Note

Evaluation of Aculeatin and Toddaculin Isolated from *Toddalia asiatica* as Anti-inflammatory Agents in LPS-Stimulated RAW264 Macrophages

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Anti-inflammatory activity of aculeatin and toddaculin, which are coumarins with a similar structure isolated from *Toddalia asiatica* (L.) LAM., was evaluated using lipopolysaccharide (LPS)-stimulated RAW264 mouse macrophage cells. Both aculeatin and toddaculin significantly inhibited mRNA expression of inflammatory mediators and nitric oxide production. Furthermore, Toddaculin suppressed LPS-induced phosphorylation of p38 and extracellular signal-regulated kinase (ERK)1/2 and inhibited LPS-induced activation of nuclear factor-kappaB (NF- κ B). However, aculeatin did not exhibit such effects, suggesting that aculeatin and toddaculin suppress LPS-induced inflammation of RAW264 cells via different mechanisms. The cellular uptake of these compounds was also evaluated. Toddaculin was detected in RAW264 cells after 4 and 24 h. However, aculeatin levels were not observed in RAW264 cells at all incubation intervals. These results indicate that de-epoxidation of a prenyl group can increase hydrophobicity of molecule and is thought to accelerate cellular uptake and/or interactions with the phospholipid bilayers of cell membranes.

Key words *Toddalia asiatica*; coumarin; aculeatin; toddaculin; anti-inflammation; macrophage

Inflammation is an adaptive response by the body to ensure the removal of detrimental stimuli as well as a healing process for repairing damaged tissue.¹⁾ However, if left uncontrolled, inflammatory mediators become involved in the pathogenesis of many inflammatory disorders.²⁾ Macrophages play a pivotal role in the host defense against pathogenic microbes by recognizing bacterial constituents resulting in the activation of a variety of antimicrobial effectors and initiation of the inflammatory cascade.³⁾ When activated by stimuli such as lipopolysaccharides (LPS), macrophages produce numerous inflammatory mediators such as nitric oxide (NO) and pro-inflammatory cytokines including tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1) and IL-6 via toll-like receptor pathways.^{4,5)} Expression of these inflammatory mediators is regulated by the activation of mitogen-activated protein kinase (MAPK) and nuclear factor-kappaB (NF- κ B).^{6,7)}

Toddalia asiatica (L.) LAM. is widely recognized as a traditional medicinal plant in Africa, India, China, and Japan and has been used for treating many diseases. This plant contains a variety of coumarin compounds, and many biological properties have been attributed to it.^{8–12)} Our recent studies have also shown that aculeatin (ACU) derived from *T. asiatica* enhances the differentiation and lipolysis of 3T3-L1 adipocytes.¹³⁾ Furthermore, toddaculin (TOD) inhibits osteoclastogenesis in RAW264 cells and enhances osteoblastogenesis in MC3T3-E1 cells.¹⁴⁾ On the other hand, another research group showed that ACU and TOD exhibit inducible NO synthase (iNOS) protein expression-inhibitory activity in LPS-stimulated mouse macrophage cells.¹⁵⁾ However, detailed mechanisms underlying these results were unclear.

Here, we focused on the anti-inflammatory activity of these coumarins. To reveal the underlying mechanisms by which ACU and TOD attenuate LPS-induced inflammation in RAW264 mouse macrophages, we investigated the mRNA

expression of these anti-inflammatory mediators and also examined the MAPK and NF- κ B signaling pathways.

MATERIALS AND METHODS

Reagents and Cell Culture Dulbecco's modified Eagle's medium (DMEM) and penicillin–streptomycin were purchased from Sigma-Aldrich (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Thermo Fisher Scientific (MA, U.S.A.). Antibodies to p38 MAPK, phospho-p38 MAPK (p-p38), p44/42 MAPK (extracellular signal-regulated kinase (ERK)1/2), phospho-p44/42 MAPK (Thr202/Tyr204; p-ERK1/2), stress-activated protein kinase/jun-amino-terminal kinase (JNK), and phospho-JNK (Thr183/Tyr185; p-JNK) were all obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). All other chemicals were reagent grade. RAW264 cells were obtained from the RIKEN cell bank (Tsukuba, Japan). They were grown in DMEM supplemented with 10% heat-incubated FBS and 1.0% penicillin–streptomycin solution in a humidified incubator with 5% CO₂ at 37°C. Test compounds were dissolved in dimethyl sulfoxide (DMSO) and added to the medium so that the final concentration of DMSO did not exceed 0.1% (v/v). Medium containing DMSO alone was prepared similarly and used as the control medium.

Isolation of Compounds Dry stem chips of *T. asiatica* were obtained from the Okinawa Medical Herb Association (Okinawa, Japan). The dry stem chips (100 g) were suspended in 1 L methanol (MeOH) and heated under reflux for 2 h at 80°C. ACU and TOD were isolated as previously reported¹³⁾ and the structures were determined by ¹H- and ¹³C-NMR and high resolution (HR)-MS (Supplementary Figs. 1–4). Complete assignment of ¹H- and ¹³C-NMR spectrum was based on interpretation of standard two-dimensional NMR (2D-NMR) data. The stereochemistry of ACU was confirmed by a spe-

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cific rotation $[\alpha]_D^{27} -14.7$ ($c=0.1$, in AcOEt) compared with the literature value $[\alpha]_D^{23} -13.95$ ($c=0.57$, in AcOEt).¹⁶⁾ HPLC purities of isolated compounds were over 98% (Supplementary Fig. 5).

Measurement of NO Production and Cell Viability

RAW264 cells were seeded in 96-well plates (1.0×10^5 cells/well). The cells were then treated with ACU or TOD at the indicated concentrations and exposed to LPS (100 ng/mL) for 24 h. After incubation, collected culture media samples were mixed with Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride, and 2.5% phosphoric acid) and incubated at 37°C for 20 min prior to determining the nitrite concentration by measuring the absorbance at 540 nm against a standard curve constructed using sodium nitrite. For the cell viability assessment, a water-soluble tetrazolium salt (WST) cytotoxicity assay was performed using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan).

DNA Microarray and Quantitative (q) RT-PCR Total RNA from RAW264 cells was extracted using TRIzol reagent (Life Technologies) and the RNeasy Mini Kit (Qiagen). A focused-DNA microarray analysis (Genopal Mouse innate immunity Chip, Mitsubishi Chemical, Tokyo, Japan) containing 183 genes related to the innate immunity system was performed following the manufacturer's protocol. For real-time qRT-PCR, cDNAs were synthesized using random primers and PrimeScript reverse transcriptase (TaKaRa Bio). Target cDNAs were amplified using Fast SYBR Green Master Mix (Life Technologies) with the following gene-specific primers: monocyte chemoattractant protein-1 (*Mcp-1*, forward: 5'-GCATCCACGTGTGGCTCA-3'; reverse: 5'-CTCCAGCCTACTCATTTGGGATCA-3'); inducible NO synthase (*NOS2*, forward: 5'-GGAATGGAGACTGTC CCA GCA-3'; reverse: 5'-GTCATGAGCAAAAGGCGCA GA-3'); *Il-6* (forward: 5'-CAACGATGATGTC ACTTGC AGA-3'; reverse: 5'-CTCCAGGTAGCTATGGTACTCCAGA-3'); *Il-1 α* (forward: 5'-TGGTTAATGACCTGCAACAGGAA-3'; reverse: 5'-AGGTCGTCTCACTACCTGTGATG-3'); *Il-1 β* (forward: 5'-TCCAGGATGAGGACATGACAC-3'; reverse: 5'-GAACGTACACCA GCA GGT TA-3'); cyclooxygenase-2 (*Cox-2*, forward: 5'-GTGTGCGACATACTCAAG CAGGA-3'; reverse: 5'-TGAAGTGGTAACCGCTCAGGT G-3'); and β -actin (forward: 5'-CATCCGTAAAGACCTCTA TGCCAAAC-3'; reverse: 5'-ATGAGCCACCGATCCAC A-3'). PCR products were measured using StepOnePlus Real-time PCR System (Life Technologies). Data were expressed as fold-differences normalized to β -actin levels.

Western Blot Analysis RAW264 macrophages were pre-treated with ACU and TOD at 100 μ M for 1 h and then exposed to LPS (100 ng/mL) for 15 and 30 min, respectively. Cells were washed twice with phosphate buffered saline (PBS) and lysed using cComplete™ Lysis-M and Phos-STOP™ (Roche Diagnostic, Tokyo, Japan). Lysates were centrifuged at 13000 rpm for 5 min and the supernatant collected. The protein content was measured using a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, U.S.A.). Each whole cell lysate was suspended in 2 $\times$ Laemmli sample buffer (Bio-Rad, Hercules, U.S.A.) containing 2% 2-mercaptoethanol and boiled at 100°C for 5 min. Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF)

membrane (Merck Millipore, MA, U.S.A.). After blocking for 1 h in 2% ECL blocking reagent (GE Healthcare, Tokyo, Japan) in TPBS (PBS and 0.1% Tween 20), the membrane was incubated overnight at 4°C with the primary antibodies. The membrane was then washed four times with TPBS and incubated at room temperature with the secondary antibody (GE Healthcare). After four washes with TPBS, protein bands were detected with ECL prime Western blotting detection reagents (GE Healthcare) and imaged on a LAS-4000 Luminescent Image Analyzer (GE Healthcare). Band intensities were measured for statistical analysis using Multi Gauge, version 3.11, software (FUJIFILM Life Science, Tokyo, Japan).

Luciferase Reporter Assay RAW264 macrophages were transfected with firefly pGL4.32/NF- κ B plasmid and Renilla pGL4.73/SV40 control plasmid (Promega, Tokyo, Japan) using Lipofectamine LTX and PLUS Reagents (Life Technologies Japan) according to the manufacturer's protocol. Transfected RAW264 cells (5.0×10^4 cells/well) were cultured in 96-well plates for 16 h. After incubation, cells were treated with different concentrations of ACU and TOD, and then LPS (0 or 100 ng/mL) was added. After 6 h, the cells were lysed and luciferase activity was measured using the dual luciferase reporter assay system (Promega). The values were normalized

Fig. 1. Chemical Structures of Aculeatin and Toddaculin (A), Effects of Aculeatin and Toddaculin on NO Production (B) and Cell Viability (C) in LPS-Stimulated RAW264 Cells

Cells were treated with compounds and LPS (100 ng/mL) for 24 h and NO production was measured using the Griess reagent (B), whereas cell viability was measured by WST-8 assay. Data shown are mean $\pm$ S.E.M. ($n=3$). The various letters indicate significant differences in Tukey–Kramer test results ($p < 0.05$).

by control luciferase activities.

Cellular Uptake Analysis RAW cells (3.0×10^6 cells) were seeded in a 25 cm^2 flask and incubated with $100\text{ }\mu\text{M}$ ACU and TOD for 4 and 24 h. After incubation, cells were washed twice with PBS, and the number of cells was counted using a hemocytometer. After centrifugation at 1000 rpm for 2 min, 1 mL of MeOH was added and the cells were sonicated. The solution was centrifuged at 10000 rpm for 10 min, supernatant collected and then dried over N_2 gas. The dried material was dissolved in $200\text{ }\mu\text{L}$ HPLC mobile phase and then filtered through a $0.2\text{-}\mu\text{m}$ membrane. Then, $20\text{ }\mu\text{L}$ of samples were injected onto a C18 column (Wakosil-II 5C18 RS-Prep, $4.6 \times 250\text{ mm}$, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The mobile phase was 50% acetonitrile (ACU) or 70% acetonitrile (TOD). The flow rate was 1 mL/min ; the compounds were detected with UV absorption at 310 nm .

Statistical Analysis Data are expressed as means $\pm$ standard error of the mean (S.E.M.). Statistical comparisons between the groups were analyzed by one-way ANOVA, and then differences among means were analyzed by Dunnett's test or Tukey–Kramer test. Statistical analysis was performed using GraphPad Prism ver. 5.02 for Windows (GraphPad Software, San Diego, California, U.S.A.). Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

In this study, we focused on anti-inflammatory mechanisms of two coumarins, ACU and TOD (Fig. 1A). We first investigated NO production in LPS-stimulated RAW264 cells in the presence and absence of ACU and TOD. Both ACU and TOD ($25\text{--}150\text{ }\mu\text{M}$) suppressed NO production in LPS-stimulated macrophages in a dose-dependent manner and to a similar extent (Fig. 1B). Our results confirm that $150\text{ }\mu\text{M}$ of ACU and TOD did not affect the conditions of the cell (Fig. 1C); there-

fore, it was decided to use a concentration of up to $100\text{ }\mu\text{M}$ for further experiments. In this concentration, ACU and TOD suppressed the production of TNF- α and IL-6 in LPS-stimulated RAW264 macrophages with a concentration-dependent manner (Supplementary Fig. 6).

To investigate whether ACU and TOD suppress the gene expression of inflammatory mediators by LPS stimulation in RAW264 cells, we conducted DNA microarray analysis. In terms of the results, treatment with $100\text{ }\mu\text{M}$ of each of the compounds over 24 h downregulated the expression of inflammatory chemokines, cytokines (*Mcp-1*, *Il-6*, *Il-1 α* , and *Il-1 β*), and inflammatory enzymes (*Cox-2* and *Nos-2*) (data not shown). We then evaluated these genes with real-time qPCR and confirmed that the expression of these genes was significantly decreased by ACU and TOD in a dose-dependent manner to the same extent (Fig. 2). These results indicated that ACU and TOD inhibited RAW264 cell inflammation stimulated by LPS at the mRNA level.

To reveal the underlying mechanisms of anti-inflammatory properties of ACU and TOD, the effects of these two compounds on MAPK phosphorylation were assessed using Western blot analysis. The results showed that TOD significantly suppressed p38 phosphorylation at 15 min but ACU did not (Fig. 3). TOD significantly suppressed ERK1/2 phosphorylation at 15 and 30 min, although ACU did not have significant effects. The acceleration was found in phosphorylation of JNK by TOD at 15 min after LPS-stimulation. This phenomenon was also observed in our previous study using receptor activator of NF- κB ligand-stimulated RAW264 cells.¹⁴⁾ However, the contribution of JNK activation to the anti-inflammatory effect of TOD was unclear and further study was required.

To examine the effects of ACU and TOD on that transcriptional activity, we conducted NF- κB luciferase reporter assays. LPS treatment markedly elevated the activity of NF- κB over 10 times. It is notable that TOD significantly suppressed the

Fig. 2. Effects of ACU and TOD on mRNA Expression [(A) *Mcp-1*, (B) *Nos-2*, (C) *Il-6*, (D) *Il-1 α* , (E) *Il-1 β* , and (F) *Cox-2*] in RAW264 Cells

Cells were treated with compounds and LPS (100 ng/mL) for 24 h, and mRNA levels were measured using qPCR. Data shown are mean $\pm$ S.E.M. ($n=3$). The various letters indicate significant differences in Tukey–Kramer test results ($p < 0.05$).

Fig. 3. Effects of ACU and TOD on LPS-Induced Phosphorylation of MAPKs in RAW264 Cells

Cells were treated with compounds (100 μ M) and LPS (100 ng/mL) for 15 and 30 min. Then, cells were lysed and analyzed by Western blotting. Data shown are means $\pm$ S.E.M. ($n=3-4$). * $p<0.05$ vs. LPS-stimulated cells by Dunnett's test.

NF- κ B activity, whereas ACU was significantly less effective (Fig. 4). These results suggest that TOD can suppress LPS-induced inflammation *via* p38, ERK1/2, and NF- κ B pathways but ACU may use a different mechanism.

Our previous study revealed that ACU can promote adipocyte differentiation better than TOD.¹³ Conversely, TOD inhibited the differentiation of osteoclasts and induced osteoblastogenesis,¹⁴ whereas ACU did not affect osteoclastogenesis nor osteoblastogenesis under the same conditions. Epoxidation of a prenyl group seems to play an important role in the various biological activities.

To evaluate the reason for the difference in the mechanisms of action of ACU and TOD, we examined cellular uptake level of these compounds. TOD was detected in RAW264 cells

after 4 h (129 pmol/ 1.0×10^6 cells) and 24 h (177 pmol/ 1.0×10^6 cells). However, ACU content in RAW264 cells were less than 20 pmol/ 1.0×10^6 cells at all incubation intervals (Fig. 5). Prenylation of flavonoids dramatically increased cellular uptake in C2C12 myotubes.¹⁷ Regarding flavonoids, prenylation can increase hydrophobicity and may accelerate cellular uptake and/or interactions with the phospholipid bilayers of cell membranes or hydrophobic target proteins.¹⁸ Epoxidation of a prenyl group decreased hydrophobicity of TOD because the log P of TOD and ACU was 3.17 and 1.91, respectively (CS Chem Draw Ultra ver. 6.0, Cambridge Soft Corporation, MA, U.S.A.). The lower accumulation in the cell and/or weak association with cell membranes could be correlated with a decrease in hydrophobicity due to epoxidation. Despite the small

Fig. 4. Effects of ACU and TOD on LPS-Induced NF-κB Activation

Luciferase reporter assay was performed as described in Materials and Methods. Data shown are means±S.E.M. ($n=3$). * $p<0.05$ vs. LPS-stimulated cells by Dunnett's test.

Fig. 5. Uptake Levels of ACU and TOD in RAW264 Cells

Cells were incubated with each compound for 4 or 24 h and then extracted and analyzed by HPLC. Data are expressed as mean±S.E.M. ($n=3$). N.D., Not detectable.

amount of ACU accumulated in the cells, biological activity in the RAW264 cells of ACU and TOD was comparable. It is of interest how ACU express anti-inflammatory its activity in the RAW264 cells, but this will require further investigation.

In conclusion, our results demonstrated that both ACU and TOD, which are coumarins with similar structures, exhibited anti-inflammatory activity in LPS-stimulated macrophages, although the specific mechanisms might be different. TOD suppressed LPS-induced phosphorylation of p38 and ERK1/2 and inhibited LPS-induced activation of NF-κB. However, ACU did not exhibit such effects under the same conditions. Our findings about cellular uptake suggested that epoxidation of the prenyl group decreases hydrophobicity of a molecule and this may be involved in the mechanisms of anti-inflammatory activity. Further research will be needed to clarify the mechanisms of anti-inflammatory activity by ACU in LPS-stimulated macrophage cells.

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Conflict of Interest Momochika Kumagai, Takashi Mishima, Izumi Yoshida, and Munetomo Nakamura are employees of Japan Food Research Laboratories. Akio Watanabe, Keisuke Nishikawa, and Yoshiki Morimoto have no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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