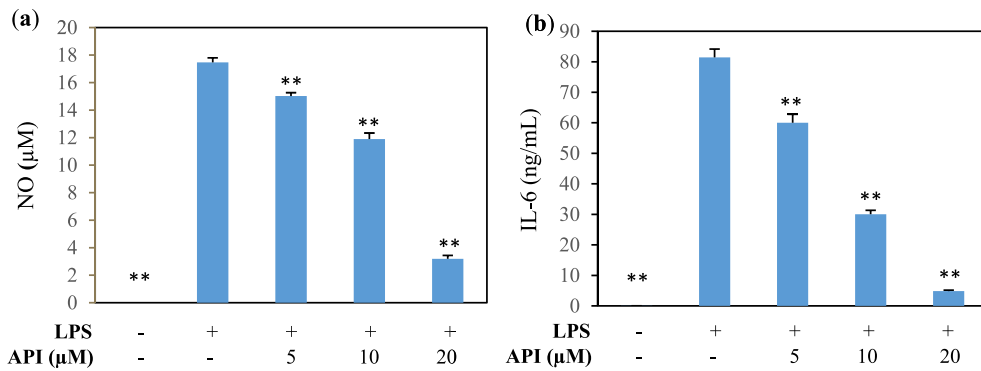
Fig. 3. Effect of API on proinflammatory mediator secretion from MG6 cells after 24 h of LPS treatment (100 ng/mL). (a) NO and (b) IL-6 secretion. Values indicate the mean ± SE (n = 6). **$p < 0.01$ vs LPS (+).
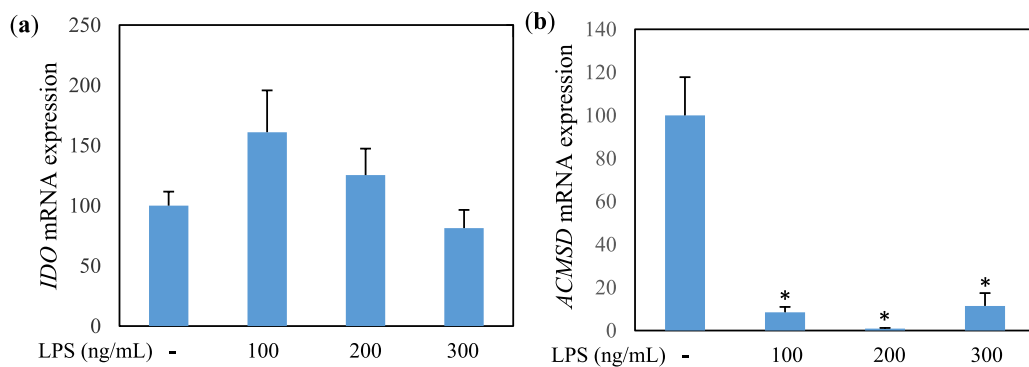

Fig. 4. *IDO* (a) and *ACMSD* (b) mRNA expression (relative value) in MG6 cells treated with LPS for 24 h. Values indicate mean ± SE (n = 5–6). *$p < 0.05$ vs LPS (−).
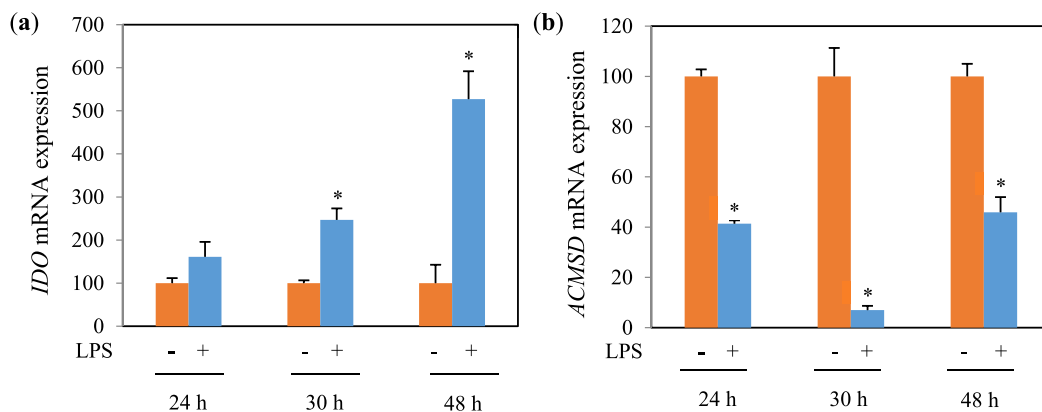

Fig. 5. *IDO* (a) and *ACMSD* (b) mRNA expression (relative value) in MG6 cells treated with 100 ng/mL of LPS for various stimulation times. Values indicate mean ± SE (n = 5–6). *$p < 0.05$ vs LPS (−).

LPS-induced TNF production (data not shown).

3.3. IDO and ACMSD mRNA expression

Since this study mainly aims to investigate the relationship of inflammation with tryptophan metabolic enzymes, particularly

ACMSD, we carried out qPCR analyses of mRNAs isolated from MG6 cells treated with LPS. To obtain the optimum experimental conditions, the preliminary experiment of LPS in several doses and stimulation time on the mRNA expression level of *IDO* and *ACMSD* was performed. LPS treatment with a dose of 100 ng/mL resulted in the optimum induction effect to increase *IDO* expression level (p < 0.0857) (Fig. 4a) followed by reduction of *ACMSD* level (Fig. 4b) in MG6 cells at 48 h (5a) and 30 h (Fig. 5b) of LPS stimulation, respectively. LPS treatment suppressed *ACMSD* mRNA expression (Figs. 5a and 6a) and induced *IDO* mRNA expression (Figs. 5b and 6b) compared with the LPS untreated group. Similarly, we examined the anti-inflammatory effect of API on tryptophan metabolism in LPS-induced microglial cells. API treatment results in a significant increment in *ACMSD* mRNA expression in a dose-dependent manner (Fig. 6a) and inhibition of *IDO* mRNA expression (Fig. 6b) in LPS-treated MG6 cells.

3.4. Expression of NF-κB and MAPK pathway proteins

NF-κB and MAPK pathways control the production of inflammatory mediators that include inflammatory cytokines and chemokines [9,30]. The cytoplasmic level of IκBα protein was measured by western blotting to determine the inhibitory effect of API on NF-κB pathways. After stimulation of MG6 cells with LPS for 30 min, cytosolic IκBα protein was degraded. The LPS-induced IκBα degradation was lightly inhibited by co-treatment of LPS and API at high doses (Fig. 7a). LPS treatment for 30 min also facilitated the phosphorylation of MAPK proteins, which included JNK, Erk and p38 in MG6 cells (Fig. 7b). API attenuated the levels of phosphorylated JNK and ERK with the highest decrease in phosphorylation intensity at 72% and 52%, respectively, in 20 μM API-treated group in comparison with those in LPS-treated group. However, LPS-induced phosphorylation of p38 was not remarkable affected by API with a 46% reduction in phosphorylation level of p38 at 20 μM.

4. Discussion

Flavonoids are abundant in vegetables, fruits, and herbs. A high intake of diets rich in flavonoids is reported to prevent numerous chronic diseases that include metabolic disorders, cardiovascular diseases, neurodegenerative diseases, cancer, and allergies [1, 31–34]. API is a natural flavonoid that has received immense attention because of its low intrinsic toxicity and various biological activities [1–5]. Previous studies have reported that API remarkably inhibits cytokine production and astrocyte activation stimulated by LPS via NF-κB, MAPK and STAT3 pathways [9]. It has also been shown that API provides protection against neuroinflammation associated with AD *in vitro* and *in vivo* [7,8]. API is proposed to be an important dietary flavonoid with potent neuroprotective, chemopreventive, and anti-inflammatory abilities [1,2,4,9,10].

LPS is produced by gram-negative bacteria, which binds to a surface receptor called Toll-like receptor 4 (TLR4) and triggers the secretion of cytokines [35]. Activating of microglial cells by LPS leads to increased secretion of inflammatory mediators that include NO, IL-6, and TNF [36,37]. In the present study, microglial cells were treated with LPS to mimic the inflammatory state in neurodegenerative diseases. API treatment led to significant reductions of LPS-induced NO and IL-6 levels in MG6 cells. However, API did not affect TNF production. LPS induced massive NO production in an immune cell that participates in pathological acute and chronic inflammatory disorders [38]. During inflammation, high production of NO causes cytotoxicity, tissue and DNA damage, and mutations [39]. Moreover, IL-6 via a specific receptor stimulates various signaling pathways activating gene [40]. In CNS disorders, various molecules elevate the IL-6 level, including LPS and TNF, which increases the central production of inflammatory cytokines [37,40]. Therefore, our results suggest that API attenuates LPS-induced activation of glial cells and the consequential production of inflammatory metabolites, whereby reducing of chronic inflammation is an effective strategy to prevent the pathological progression of chronic diseases.

Several studies have demonstrated the significance of kynurenine metabolism in aging and neurodegenerative diseases through the
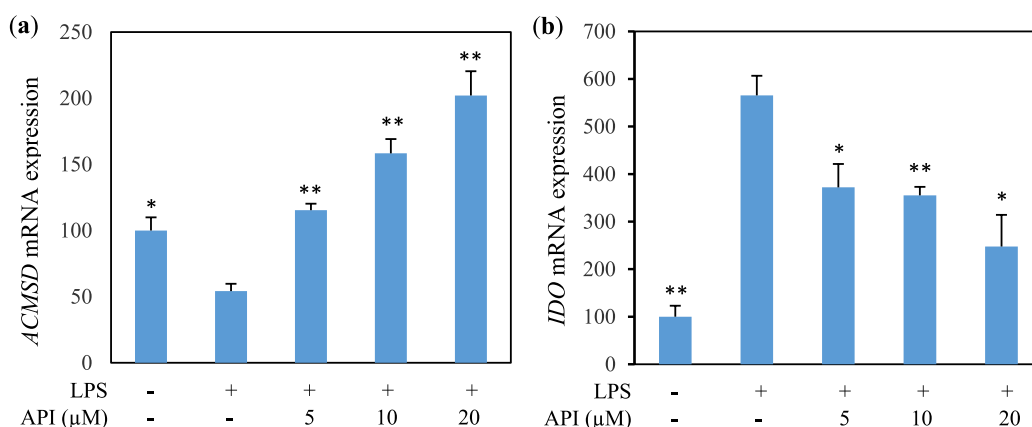

Fig. 6. Effect of API on mRNA expression in MG6 cells treated with LPS (100 ng/mL). (a) *ACMSD* (30 h) and (b) *IDO* (48 h) expression (relative value). Values indicate the mean ± SE (n = 6). *p < 0.05 and **p < 0.01 vs LPS (+).

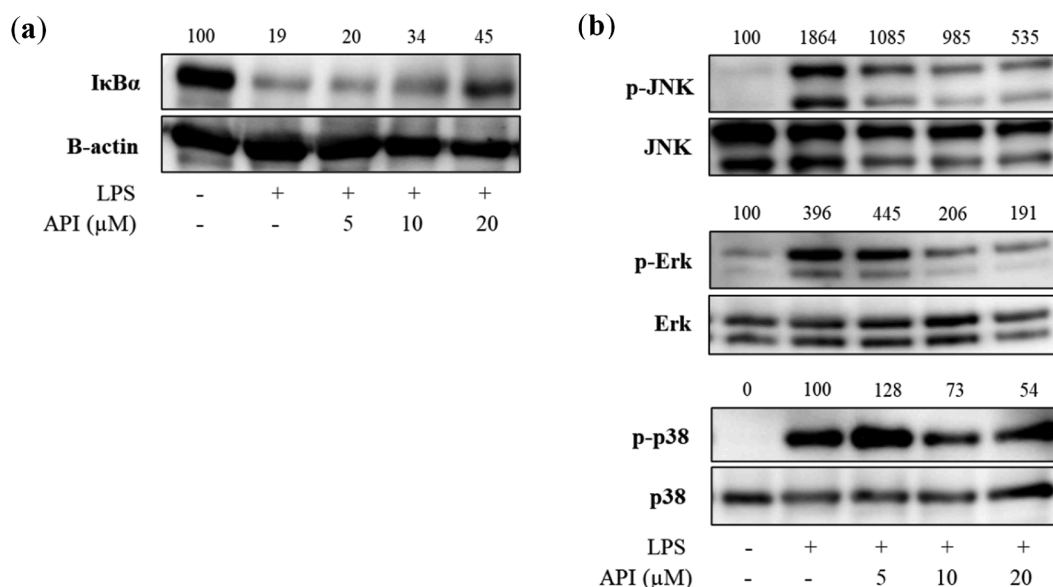expression of ACMSD. Furthermore, suppression of NF-κB activation enhances PGC1α expression [62], which is positively implicated in regulating ACMSD expression in cooperation with HNF4α [63]. Thus, controlling the transcriptional regulatory region of *IDO* and *ACMSD* genes may modulate the expression level of these enzymes.

Taken together, our results indicated that API recovered the LPS-induced decrease in *ACMSD* and increased *IDO* mRNA expression. These actions may be mediated by attenuation of IL-6 and NO production via inhibition of Erk and JNK MAPK phosphorylation as well as nuclear translocation of NF-κB.

5. Conclusion

ACMSD and *IDO* tryptophan metabolic key enzyme's gene expressions are altered under the inflammatory condition induced by LPS. Additionally, API recovered the gene expression of tryptophan metabolic enzymes and suppressed the production of IL-6 and NO. The anti-inflammatory effect of API may be mediated by inhibiting pro-inflammatory mediators via inactivation of Erk, JNK MAPK, and NF-κB pathways in LPS-stimulated microglial cells, which prevents a vicious cycle of generating inflammatory metabolites. Ultimately, API is a promising therapeutic target for inflammatory diseases related to neurodegeneration.

Credit author statement

Dian Kurniati: Performed the experiments; Analyzed and interpreted the data; Wrote the paper; Shizuka Hirai: Contributed reagents, materials, analysis tools or data; Yukari Egashira: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supp. Material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2022.e12743.

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