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The hyaluronan synthesis inhibitor 7-hydroxy-4-methylcoumarin inhibits LPS-induced inflammatory response in RAW 264.7 macrophage cells

Gwan Bo Kim[1] · **Chang-Gu Hyun**[1]

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Abstract 7-Hydroxy-4-methylcoumarin (7H-4MC) inhibits hyaluronan production in multiple cell lines and tissue types both in vitro and in vivo. It is a commercially available drug approved for human use, called hymecromone, in European and Asian countries to prevent biliary spasms. Nevertheless, as the pharmacological efficacy of 7H-4MC has not yet been reported in macrophages, this study investigated its anti-inflammatory effects and mechanism of action using lipopolysaccharide (LPS)-induced RAW 264.7 macrophages. LPS-induced RAW 264.7 cells were treated with various concentrations of 7H-4MC (62.5, 125, 250, and 500 μM). The application of 7H-4MC significantly reduced nitric oxide and prostaglandin E_2 production without cytotoxic effects. Additionally, 7H-4MC strongly decreased the expression of inducible nitric oxide synthase and cyclooxygenase. Furthermore, 7H-4MC reduced the production of proinflammatory cytokines, such as tumor necrosis factor-α, interleukin (IL)-1β, and IL-6. Finally, 7H-4MC exerted its potent anti-inflammatory actions via the upregulation of IκB-α production, which led to the inhibition of nuclear factor-κB (NF-κB) activity. These results, obtained in macrophage cell lines, suggest that 7H-4MC prevents inflammatory diseases via the NF-κB signaling pathway and that its use could be beneficial for human health. Ultimately, this is the first report describing the anti-inflammatory activity of 7H-4MC in a macrophage cell line.

Keywords Inflammation · IκB-α · 7-Hydroxy-4-methylcoumarin · Hyaluronan · Macrophage · Nuclear factor-κB

Chang-Gu Hyun (✉)
E-mail: cghyun@jejunu.ac.kr

[1]Jeju Inside Agency and Cosmetic Science Center, Department of Chemistry and Cosmetics, Jeju National University, Jeju 63243, Republic of Korea

Introduction

Coumarins (benzo-α-pyrones) are oxygen heterocycles and naturally occurring benzopyrene derivatives that have been identified in plants, bacteria, and fungi. They play a pivotal role as major lead compounds in drug research and development because they have low molecular weight, high solubility in most organic solvents, low toxicity, high bioavailability, and various pharmacological effects as anticoagulants, antimicrobial agents, anti-inflammatory agents, neuroprotectants, anti-diabetic agent, and anticonvulsants [1-3]. 7-Hydroxy-4-methylcoumarin (7H-4MC) recently began to attract our attention in an ongoing screening program designed to develop cosmetic ingredients from coumarin and its derivatives (Fig. 1).

7H-4MC is a commercially available drug that has been approved for human use; it is one of few prescription drugs directly derived from coumarin. It is called hymecromon and is prescribed in European and Asian countries to prevent biliary spasms. Currently, 7H-4MC is being investigated in human clinical trials as a treatment for hyaluronan-related fibrotic liver and autoimmune biliary disease [4]. 7H-4MC is widely used as a hyaluronan synthesis inhibitor and protects against lipopolysaccharide (LPS)-induced lung injury, oxidative damage, and tumors [5-7]. Its use significantly reduces hepatic lipid content in mice fed a choline-deficient amino acid diet [8]. Recently, 7H-4MC has been shown to exert effects against coronavirus disease 2019 (COVID-19) infection. Infection with the virus that causes COVID-19, severe acute respiratory syndrome coronavirus 2, results in viral pneumonia. According to a recent review by Ontong and Prachayasittikul, 7H-4MC treatment may prevent acute respiratory syndrome via the induction of hyaluronan deficiency in patients with severe COVID-19 infection and, ultimately, improve the survival rate of patients with COVID-19 [9]. Nevertheless, the anti-inflammatory activity of 7H-4MC remains uncharacterized. Therefore, we conducted a detailed study to investigate the anti-inflammatory effects of 7H-4MC in murine RAW 264.7 cells.

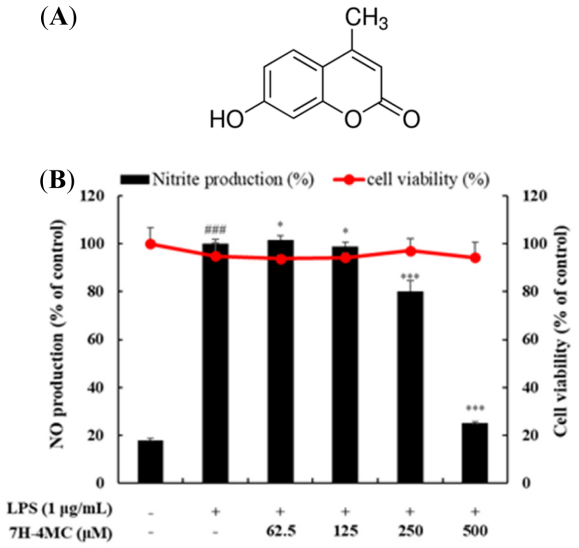

Fig. 1 Structure of 7-hydroxy-4-methylcoumarin (7H-4MC, A) and effect of 7H-4MC on nitric oxide production in LPS-induced RAW 264.7 cells (B). Cells were seeded in 24-well plates, incubated for 24 h, and treated with 7H-4MC (62.5, 125, 250, and 500 μM) and LPS for 24 h. Cytotoxicity of 7H-4MC was evaluated using MTT assays. Amount of nitric oxide in the medium was measured using Griess reagent. Results are presented as the mean ± SD of three independent experiments. $^{###}p$ <0.001 vs. control. $^{*}p$ <0.05, $^{***}p$ <0.001 vs. LPS alone

Inflammation is a complex process mediated by the activation of various immune cells, such as macrophages. Macrophages play an important role in the modulation of inflammatory responses through the release of inflammatory mediators and pro-inflammatory cytokines such as nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 [10-12]. LPS is the most abundant component of the gram-negative bacterial cell wall. It can stimulate the release of inflammatory mediators and pro-inflammatory cytokines in murine RAW 264.7 cells, which leads to an acute inflammatory response towards tissue injuries and pathogens. Therefore, LPS-induced RAW 264.7 cells are an excellent model for drug screening and the evaluation of potential inhibitors against inflammatory responses [13-15]. The present study determined whether 7H-4MC inhibited the release of inflammatory mediators and pro-inflammatory cytokines in LPS-induced RAW 264.7 cells.

Materials and Methods

Chemicals and reagents

7H-4MC was obtained from ChemFaces Biochemical Co., Ltd. (Wuhan, China). The bicinchoninic acid (BCA) protein assay kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were obtained from Gibco (Grand Island, NY, USA). The protease inhibitor cocktail, LPS, and Griess reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). The PGE_2 enzyme-linked immunosorbent assay (ELISA) kit was obtained from Abcam (Cambridge, UK). The mouse IL-1β ELISA kit was purchased from R&D Systems (St. Louis, MO, USA). The mouse IL-6 ELISA kit, mouse TNF-α ELISA kit, and anti-cyclooxygenase (COX)-2 antibody were obtained from BD Biosciences (San Diego, CA, USA). The primary antibodies against, IκB-α and β-actin and secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-inducible nitric oxide synthase (iNOS) antibody was obtained from Novus Biologicals (Littleton, CO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), the enhanced chemiluminescence (ECL) kit, the radioimmunoprecipitation (RIPA) buffer, dimethyl sulfoxide (DMSO), 20× Tris-buffered saline (TBS), and phosphate-buffered saline (PBS) were obtained from Biosesang (Sungnam, Korea). The 2× Laemmli sample buffer was obtained from Bio-Rad (Hercules, CA, USA).

Cell culture

RAW 264.7 cells were purchased from the Korea Cell Line Bank (Seoul, Korea). The cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in an incubator maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were passaged at 2-day intervals.

Cell viability assay

The MTT assay was used to evaluate the viability of RAW 264.7 cells after 7H-4MC application. The cells (1.5×10^5 cells/well) were seeded in 24-well plates (SPL, Gyeonggi-do, Korea). After a 24-h culture, the cells were treated with the 7H-4MC (62.5, 125, 250, and 500 μM) and LPS (1 μg/mL) for 24 h. Then, 400 μL of MTT solution (0.4 mg/mL) was added to each well, which were incubated for 4 h. The supernatant was removed, the formazan crystals were dissolved in DMSO (800 μL), and the optical density was measured at 570 nm using a microplate reader (Tecan, Männedorf, Switzerland).

NO production

Griess reagent was used to measure nitrite production by RAW 264.7 cells. Cells (1×10^5 cells/well) were seeded into 24-well plates. After a 24-h culture, the cells were treated with 7H-4MC (62.5, 125, 250, and 500 μM) and LPS (1 μg/mL) for 24 h. Griess reagent (100 μL) was mixed with an equal volume of culture supernatant, which were incubated for 15 min in a 96-well plate (SPL, Gyeonggi-do, Korea). Optical density was measured at 540 nm using a microplate reader.

Measurement of inflammatory cytokine

The cells were treated with 7H-4MC and LPS in a manner similar to that used to measure nitrite production. The supernatant was

collected from each well and the levels of PGE_2, IL-1β, IL-6, and TNF-α were measured using ELISA kits, following the manufacturers' protocols. Optical density was measured using a microplate reader (Tecan).

Western blotting assay

RAW 264.7 cells (8×10^5 cells/well) were seeded in 60-mm culture dishes (Corning, Acton, MA, USA) and incubated for 24 h. The cells were treated with 7H-4MC (62.5, 125, 250, and 500 μM) and LPS (1 μg/mL) for the indicated times. After removing the supernatant and washing with cold PBS, cells were lysed with RIPA buffer (150 mM sodium chloride, 50 mM Tris-HCl, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing 1% protease inhibitor cocktail. The mixture was centrifuged at 15,000 rpm for 20 min at −8 °C, and protein concentrations were measured using the BCA protein assay kit. Then, the protein was mixed with 2× Laemmli sample buffer and heated at 100 °C for 5 min. The proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes, after which, the membranes were blocked with 1× TBS (3 M sodium chloride, 0.4 M Tris-HCl, pH 7.4) containing 0.1% Tween 20 (TBST) and 3% non-fat skim milk for 2 h. The membranes were washed three times with TBST for 10 min. Primary antibodies were diluted in TBST; the diluted antibody and the membrane were incubated overnight at 4 °C and washed three times with TBST. The secondary antibodies were diluted in TBST; the diluted antibody and the membrane were incubated at room temperature for 2 h and washed three times with TBST. After washing, the bands were visualized using an ECL kit.

Statistical analysis

All results are presented as the mean ± standard deviation of three replicates. One-way analysis of variance (ANOVA) was performed to evaluate the differences between the control and treatment groups. Statistical significance was set at $p<0.05$.

Results and Discussion

7H-4MC reduces LPS-induced NO production

LPS, a major cell wall component of gram-negative bacteria, is used to induce NO synthesis in screening systems to determine the anti-inflammatory effects of a compound as it elicits strong innate and adaptive immune responses [16]. Owing to the short half-life of NO, nitrate production is used as an indicator of NO release by LPS-induced macrophages. Additionally, NO release from LPS-induced macrophages is detected and quantified photometrically as its stable product, nitrite, by a simple colorimetric reaction, as described in the Materials and Methods section [17]. To investigate the effects of 7H-4MC on NO production, RAW 264.7 cells were treated with various concentrations of 7H-4MC in the presence of LPS (1 μg/mL) for 24 h. The supernatants were separated and nitrite levels were measured. NO production was 5.6 fold higher in LPS-induced macrophages than in normal macrophages (1±0.018 vs. 0.18±0.009, $p<0.001$; Fig 1B). 7H-4MC treatment reduced LPS-induced NO production in a dose-dependent manner; at 7H-4MC concentrations of 125, 250, and 500 μM, the production of NO by LPS-induced macrophages decreased by 1.1% (0.98±0.017, $p<0.05$), 20% (0.8±0.045, $p<0.001$), and 75.1% (0.25±0.006, $p<0.001$), respectively, compared with that by LPS-induced macrophages not treated with 7H-4MC. The vehicle control, DMSO, did not affect NO production, reaffirming its immunological activity (data not shown). Furthermore, 7H-4MC caused minimal changes in NO production in resting macrophages. Additionally, the potential cytotoxicity of 7H-4MC was evaluated using an MTT assay after the cells were incubated for 24 h in the presence or absence of LPS. The result showed that 7H-4MC had no cytotoxicity on cells within the range of 500 μM (Fig. 1B). These results indicate that the inhibitory effects of 7H-4MC are not attributable to cytotoxicity.

7H-4MC reduces the release of inflammatory mediators and pro-inflammatory cytokines

PGE_2, an important inflammatory mediator, is produced from arachidonic acid by COX-2. In a variety of inflammatory cells, including macrophages, COX-2 expression is induced by cytokines and other activators, such as LPS, which results in the release of a large amount of PGE_2 at inflammatory sites [18-19]. We examined the effects of 7H-4MC on PGE_2 production, which was quantified in the supernatants of LPS-induced RAW 264.7 macrophages. RAW 264 cells were pre-incubated with 7H-4MC for 1 h, followed by stimulation with 1 μg/mL LPS for 24 h. The control group was not treated with LPS or 7H-4MC. Cell culture media were collected and PGE_2 levels were determined. PGE_2 levels were 4.6-fold higher in LPS-induced macrophages than in unstimulated macrophages. With the addition of 7H-4MC (62.5, 125, 250, and 500 μM), a dose-dependent reduction in PGE_2 level was observed: 7.7±0.6, 19.8±0.6, 52.2±5.0, and 79.7±1.6%, respectively (Fig 2A).

When macrophages are stimulated with LPS, they secrete several pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α. Because 7H-4MC potently inhibited the secretion of pro-inflammatory mediators, we next investigated its effects on LPS-induced IL-1β, IL-6, and TNF-α secretion in culture supernatants using ELISAs. 7H-4MC suppressed LPS-induced IL-1β, IL-6, and TNF-α production in a con-centration-dependent manner (half-maximal inhibitory concentrations: 237.7 μM for IL-1β and 106.3 μM for IL-6; Fig. 2). A housekeeping protein, β-actin, was constitutively expressed and unaffected by 7H-4MC treatment. Thus, 7H-4MC modulates the LPS-induced production of TNF-α, IL-lβ, and IL-6 in RAW 267.4 macrophages.

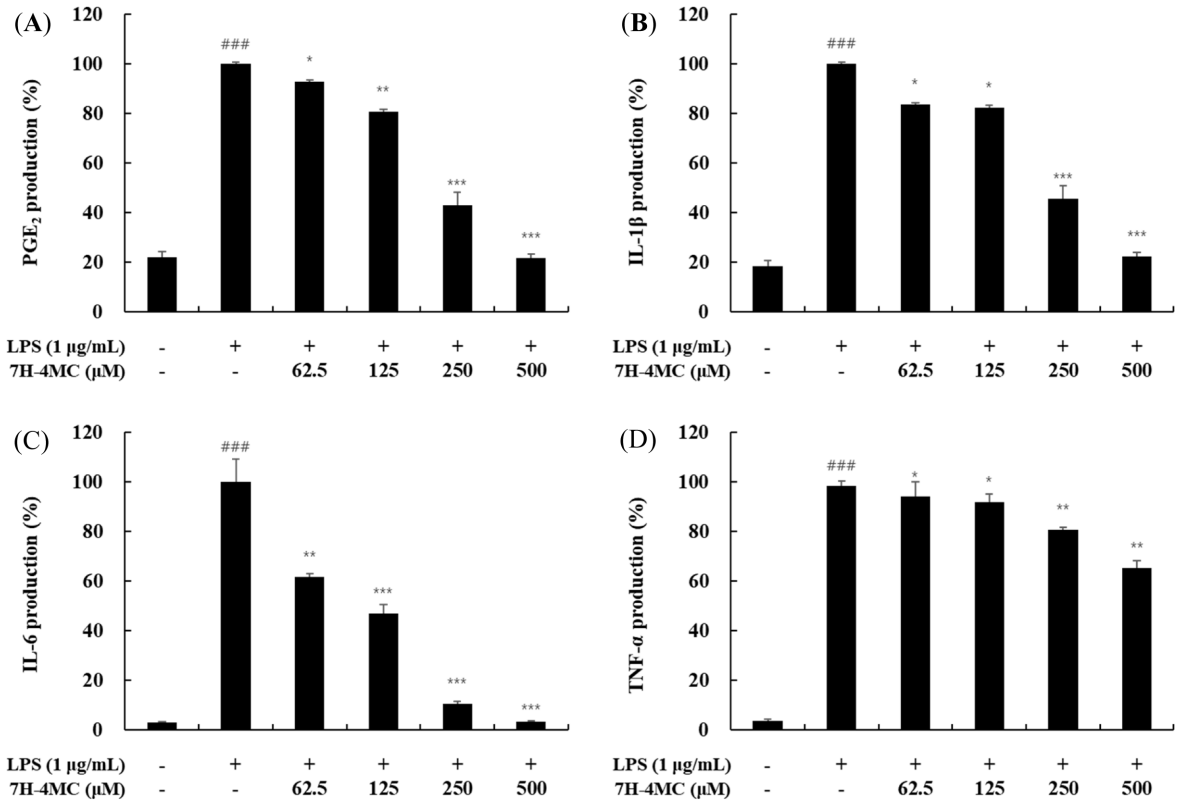

Fig. 2 Effects of 7H-4MC on the production of pro-inflammatory cytokines in LPS-induced RAW 264.7 cells. Cells were treated with 7H-4MC (62.5, 125, 250, and 500 μM) and LPS for 24 h. PGE_2 (a), IL-1β (b), IL-6 (c), and TNF-α (d) pro-duction was determined by ELISA. Results are presented as the mean±SD of three independent experiments. $^{###}p$ <0.001 vs. control. *p <0.05, **p <0.01, ***p <0.001 vs. LPS alone

7H-4MC inhibits LPS-induced iNOS and COX-2 protein expression

LPS has been reported to induce the production of NO, a second messenger, through the activation of iNOS expression. LPS induces iNOS expression by the transcriptional regulation of inflammation-related factors nuclear factor-κB (NF-κB) and activator protein 1. Additionally, several genes, encoding COX-2, vascular endothelial growth factor, pro-inflammatory cytokines, and chemokines, to which the transcription factors are directly linked, are known [20]. Therefore, we evaluated whether exogenous 7H-4MC regulates the expression of these genes. To investigate the mechanism underlying the inhibitory effect of 7H-4MC on the LPS-induced secretion of NO and PGE_2 in RAW 264.7 cells, we analyzed the expression of iNOS and COX-2 proteins in the inflammation model by western blotting. Western blotting analysis showed little iNOS and COX-2 expression in unstimulated RAW 264.7 macrophages, but strong iNOS and COX-2 expression was observed after stimulation with LPS for 20 h. In contrast, 7H-4MC significantly inhibited the induction of iNOS and COX-2 expression in LPS-induced cell cultures in a concentration-dependent manner. These data indicate that the inhibition of NO and PGE_2 production by 7H-4MC, as shown in Fig. 1B and 2A, results from the inhibition of LPS-induced iNOS and COX-2 protein expression in RAW 264.7 macrophages.

7H-4MC suppresses LPS-induced degradation of IκB-α protein In the NF-κB signaling pathway, NF-κB binds to its inhibitor protein, IκB-α, and resides in the cytoplasm of unstimulated cells. Its activation is regulated by the degradation of IκB-α, which frees NF-κB and allows its translocation to the nucleus [18-20]. To better demonstrate the mechanisms involved in the inhibitory effect of 7H-4MC on the LPS-induced release of inflammatory mediators, the protein levels of IκB-α were examined by western blotting. LPS stimulation accelerated the degradation of IκB-α and, under the same conditions, 7H-4MC reversed these effects in a dose-dependent manner (Fig. 4). This result suggests that LPS-induced IκB-α degradation is significantly blocked by pre-treatment with 7H-4MC.

In this report, we provide evidence that 7H-4MC has anti-inflammatory properties. Herein, we first investigated the effect and mechanism of 7H-4MC on the LPS-induced release of inflammatory mediators and pro-inflammatory cytokines, including NO, PGE_2, TNF-α, IL-1β, and IL-6. In the present study, we demonstrated that 7H-4MC significantly inhibited the LPS-induced release of inflammatory mediators and pro-inflammatory cytokines in macrophages via the downregulation of iNOS and COX-2 expression. Moreover, the NF-κB pathway is involved in

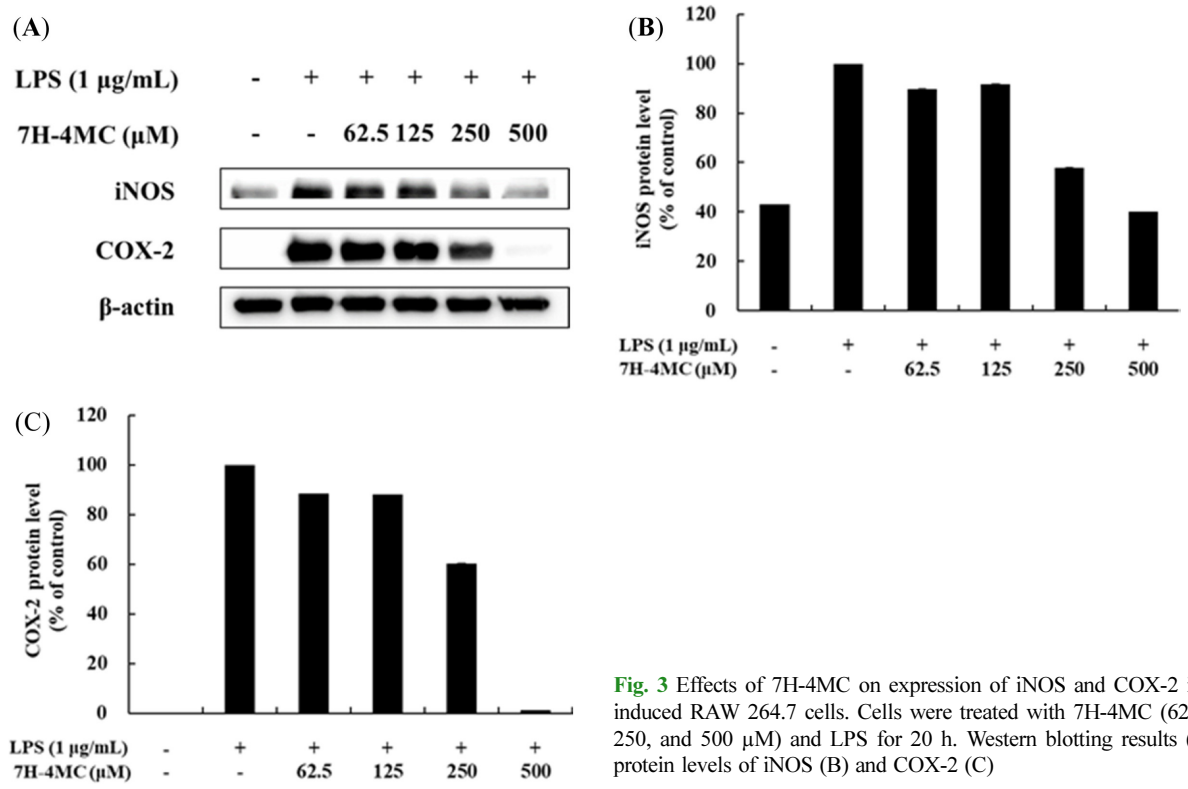

Fig. 3 Effects of 7H-4MC on expression of iNOS and COX-2 in LPS-induced RAW 264.7 cells. Cells were treated with 7H-4MC (62.5, 125, 250, and 500 μM) and LPS for 20 h. Western blotting results (A) and protein levels of iNOS (B) and COX-2 (C)

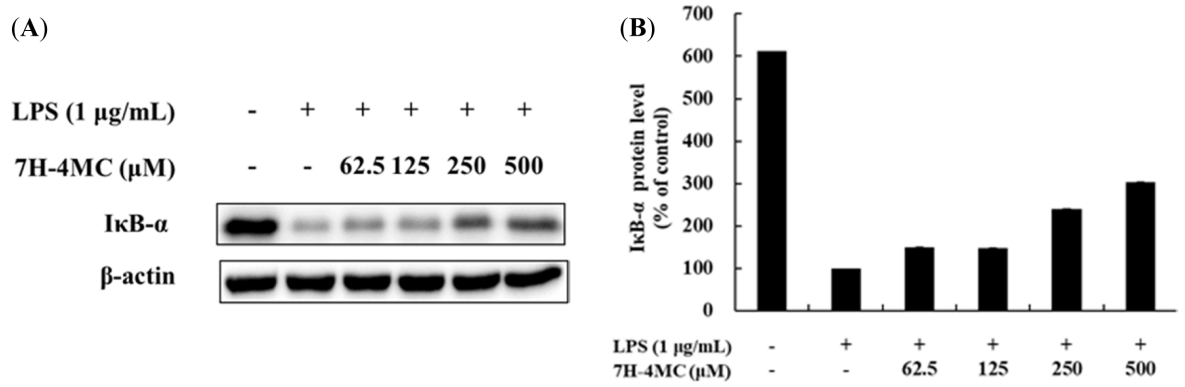

Fig. 4 Effects of 7H-4MC on the expression of IκB-α in LPS-induced RAW 264.7 cells. Cells were treated with 7H-4MC (62.5, 125, 250, and 500 μM) and LPS for 20 min. Western blotting results (A) and protein levels of IκB-α (B)

pro-inflammatory signaling cascades, and the degradation of IκB-α is involved in the increased release of inflammatory mediators and pro-inflammatory cytokines in LPS-induced macrophages. Thus, we assessed whether 7H-4MC inhibited the inflammatory response via the overproduction of IκB-α protein in LPS-induced macrophages. In 7H-4MC pre-treatment, 7H-4MC blocked the LPS-induced degradation of IκB-α. This result suggests that 7H-4MC exerts anti-inflammatory actions via the inhibition of NO, PGE_2, TNF-α, IL-1β, and IL-6 release through the suppression of the NF-kB signaling pathway. We have provided a molecular basis for the anti-inflammatory mechanism of 7H-4MC, which has great potential as a novel functional ingredient for human health.

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