Anti-inflammatory Activities of 7,8-Dihydroxy-4-Methylcoumarin Acetylation Products via NF-κB and MAPK Pathways in LPS-Stimulated RAW 264.7 Cells

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Abstract

Coumarins are phenolic compounds that are characterized by fused benzene and α-pyrene rings. Among coumarin-based compounds, 7,8-dihydroxy-4-methylcoumarin (DHMC) has anti-inflammatory activities, but whether the level of this activity varies according to the degree of acetylation remains unknown. Therefore, we acetylated DHMC to yield monoacetylated 8-acetoxy-4-methylcoumarin (8AMC) and 7,8-diacetoxy-4-methylcoumarin (DAMC). We then compared the anti-inflammatory activities of DHMC with its acetylated derivatives and discovered a novel anti-inflammatory agent. We evaluated whether DHMC, 8AMC, and DAMC could inhibit lipopolysaccharide (LPS)-induced stimulation in RAW 264.7 cells. We found that DHMC, 8AMC, and DAMC induced a dose-dependent downregulation of nitric oxide (NO), prostaglandin E2 (PGE2), pro-inflammatory cytokine, inducible NO synthase (iNOS), and cyclooxygenase-2 (COX-2) expression at the mRNA and protein levels. Western blotting showed that DHMC, 8AMC, and DAMC inhibited phosphorylated mitogen-activated protein kinase (MAK), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38, and nuclear factor-kappa B (NF-κB) expression in a concentration-dependent manner. Furthermore, 8AMC was the most effective inhibitor with powerful anti-inflammatory activity. These results indicate that acetylation can improve the anti-inflammatory activity of natural precursors. We also discovered the new anti-inflammatory compounds 8AMC and DAMC.

Keywords
kinase pathways, anti-inflammation, coumarin, monoacetylation, bioactivity, nitric oxide

Introduction

Inflammation is the main defense mechanism that aids the regeneration of various damaged tissues or organs in response to physical and biological stresses in humans. However, continuous inflammatory responses can promote mucosal damage and lead to diseases, such as cancer. An inflammatory reaction arises when lipopolysaccharide (LPS), an endotoxin that stimulates macrophages, binds to the outer cell membrane of Gram-negative bacteria and activates a series of reactions. LPS induces the expression of tumor necrosis factor α (TNF-α) and inflammatory cytokines, such as interleukin-1β (IL-1β) and nitric oxide (NO). Activated macrophages also stimulate the expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), which function as inflammatory mediators and immune modulators. The activated enzymes thus induce various inflammatory responses and increase the severity of the inflammatory response. For example, iNOS catalyzes the production of NO from l-arginine, during which reactive nitrogen species are produced that disrupt apoptosis and homeostasis in surrounding tissues. COX-2 converts arachidonic acid into prostaglandin E2 (PGE2), and the higher modulating factor nuclear factor-kappa B (NF-κB) might be stimulated. Mitogen-activated protein kinases (MAPKs) and

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NF-κB are inflammatory response mediators that modulate many aspects of the immune system. When the inhibitor of NF-κB (IκB) is dephosphorylated, p65 translocation to the nucleus and NF-κB activity is inhibited; these phenomena are associated with the regulation of the secretion of various cytokines as well as several pro-inflammatory mediators that are involved in immune regulation. Furthermore, the MAPK pathway is initiated by MAPK dimerization, which is activated when ligands, such as cytokines and hormones, bind to and phosphorylate intracellular receptors, such as rat sarcoma virus (RAS) guanosine triphosphate hydrolases (GTPases). Activated RAS/RAC GTPases regulate the expression of various genes, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, via the stepwise activation of MAPK proteins that are also involved in NF-κB activation. These two pathways play an important role in eliciting anti-inflammatory effects.

Coumarin-based compounds in plants, fungi, and bacteria have various physiological activities. Among coumarin-based compounds, 7,8-dihydroxy-4-methylcoumarin (DHMC) has antioxidant and anti-inflammatory activities. However, the anti-inflammatory activities of its acetyl derivatives, 8-acetoxy-4-methylcoumarin (8AMC) and 7,8-diacetoxy-4-methylcoumarin (DAMC), have remained unknown. Therefore, the present study aimed to derive potent anti-inflammatory agents from existing coumarins simply by acetylation.

Results

Acetylation of DHMC

A synthetic method using an acetic acid reagent was used to acetylate the hydroxyl groups of DHMC. The optimal acetylation conditions were determined by reacting for 30 min, 1 h, and 2 h. The yields of DHMC, 8AMC, and DAMC within 1 h were 35.8%, 35.5%, and 7.6%, respectively. The yield of DAMC at ≥2 h was ≥80%. The compounds were purified by preparative high-performance liquid chromatography (HPLC), then peaks with retention times that differed from those of DHMC were analyzed. The results of electrospray ionization mass spectrometry (ESI-MS) inferred that the hydrogen group of DHMC was substituted with an acetyl group.

NMR Spectroscopy

The molecular structures of the acetylated products were identified using 1H- and 13C-NMR spectroscopy. The results confirmed that the acetylated products were 8AMC and DAMC, which is consistent with previously published nuclear magnetic resonance (NMR) results. Acetylation of 7,8-Dihydroxy-4-methylcoumarin through the addition of acid anhydride resulted in the synthesis of 8-Acetoxy-7-hydroxy-4-methylcoumarin.

1H-NMR (400 MHz, DMSO-d6) δ: 10.78 (s, 1H, OH), 7.50 (d, J = 8.79 Hz, 1H, ArH), 6.93 (d, J = 8.79 Hz, 1H, ArH), 6.17 (s, 1H, H-3), 2.37 (s, 3H, C-4 CH3), 2.34 (s, 3H, COCH3). 13C-NMR (100.5 MHz, DMSO-d6) δ: 168.25, 159.43, 154.01, 152.90, 146.85, 125.05, 123.05, 112.97, 112.64, 110.53, 20.31, 18.27.

Effects of DHMC, 8AMC, and DAMC on the Viability of RAW 264.7 Cells and Their Ability to Reduce NO Production

RAW 264.7 cells stimulated with LPS were incubated with various concentrations of DHMC, 8AMC, and DAMC to determine their cytotoxicity. The results of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays revealed that no compound was cytotoxic up to a concentration of 25 µM (Figure 1A). Furthermore, DHMC, 8AMC, and DAMC inhibited NO activity at nontoxic concentrations (in a concentration-dependent manner). The acetylated derivatives, 8AMC and DAMC, respectively, inhibited NO production 7% and 4% more than DHMC (Figure 1B). Therefore, the suppression of NO production did not significantly differ. We then investigated the effects of the coumarin compounds on the expression of other pro-inflammatory cytokines and proteins.

Effects of DHMC, 8AMC, and DAMC on Pro-Inflammatory Cytokine Production

The levels of inflammatory cytokines, TNF-α, IL-1β, and IL-6, which were elicited in response to DHMC, 8AMC, and DAMC treatment, were evaluated using appropriate enzyme-linked immunosorbent assays (ELISAs). We stimulated RAW 264.7 cells with LPS and incubated them with 5, 10, and 25 µM each of DHMC, 8AMC, and DAMC. The results showed that all 3 compounds decreased IL-1β and IL-6 production in a concentration-dependent manner, confirming the significant inhibitory activity. The maximum concentration of 8AMC (25 µM) reduced the abundance of IL-1β and IL-6 by 59% and 74%, respectively (Figure 2A and B). The levels of TNF-α were decreased following treatment with only 25 µM of 8AMC (Figure 2C). These findings suggested that the acetyl derivatives inhibited the expression of pro-inflammatory factors more effectively than DHMC.

Effects of DHMC, 8AMC, and DAMC on the Expression of iNOS and COX-2 at the Protein and mRNA Levels

We investigated the effects of DHMC and its acetylated derivatives on the expression of iNOS and COX-2 at the protein and mRNA levels. RAW 264.7 cells were stimulated with LPS and incubated with 5, 10, and 25 µM each of DHMC, 8AMC, and DAMC for 20 h. The production of PGE2 was inhibited in these cells in a concentration-dependent manner. In particular, 5, 10, and 25-µM DAMC inhibited cellular PGE2 secretion by 59%, 68%, and 80%, respectively (Figure 3A). The expression of iNOS and COX-2 regulates NO and PGE2 production. Therefore, we investigated...
whether the effects of DHMC, 8AMC, and DAMC on NO and PGE2 production were due to the inhibition of the expression of iNOS and COX-2 at the mRNA and protein levels. All 3 compounds inhibited the expression of iNOS and COX-2 in a concentration-dependent manner compared with LPS (Figure 3B-E), and 8AMC and DAMC were the most powerful inhibitors. These results were consistent with the suppression of NO and PGE2 production and indicated that iNOS and COX-2 regulate the secretion of inflammatory factors.

**Effects of DHMC, 8AMC, and DAMC on the MAPK and NF-kB Pathways**

We assessed inflammatory signals in LPS-stimulated RAW 264.7 cells by evaluating the expression of MAPK and NF-kB pathway intermediates. The phosphorylation of ERK was significantly reduced by DHMC, 8AMC, and DAMC (Figure 4A). The phosphorylation of p38 and JNK was also reduced in a dose-dependent manner by DHMC, 8AMC, and DAMC (Figure 4B and C). Among the 3 compounds, MAPK expression was the most effectively suppressed by 8AMC. These results showed that 8AMC inactivates MAPK by inhibiting the phosphorylation of MAPK pathway intermediates and exerting anti-inflammatory activity. We also showed that DHMC, 8AMC, and DAMC at concentrations of 5 to 25 μM increased IκBα expression in the cytoplasm and suppressed NF-kB phosphorylation, thus regulating the activity of the NF-kB signaling pathway (Figure 4D and E). Therefore, we showed that DHMC, 8AMC, and DAMC alleviate the inflammatory response by inhibiting the expression of pro-inflammatory cytokines and inflammatory mediators at least via MAPK and NF-kB signaling pathways.

**Discussion**

Many acetylated derivatives exert marked antithrombotic, antioxidant, and anti-inflammatory effects. This suggests that powerful anti-inflammatory agents can be derived from known coumarins by acetylation.

We, therefore, speculated that acetylated derivatives would regulate major cellular processes through intracellular signal transduction. Consequently, we evaluated the anti-inflammatory activity of these compounds by analyzing their structure–activity relationships. The order of inhibitory activity on the MAPK and NF-kB signaling pathways in LPS-stimulated RAW 264.7 cells was 8AMC > DAMC > DHMC. Thus, acetylated 8AMC and DAMC had more powerful anti-inflammatory activity than DHMC. Physiological activity depends not only on the structural function of a compound but also on its ability to cross the plasma membrane, which comprises hydrophilic and hydrophobic compartments. The permeability of flavonoids depends on their lipophilicity, and substitution with an acetyl group renders them lipophilic. In addition, lipophilic compounds with an acetyl group are more physiologically active than hydrophilic compounds, and their anti-inflammatory activities are exerted by regulating the STAT1 and NF-kB pathways. Therefore, the present results support the finding that conversion to lipophilicity after acetyl group substitution can enhance physiological activity.

In addition, the number of substituted acetyl groups is associated with differences in the enhancement of physiological activities. This might be caused by specific interactions among proteins during intracellular regulation by an acetylated compound.

We showed that acetylated 8AMC and DAMC derivatives inhibited the overexpression of pro-inflammatory mediators more effectively than DHMC. This suggested that anti-inflammatory activity could be improved through acetylation.
Furthermore, acetylated derivatives might be useful for treating or preventing chronic inflammatory diseases.

Materials and Methods

Acetylation of DHMC

We acetylated 0.95% (w/v) DHMC (Sigma-Aldrich) using 25% (v/v) acetic anhydride (Samchun Pure Chemical Co., Ltd) and 75% (v/v) pyridine (Junsei Chemical Co., Ltd) in round-bottomed flasks with a magnetic stir bar. Flasks were placed in an oil bath at 90 °C and agitated using an electromagnetic stirrer for 30 min, 1 h, and 2 h.

HPLC Analysis and Purification of DHMC, 8AMC, and DAMC

We used an LC-2030C PLUS UV HPLC system (Shimadzu) with a Shim-pack GIS C18 column (5 μm ODS, i.d. 250 × 4.6 mm; Sigma-Aldrich). Derivatives were separated and purified using preparative HPLC (Shimadzu) and a Luna® 5 μm C18 (2) 100A, 250 × 10 mm column (Phenomenex). Flow rates for HPLC analysis and purification were 1.0 and 5.0 mL min⁻¹, respectively. The column temperature was 40 °C, and the mobile phases were 0.1% trifluoroacetic acid (TFA, SAMCHUN) in water (Solvent A) and acetonitrile (Solvent B, Sigma-Aldrich). The derivatives were compared and analyzed by increasing solvent B from 10% to 100% over a period from 0 to 30 min. All derivatives used in the present study were purified using preparative HPLC.

LC-MS and NMR Analysis of 8AMC and DAMC

We measured the mass of various peaks by HR-Q TOF ESI/MS using an Acquity UPLC (Waters Corp.) system coupled with SYNAPT G2-Si. Mass data were then processed using MassLynx v. 4.1. We accurately identified molecular structures by heteronuclear single quantum coherence spectroscopy (HSQC) using the Varian VNMRS-500 MHz NMR system (Agilent Technologies Inc.). Spectral expansion was analyzed using a deuterated NMR solvent (Sigma-Aldrich) as the reference.

Cell Culture and Viability Assay

RAW 264.7 macrophages, purchased from Korea Cell Line Bank (Seoul, Korea), were cultured in Dulbecco’s modified...
Eagle medium (Thermo Fisher Scientific Inc.) supplemented with 10% heat-inactivated fetal bovine serum containing 1% penicillin/streptomycin. Cells (8.0 × 10^4 cells/well) were seeded onto 24-well plates and incubated at 37 °C for 24 h in a 5% CO₂ atmosphere. The cells were stimulated with LPS (1 μg/mL) and incubated with 5, 10, and 25 μM each of DHMC, 8AMC, and DAMC, as determined by ELISA. Expression of iNOS and COX-2 at the (B, C) mRNA and (D, E) protein levels, as determined by qRT-PCR and Western blotting, respectively. All results are shown as the mean ± SD of triplicate experiments. *P < .05, **P < .01, ***P < .001 versus LPS.

Abbreviations: DAMC: 7,8-diacetoxy-4-methylcoumarin; DHMC: 7,8-dihydroxy-4-methylcoumarin; ELISA: enzyme-linked immunosorbent assay; iNOS: inducible NO synthase; LPS: lipopolysaccharide; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; 8AMC: 8-acetoxy-4-methylcoumarin.

Figure 3. Effects of DHMC, 8AMC, and DAMC on PGE₂ production and the expression of iNOS and COX-2 at the mRNA and protein levels in RAW 264.7 cells stimulated with LPS. (A) Levels of PGE₂ in the culture medium of RAW cells stimulated with LPS (1 μg/mL) and incubated with 5, 10, and 25 μM each of DHMC, 8AMC, and DAMC, as determined by ELISA. Expression of iNOS and COX-2 at the (B, C) mRNA and (D, E) protein levels, as determined by qRT-PCR and Western blotting, respectively. All results are shown as the mean ± SD of triplicate experiments. *P < .05, **P < .01, ***P < .001 versus LPS.
Figure 4. Effects of DHMC, 8AMC, and DAMC on the phosphorylation of kinases in RAW 264.7 cells stimulated with LPS. (A) ERK, (B) p38, (C) JNK, (D) NF-κB, and (E) IκB kinases. Cells (6.5 × 10⁵/mL) were stimulated with LPS (1 μg/mL) and incubated with 5, 10, and 25 μM each of DHMC, 8AMC, and DAMC for 30 min. Expression of target molecules was determined in culture supernatants by Western blotting. *P < .05, **P < .01, ***P < .001 versus LPS.

Abbreviations: DAMC: 7,8-diacetoxy-4-methylcoumarin; DHMC: 7,8-dihydroxy-4-methylcoumarin; ERK: extracellular signal-regulated kinase; ELISA: enzyme-linked immunosorbent assay; IκB: inhibitor of NF-κB; iNOS: inducible NO synthase; JNK: c-Jun N-terminal kinase; LPS: lipopolysaccharide; NF-κB: nuclear factor-kappa B; 8AMC: 8-acetoxy-4-methylcoumarin.
DHMC, 8AMC, and DAMC for 24 h. Cell viability was measured using MTT assays. Formazan crystals formed by incubating the cells for 3 h at 37 °C with the MTT reagent (Sigma-Aldrich) were dissolved in DMSO. The cells were transferred to 96-well plates, following which absorbance was measured at 570 nm using a microplate reader (Thermo Fisher Scientific Inc.).

**Measurement of NO Production**

We seeded RAW 264.7 cells (8.0 × 10⁶/well), stimulated them 24 h later with LPS (1 µg/mL), and incubated them with 5, 10, and 25 µM each of DHMC, 8AMC, and DAMC for 24 h. A 1:1 ratio of cell culture supernatant to Griess reagent (Sigma-Aldrich) was reacted for 10 min as previously described, following which absorbance was measured at 540 nm.

**Measurement of IL-1β, IL-6, TNF-α, and PGE₂ Production**

Cells stimulated with LPS (1 µg/mL) were incubated with 5, 10, and 25 µM each of DHMC, 8AMC, and DAMC for 24 h to measure IL-1β, IL-6, TNF-α, and PGE₂ levels using the following mouse ELISA kits: IL-1β/IL-1 F2 Quantikine and PGE₂ (R&D Systems), IL-6 (Becton Dickinson and Co.), and TNF-α (Invitrogen).

**Quantitative Reverse Transcription-Polymerase Chain Reaction**

Total RNA (1 µg) extracted from RAW 264.7 cells using RNeasy Mini Kits (Qiagen) was reverse-transcribed using PrimeScript first-strand cDNA Synthesis Kit (Takara Bio Inc.). Relative gene expression was determined by quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR Green Real-Time PCR with TB Green Premix Ex Taq II (Takara Bio Inc.) Data were normalized using the housekeeping gene GAPDH. The target gene was amplified using the following specific forward and reverse (5′-3′) primers:

- iNOS: AATGGCAACATCAGTGCCGACACT and GGTGTTGTGCTACAGAAGTCTCTGACTC;
- COX-2: GGAGAGACTATCAAGATTGAC and ATGGTCTAGACTTTTACA;
- GAPDH: GGTTCCTCCAGCCGGCA and GGCATGGCCCTCCGTGT.

**Western Blotting**

Intracellular inflammatory proteins were detected by Western blotting. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 100 mM phenylmethylsulfonyl fluoride (PMSF), 200 mM Na₃VO₄ following which lysates were centrifuged at 15,928 x g for 30 min. Proteins in the supernatant were quantified using BCA protein assay kits (Thermo Fisher Scientific Inc.) and then resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes (Bio-Rad Laboratories Inc.). The membranes were immersed in Tris-buffered saline containing 5% skim milk (Becton Dickinson and Co.) and 0.1% Tween 20 (TBST) for 90 min at room temperature (∼21 °C-23 °C) to block nonspecific antigen binding. The membranes were washed 4 times at 10-minute intervals using TBST. The membranes were then incubated overnight at 4 °C with primary antibodies (all from Cell Signaling Technology) against the following antigens: iNOS, COX-2, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), phospho-SAPK/JNK (Thr183/Tyr185), p44/42 MAPK (Erk1/2), p38 MAPK, SAPK/JNK, phospho-NF-kB p65 (Ser536) (93H1), rabbit mAb (1: 1000), and IκBα (L35A5) mouse mAb amino-terminal antigen (1:1000). After 4 washes with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (H&L) (Rockland Immunochemicals Inc.) for 2 h at room temperature and then washed 4 times with TBST. Proteins were identified and quantified using enhanced chemiluminescence (ECL) kits and a GS-700 densitometer (both from Bio-Rad Laboratories Inc.). Protein expression data were analyzed using Image J (NIH).

**Statistical Analysis**

All experimental results are expressed as the mean ± SD, and the statistical significance was evaluated using Student’s t-test. Values with P < .05 were considered statistically significant.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Ethical Approval**

Not applicable, because this article does not contain any studies with human or animal subjects.

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**Informed Consent**

Not applicable, because this article does not contain any studies with human or animal subjects.
Supplemental Material

Supplemental material for this article is available online.

Trial Registration

Not applicable, because this article does not contain any clinical trials.

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