Anti-inflammatory effects of 6-formyl umbelliferone via the NF-κB and ERK/MAPK pathway on LPS-stimulated RAW 264.7 cells

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Abstract. Inhibition of over-activated inflammation has been demonstrated as one of the most efficient strategies for treating inflammatory diseases. In the present study, 6-formyl umbelliferone (6FU) was used to evaluate its anti-inflammatory effects on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. 6FU inhibited chronic inflammatory processes, including increasing nitric oxide levels, and the expression of pro-inflammatory genes and producing cytokines was investigated by a nitrite assay and reverse transcription-polymerase chain reaction, respectively. Nitric oxide and pro-inflammatory cytokines, including tumor necrosis factor-α, interleukin (IL)-1β and IL-6 were decreased by treatment with 6FU, without cell cytotoxicity in LPS-stimulated RAW 264.7 cells, which was measured by a WST-1 assay. In the western blot analysis, the expression levels of phosphorylated extracellular signal-regulated kinase (ERK)1/2 was downregulated in 6FU-treated cells. Furthermore, in the western blotting and immunofluorescence staining results, translocation activities of ERK1/2 and NF-κB from the cytoplasm to the nucleus were suppressed, which may inhibit translation of numerous proteins associated with pro-inflammation, including inducible nitric oxide synthase and cyclooxygenase-2. Therefore, based on these results, it was suggested that 6FU may be a potential candidate for the development of agents against chronic inflammation.

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Abbreviations: COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells

Key words: 6-formyl umbelliferone, anti-inflammation, COX-2, iNOS, NF-κB

Introduction

Inflammation, an innate response in the immune system, is triggered through the release of specific cytokines, noxious stimuli and tissue injury (1). An inflammatory response may occur due to damage in tissues or organs, and diseases, including cancer, cardiovascular disease, diabetes, obesity, rheumatoid arthritis, depression and Parkinson's disease (2,3). Therefore, the inhibition of overactivated inflammation is considered one of the most efficient strategies for treating inflammatory diseases (2,3).

In inflammation, primary mediators, including nitric oxide (NO) and chemotactic cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6, are stimulated by lipopolysaccharide (LPS), which is a component of the gram-negative bacterial cell wall (3). These mediators react as a toxic agent against infectious organisms and are associated with modulation of cellular functions and homeostasis in the innate immune response. However, the overproduction of NO leads to the production of a number of proteins, including the mitogen-activated protein kinases (MAPKs) and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) pathway, which are implicated in chronic inflammatory reactions (4,5).

MAPKs consist of three principal components, including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase/stress activated protein kinases (JNK/SAPK) and p38 (6). These proteins are closely associated with a wide range of signaling cascades and serve a key role in the regulation of synthesis of inflammation mediators at the transcriptional and translational levels. Therefore, regulation of MAPK activity is

considered a target for anti-inflammatory therapeutics (6,7). NF-κB, one of the downstream components in the MAPK signaling pathway, is translocated to the nucleus by upstream stimuli. It promotes the transcription of pro-inflammatory genes producing the pro-inflammatory enzymes, including cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (8,9). COX-2 is one of the pro-inflammatory enzymes, which converts arachidonic acid to prostaglandins E2 (PGE2) and contributes to the progression of chronic inflammatory diseases (10). In addition, the expression of COX-2 is implicated in the generation of reactive oxygen species in response to LPS stimulation (10,11).

Angelica decursiva is a traditional medicinal plant in Korea, which demonstrates curative effects for cough, thick phlegm and asthma (12). 6-Formyl umbelliferone (6FU), isolated from *Angelica decursiva*, is one of the uncommon coumarin derivatives in nature. It has been demonstrated that coumarin and its derivatives have numerous pharmacological activities, including anticoagulant, vasodilator, anthelmintic, antimicrobial and antifungal capacity; however, at present, the biological activities of 6FU have not been extensively studied (12).

The aim of the present study was to investigate the therapeutic potential of 6FU in inflammation. LPS-stimulated RAW 264.7 murine macrophages were used to monitor anti-inflammatory activity by regulating the production of inflammatory mediators by suppressing expression of the MAPKs and NF-κB signaling pathway.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin solution were purchased from Corning, Inc. (Corning, NY, USA). LPS from *Escherichia coli* O111:B4, Griess reagent, Triton X-100 and 2-mercaptoethanol were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Rabbit primary antibodies [iNOS (cat. no. 13120), COX-2 (cat. no. 12282), phosphorylated (p)-ERK1/2 (Thr202/Tyr204; cat. no. 4370), p38 (cat. no. 8690), p-p38 (Thr180/Tyr182; cat. no. 4511), JNK/SAPK (cat. no. 9252), p-JNK/SAPK (Thr183/Tyr185; cat. no. 9255), NF-κB p65 (cat. no. 8242) and GAPDH (cat. no. 5174)], horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) secondary antibody (cat. no. 7074) and anti-rabbit IgG (Heavy+Light; H+L), F(ab')2 fragment (Alexa Fluor® 488 conjugate; cat. no. 4412) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-ERK1/2 rabbit primary antibody (cat. no. sc-514302) and rabbit normal serum were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). DAPI was purchased from Roche Diagnostics GmbH (Mannheim, Germany) and formaldehyde was bought from Junsei Chemical Co., Ltd. (Tokyo, Japan). ProLong® Gold Anti-fade Reagent was obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Isolation of 6FU from A. decursiva. Isolated 6FU was provided by Ali *et al* (12); the isolation process was performed as previously described. Whole *A. decursiva* powder was refluxed in methanol for 3 h and filtered. Subsequently, the filtrate was dried in a vacuum at 40˚C for concentrating, followed by suspension in distilled water. This extract was partitioned by ethyl acetate (EtOAc), and the EtOAc fraction was served to silica gel chromatography using dichloromethane (CH_2Cl_2)-MeOH (10:1→0:1, gradient). Following chromatography, 20 subfractions (F-1 to F-20) were obtained, F-6 was partitioned with a silica gel chromatography using CH_2Cl_2-MeOH column (20:1→0:1, gradient). Following these processes, 6FU was obtained. The presence of a single compound that was 6FU were confirmed by nuclear magnetic resonance studies.

Cell culture. The murine RAW 264.7 macrophage cell line and the 293 human kidney cell line were obtained from American Type Culture Collection (Manassas, VA, USA) and incubated with DMEM containing 10% FBS and 1% penicillin/streptomycin solution at 37˚C with 5% CO_2 and a humidified atmosphere.

Cell viability. The cell viability of 6FU in RAW 264.7 and 293 cells were measured by a WST-1 assay (13-15). In total, $1x10^4$ RAW 264.7 and 293 cells were seeded in each well of 96-well cell culture plates. RAW 264.7 cells were cultured for 24 h with or without 1 μg/ml LPS and 50 or 100 μM 6FU. 293 cells were incubated with or without 25, 50 and 100 μM 6FU for 24 h. Following incubation, 10 μl EZ-cytox Cell Viability Assay Solution WST-1® (Daeil Lab Service Co., Ltd., Seoul, Korea) was added to each well and incubated for 3 h. Subsequently, the absorbance was measured using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at 460 nm.

Nitrite assay. The nitrite concentration in the medium was measured with the Griess reaction (13-15). RAW 264.7 cells were seeded in 24-well cell culture plates ($5x10^4$ cells/well) and pre-treated with 10, 25 and 50 μM 6FU for 2 h and further incubated with 1 μg/ml LPS for 24 h. The supernatant of each well (100 μl) was transferred to 96-well plates and Griess reagent was added in the dark. Absorbance was measured at 540 nm and the calculated nitrate concentration was considered as an indicator of NO production.

Western blot analysis. To perform western blot analysis, RAW 264.7 cells were pre-treated with 6FU for 2 h and stimulated with or without LPS (1 μg/ml) for 6 and 24 h. Whole cells were harvested and lysed with the cell lysis buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 1% Triton X-100, 1% deoxycholate and 0.1% SDS; Intron Biotechnology, Inc., Seongnam, Korea] and the lysates were subsequently centrifuged at 18,000 x g for 20 min. Separate nuclear and cytoplasmic proteins were obtained using NE-PER® nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The protein concentration in the cell lysates were measured using Bradford reagent (Biosesang, Inc., Seongnam, Korea). An equal amount (20 μg) of the prepared proteins were separated by 12% SDS-PAGE and subsequently transferred to a nitrocellulose membrane (Pall Life Sciences, Ann Harbor, MI, USA). Following blocking with 1X PBST buffer containing 5% skim milk for 2 h at room temperature, the membranes were incubated

Table I. Primer sequences for reverse transcription-polymerase chain reaction.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
TNF-α	ACGGCATGGATCTCAAAGAC	CGGACTCCGCAAAGTCTAAG
IL-1β	CAGGCAGGCAGTATCACTCA	AGGCCACAGGTATTTTGTCG
IL-6	AACGATGATGCACTTGCAGA	CTCTGAAGGACTCTGGCTTTG
GAPDH	AACTTTGGCATTGTGGAAGG	CACATTGGGGGTAGGAACAC

TNF-α, tumor necrosis factor-α; IL, interleukin.

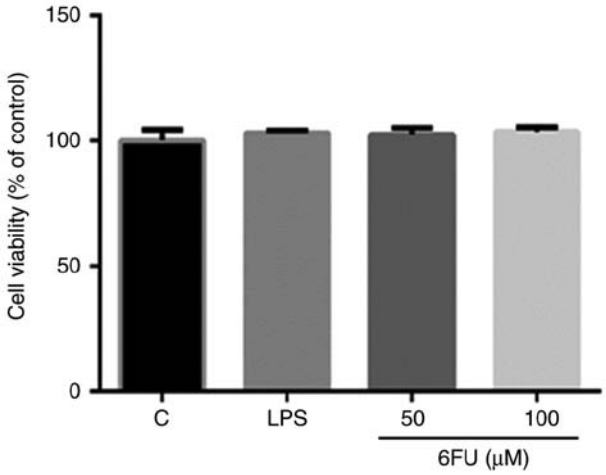

Figure 1. Effects of 6FU and LPS on cell viability in RAW 264.7 cells. Cell viability was determined by a WST-1 assay; cells were treated with 50 and 100 μM 6FU and 1 μg/ml LPS for 24 h. 6FU, 6-formyl umbelliferone; LPS, lipopolysaccharide; C, control.

overnight with primary rabbit antibodies [1:1,000 with 1X PBS containing 5% bovine serum albumin (BSA; BioShop Canada, Inc., Burlington, ON, Canada) and 0.1% Tween-20] at 4˚C. The membranes were washed three times with PBST, followed by incubation with HRP-conjugated anti-rabbit IgG secondary antibodies (1:1,000 with 1X PBS containing 5% BSA and 0.1% Tween-20) for 1 h at room temperature. The membranes were developed on X-ray film for visualization using an enhanced chemiluminescent detection solution (Pierce; Thermo Fisher Scientific, Inc.).

Immunofluorescence (IF) staining. In total, $1x10^5$ cells were plated at cover-glass bottom dishes (SPL Life Sciences, Pocheon, Korea) and pre-treated with 25 μM 6FU for 30 min (16). To investigate the nuclear translocation activity of p-ERK1/2 or NF-κB, LPS was treated for 6 or 24 h respectively. Subsequently, for pre-fixing, cells were stained with 1 μg/ml DAPI diluted in methanol (99.8%) and incubated for 15 min at 37˚C, followed by washing with PBS buffer and fixed with 4% formaldehyde for 15 min at room temperature. Following incubation, these cells were blocked with 5% rabbit normal serum containing 0.3% Triton X-100 in 1x PBS for 1 h in the dark and incubated with the anti-ERK1/2 (Thr202/Tyr204) or -NF-κB p65 primary antibody (1:2,000 with 1x PBS containing 0.3% Triton X-100) at 4˚C overnight. Following the reaction, the cells were washed with 1x PBS and incubated for 50 min with anti-rabbit IgG (H+L), F(ab')2 fragment (Alexa Fluor® 488 conjugate) as secondary antibodies (0.5 μg/ml with 1x PBS containing 0.3% Triton X-100) at room temperature in the dark. Following staining, cells were mounted using ProLong® Gold Anti-fade Reagent. Stained cells were observed using Carl Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany; magnification, x400).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). For total RNA extraction, RAW 264.7 cells were pre-treated with 6FU (25 and 50 μM) prior to stimulation with LPS (1 μg/ml), subsequently RNA was extracted with 2-mercaptoethanol the and RNeasy plus mini kit, according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). The concentration of total RNA was measured using a nanodrop (Mecasys Co., Ltd., Daejeon, Korea) and 2 μg RNA was synthesized to cDNA using a SuPrimeScript RT Premix (GeNet Bio, Inc., Daejeon, Korea) under the following conditions; 50˚C for 60 min and 70˚C for 10 min. The cDNA was amplified using Prime Taq Premix (GeNet Bio, Inc.) with specific primers presented in Table I, according to the manufacturer's protocols (95˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec, 30 cycles). Amplified PCR products were stained with ethidium bromide (Sigma-Aldrich; Merck KGaA) and visualized in a 2% agarose gel using ImageMaser® VDS Software version 3.0 in ImageMaster® VDS GE Healthcare, Chicago, IL, USA).

Statistical analysis. One-way analysis of variance with Dunnett's multiple comparison tests were used for determining the statistical significance of differences between experimental and control groups. Analysis was performed using Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Results are presented as the mean ± standard deviation and all experiments were performed in triplicates independently. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of 6FU on cell viability in RAW 264.7 and 293 cells. The cell viability of RAW 264.7 and 293 cells was measured by a WST-1 assay. RAW 264.7 cells were treated with or without 6FU (50 and 100 μM) and LPS (1 μg/ml) for 24 h. 293 cells were incubated with or without various concentrations of 6FU (25, 50 and 100 μM) for 24 h. As presented in Fig. 1, 6FU and LPS did not demonstrate any cytotoxicity on RAW 264.7 cells.

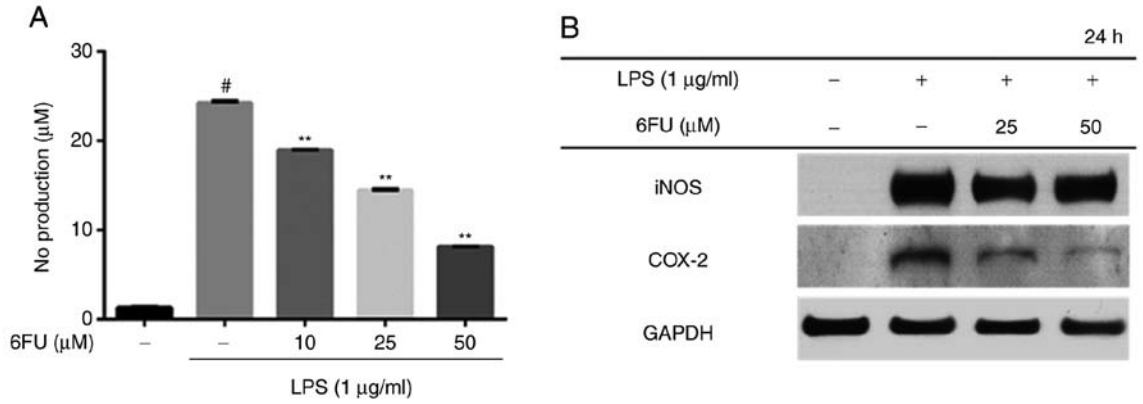

Figure 2. Effects of 6FU on NO secretion, and the expression level of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. (A) Production of NO was measured using Griess reagent. (B) Expression levels of iNOS and COX-2 were determined by western blotting in whole cell lysates. Data are presented as the mean ± standard deviation. n=3. $^{\#}$P<0.01 vs. non-treated group; **P<0.01 vs. LPS-stimulated cells. 6FU, 6-formyl umbelliferone; LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2.

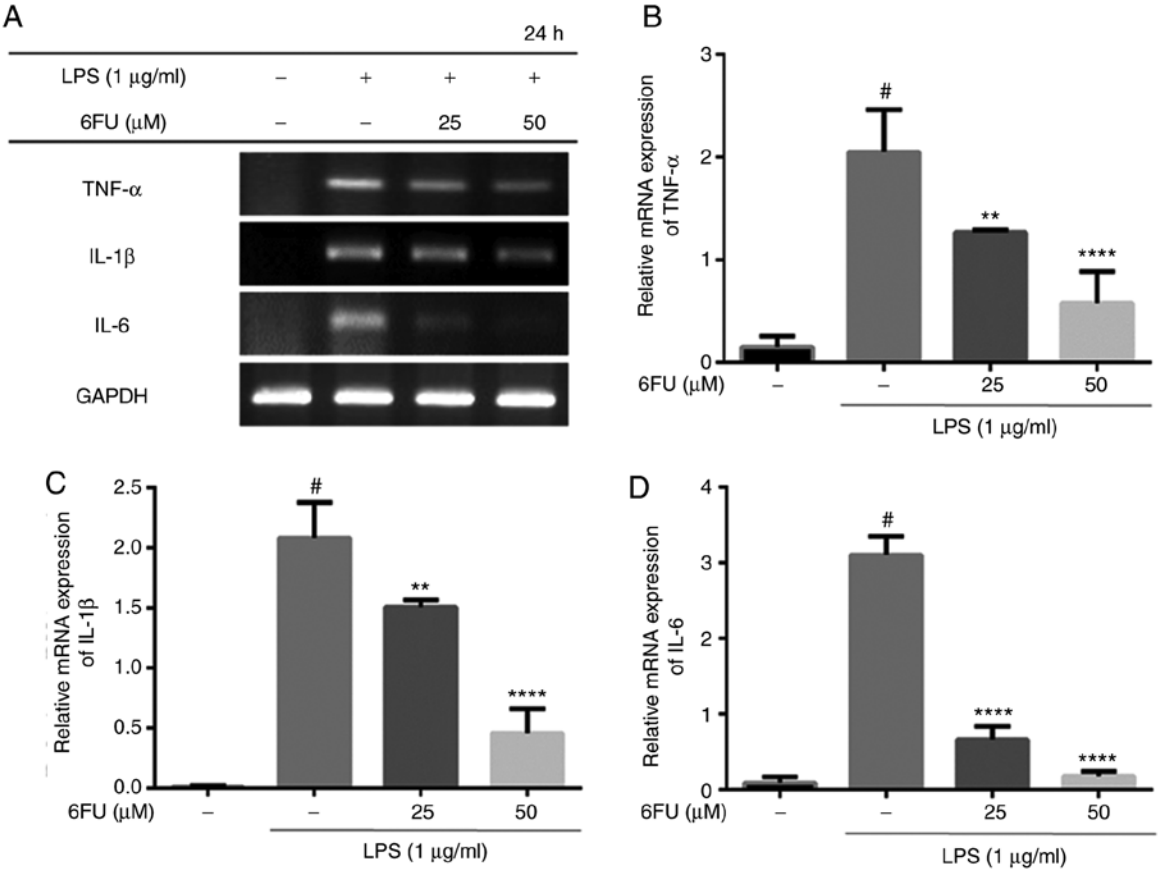

Figure 3. Regulation of the mRNA expression on pro-inflammatory cytokines. cDNA was synthesized from total RNA with specific primers, including TNF-α, IL-1β and IL-6. GAPDH was used as a loading control. (A) The expression levels of mRNA were detected by RT-PCR. Relative mRNA expressions of (B) TNF-α, (C) IL-1β (D) and IL-6. Data are presented as the mean ± standard deviation. n=3. $^{\#}$P<0.01 vs. non-treated group; **P<0.01, ****P<0.00001 vs. LPS-stimulated cells. TNF-α, tumor necrosis factor-α; IL, interleukin; 6FU, 6-formyl umbelliferone; LPS, lipopolysaccharide.

Additionally, in 293 cells, the cell viability of 6FU treated cells was 99.8, 93.9 and 88.1% at 25, 50 and 100 μM concentrations, respectively (data not shown). Therefore, <100 μM 6FU was used for investigating its anti-inflammatory capacity in the absence of cytotoxicity.

Effect of 6FU on NO production level in RAW 264.7 cells. RAW 264.7 cells were pretreated with or without 6FU for 2 h and subsequently stimulated with LPS for 24 h in order to evaluate the NO production level. The level of NO secretion was significantly increased in LPS-stimulated cells compared with non-stimulated cells (Fig. 2A; P<0.01). However, the expression level of NO was decreased by treatment with 6FU in a dose-dependent manner (Fig. 2A). Western blot analysis was performed to investigate whether 6FU has an ability to modulate the expression of pro-inflammatory enzymes,

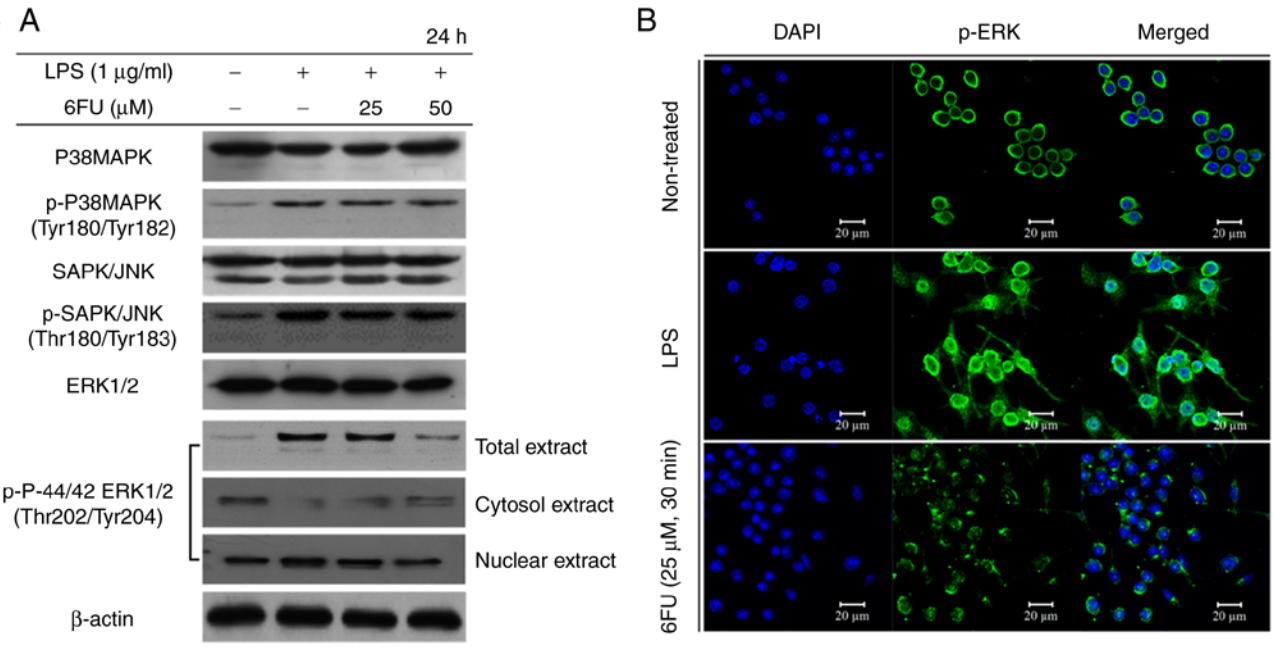

Figure 4. Detection of MAPKs expression and phosphorylation in LPS-activated RAW 264.7 macrophages. (A) Cells were pre-treated with 25 μM 6FU for 30 min prior to stimulation with 1 μg/ml LPS for 6 h. MAPK proteins and its phosphorylated forms were detected using western blotting in whole cell lysates. Only p-ERK1/2 was investigated for expression in cytoplasmic and nuclear levels. β-actin was used as a loading control. (B) Effect of 6FU on nuclear translocation of p-ERK1/2, detected by immunofluorescence staining. Blue indicates DAPI and green indicates p-ERK1/2. MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide; 6FU, 6-formyl umbelliferone; p, phosphorylated; ERK, extracellular signal-regulated kinase; SAPK, stress activated protein kinase; JNK, c-Jun N-terminal kinase.

including iNOS and COX-2. The results demonstrated that 6FU downregulated the expression of iNOS and COX-2 in contrast with LPS only-treated cells (Fig. 2B).

Effect of 6FU on mRNA expression of pro-inflammatory cytokines. As presented in Fig. 3, 6FU significantly suppressed the mRNA expression of pro-inflammatory cytokines, including IL-6, TNF-α and IL-1β, compared with LPS-stimulated RAW264.7 cells ($P<0.01$).

Effect of 6FU on LPS-induced phosphorylation and activation of MAPKs. The LPS-induced phosphorylation level of MAPKs, including p-ERK, p38 and JNK were measured by western blot analysis. In LPS only-treated RAW 264.7 cells, the phosphorylation level of ERK, p38 and JNK were increased. However, only the expression of p-ERK1/2 was markedly decreased in the 6FU-treated LPS-stimulated RAW264.7 cells in a dose dependent manner compared with the phosphorylation level of p38 and JNK (Fig. 4A). In addition, the translocation of phosphorylated ERK1/2 to the nucleus was inhibited following pretreatment with 6FU in LPS-stimulated RAW 264.7 cells by western blotting and IF staining (Fig. 4).

Effect of 6FU on LPS-induced activation and translocation of NF-κB. To investigate the activity of 6FU on nuclear translocation of NF-κB, western blot analysis and IF staining were performed. Fig. 5A demonstrated that 6FU decreased the concentration of NF-κB in the nucleus in LPS-stimulated RAW 264.7 macrophages. In contrast, the expression level of NF-κB in the cytoplasm was upregulated by 6FU. Furthermore, 6FU inhibited nuclear translocation activity of NF-κB in LPS-treated cells (Fig. 5B). Therefore, 6FU decreased the expression and nuclear translocation of NF-κB in LPS-stimulated macrophages.

Discussion

The aim of the present study was to investigate the anti-inflammatory properties of 6FU. The cytotoxicity of 6FU on RAW 264.7 and 293 cell lines was determined. The results demonstrated that 6FU did not exhibit any cytotoxicity on RAW 264.7 cells ≤100 μM. In 293 cells, 6FU did not demonstrate any significant cytotoxic effect ≤50 μM. However, 100 μM 6FU in 293 cells resulted in 88.1% cell viability. These results suggested that ≤50 μM 6FU did not demonstrate any cytotoxicity on the murine and human cell lines, thus, ≤50 μM 6FU was used for further investigation. It was investigated whether 6FU may regulate production of NO in LPS-stimulated RAW 264.7 murine macrophages, as NO is one of the principal contributors to the formation of reactive nitrogen species and mediates the inflammatory response (17,18). NO production was decreased by 6FU without cytotoxic effects, compared with LPS-only-treated RAW264.7 cells.

It has been demonstrated that iNOS catalyzes the formation and release of a large amount of NO, and COX-2 serves an essential role in the inflammatory response as a precursor of various biological active mediators, including PGE2 (19,20). The present results demonstrated that 6FU markedly inhibited the protein expression level of iNOS and COX-2 against a stimulus of inflammation in RAW 264.7 cells. Therefore, it was suggested that 6FU has an ability to suppress production of NO and PGE2 through downregulation of iNOS and COX-2 expression. Additionally, endotoxins, including

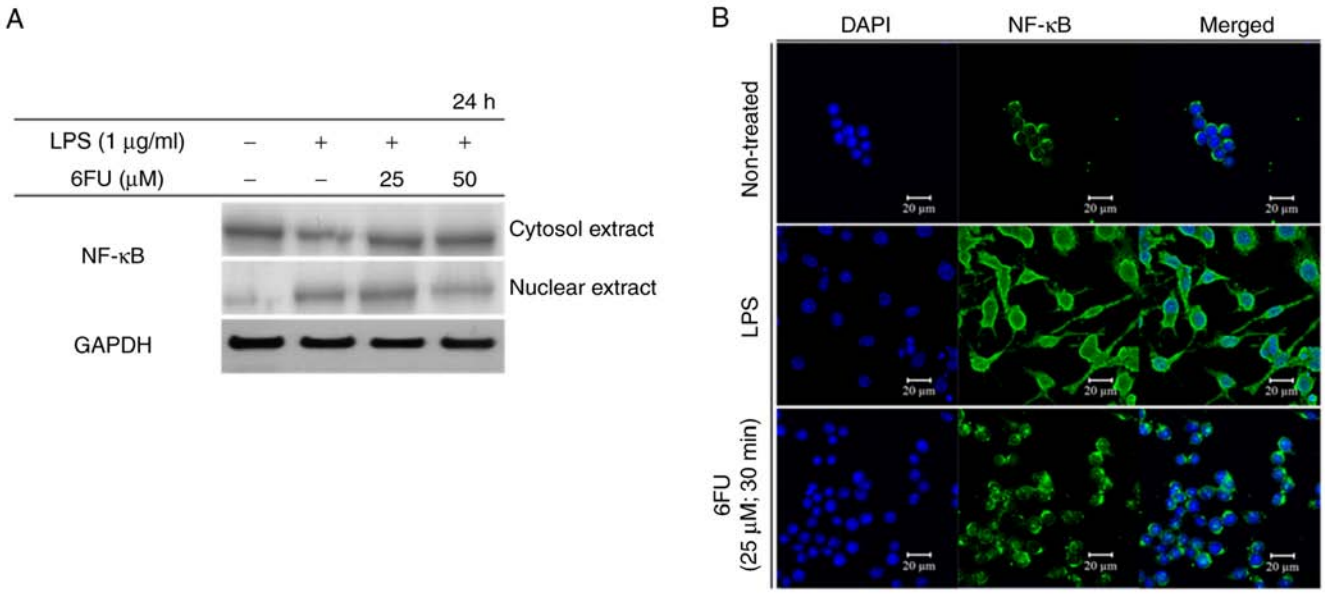

Figure 5. Translocation of NF-κB to the nucleus in LPS-stimulated RAW 264.7 cells. Effect of 6FU on nuclear translocation of NF-κB in LPS-stimulated macrophages. Cells were pretreated with 25 μM 6FU for 30 min and subsequently stimulated with LPS for 24 h. (A) Expression levels of cytoplasmic and nuclear NF-κB were investigated by western blot analysis and GAPDH was used as the loading control. (B) Nuclear translocation activity of NF-κB was detected using immunofluorescence staining. Blue indicates DAPI and green indicates NF-κB. NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; LPS, lipopolysaccharide; 6FU, 6-formyl umbelliferone.

LPS in the present study, stimulate macrophages to express cytokines, including TNF-α, IL-1β and IL-6, which activate inflammation-associated signaling pathways (21,22). It was demonstrated that the mRNA expression levels of TNF-α, IL-1β and IL-6 were significantly decreased by 6FU compared with LPS-stimulated cells. These results suggested that 6FU attenuated the inflammatory response by regulating expression of iNOS, COX-2 and numerous pro-inflammatory cytokines.

Based on these results, it was hypothesized that 6FU may regulate the cellular signaling pathway, which is associated with the production of NO and pro-inflammatory cytokines in macrophages. To further investigate the mechanisms of NO and cytokine production, the expressions of MAPK signaling proteins were examined, which have been demonstrated to regulate various cellular activities, cell proliferation, differentiation, migration and the inflammatory response (6). MAPK signaling pathway proteins consist of ERK1/2, JNK/SAPK and p38, which mediate intracellular signaling initiated by extracellular stimuli. Among them, activated ERK1/2 serves an essential role in the regulation of the inflammatory response by promoting phosphorylation of its downstream proteins (23,24). It was identified that p-ERK1/2 was markedly decreased by treatment with 6FU; however, expression of p-p38 and p-JNK/SAPK did not demonstrate any difference. Furthermore, the nuclear translocation activity of p-ERK1/2 was inhibited by 6FU in LPS-stimulated RAW 264.7 macrophages. These results suggested that 6FU inhibits the ERK-mediated inflammatory response by suppressing phosphorylation and translocation of ERK1/2.

NF-κB additionally serves as one of the key regulators of the inflammatory gene expression, which induces the synthesis of pro-inflammatory cytokines, including iNOS and COX-2 (8). It has been investigated that inflammatory stimuli activate NF-κB translocation from the cytoplasm to the nucleus, and its transcriptional activity through degradation of inhibitor of NF-κB by proteasomes (25,26). It was observed that 6FU suppressed the translocation activity of NF-κB from the cytoplasm to the nucleus by western blot analysis and IF staining, which demonstrated the anti-inflammatory capacity of 6FU.

In conclusion, the present results demonstrated that 6FU downregulates the production of NO and pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 by inhibition of the inflammation-associated signaling pathways, including ERK1/2 and NF-κB. Future *in vivo* animal studies are required to further elucidate the detailed protein expression associated with inflammation. In the present study, it was identified that 6FU has potential as one of the therapeutic candidates for chronic inflammation.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JSC and GDK contributed to the design of the study and managed the experiments. HAJ analyzed the experimental data. C-WK conducted the nitrite assay. N-HK performed

the immunofluorescence staining. HWC conducted the cell viability assay. S-BK and M-JK conducted the western blot analysis and reverse transcription-polymerase chain reaction assays, and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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