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1,1-Dimethylallylcoumarins Potently Supress both Lipopolysaccharide- and Interferon-γ-induced Nitric Oxide Generation in Mouse Macrophage RAW 264.7 Cells

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Abstract—We investigated the suppressive effects of 16 coumarin-related compounds on both lipopolysaccharide (LPS)- and interferon (IFN)-γ-induced nitric oxide (NO) generation in a mouse macrophage cell line, RAW 264.7. Notably, coumarins possessing prenyl unit(s) were found to be highly active, a tendency consistent with our previous study. Among the coumarins tested, 1,1-dimethylallylcoumarins showed the highest inhibitory activity. Western blotting analysis revealed that they inhibited NO generation by suppressing inducible NO synthase (iNOS) protein expression. Our ongoing studies suggest that coumarins are prominent natural compounds that attenuate excessive and prolonged NO generation at inflammatory sites.

A gaseous free radical, nitric oxide (NO), has attracted attention, since its excessive and prolonged production plays important roles in lifestyle-related diseases, including inflammation and carcinogenesis.[1] Some naturally occurring compounds have been suggested as suppressants of such diseases by counteracting NO generation.[2,3] In the previous study, coumarins isolated from *Citrus hystrix* DC fruit showed a notable inhibitory activity toward both lipopolysaccharide (LPS)- and interferon (IFN)-γ-induced NO generation in mouse macrophage RAW 264.7 cells.[4] Concurrently, a structure–activity relationship study revealed that coumarins which possess prenyl unit(s) were categorized as a conspicuous class of NO generation inhibitors.[4] In the present study, we further examined the suppressive effects of 16 naturally occurring or synthetic coumarin-related compounds. Of the coumarins, 1,1-dimethylallylcoumarins were indicated to be distinguished inhibitors, blocking both LPS- and IFN-γ-induced NO generation by suppressing inducible NO synthase (iNOS) protein expression.

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Isolation and Synthesis of Coumarin-related Compounds

The tested coumarins had been isolated or synthesized as previously reported.[5–17] The natural coumarins were: osthenol (**1**, from *Citrus* sp.)[5]; nordentatin (**4**, from *Citrus* sp.)[5]; clausarin (**5**, *Citrus* sp.)[5]; ponfolin (**6**, *Citrus* sp.)[6]; clausenidin (**7**, *Clausena excavata*)[7]; prangenin (**8**, *Clausena lansium*)[8]; microminutinin (**9**, from *Micromelum minutum*)[9]; microminutin (**11**, from *M. minutum*)[10]; phebalosin (**15**, *M. exotica*)[11]; and micromarin-A (**16**, *M.* sp.).[12] Those synthesized were: umbelliprenin (**2**)[13]; 7-isopentenyloxycoumarin (**3**)[14]; columbianetin (**10**)[15]; 7-propenyloxycoumarin (**12**)[16]; 8-propenylumbelliferone (**13**)[16]; and 5,7-dihydroxycoumarin (**14**).[17]

Bioassay Methods

NO generation test

LPS- and IFN-γ-induced NO generation tests were performed as previously reported.[4] Briefly, RAW 264.7 cells (2×10^5 cells/mL), a murine macrophage cell line,

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grown in 1 mL of RPMI medium on a 24-well plate were treated with LPS (100 ng/mL), tetrahydrobiopterin (BH_4, 10 mg/mL), IFN-γ (100 U/mL), L-arginine (2 mM), and a test compound dissolved in dimethylsulfoxide (DMSO, 0.5%, v/v) at a concentration of 50 or 10 μM. Cells treated without any test compounds were used as a positive control. After 24 h, the levels of nitrite (NO_2^-) were measured by Griess assay. Each experiment was done in triplicate, and the data are shown as mean ± standard deviations.

Western blotting

Confluent RAW 264.7 cells on a 6-well plate were stimulated and incubated in the same manner as described above. After the cells were washed, a boiling lysis solution was added. Ten micrograms of protein were separated on a 10% polyacrylamide gel and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, MA). After blocking with Block Ace (UK-B25, Dainippon Pharmaceutical Co., Ltd.) overnight at 4 °C, the membranes were incubated with a primary antibody (anti-mouse iNOS, 1:1000 dilution, Affinity Bioreagents, Inc.), and then with a secondary antibody (peroxidase-conjugated swine anti-rabbit IgG, 1:1000 dilution, Dako). The blots were developed using an ECL detection kit (Amersham Life Science). The antibodies were stripped and the blots were reprobed with rabbit polyclonal anti-β-actin antibody (1:1000 dilution, Biochemical Technologies). The membrane was then incubated with the secondary antibody described above. iNOS band levels were corrected by those of β-actin as an internal standard. Each experiment was done in triplicate, and the data are shown as mean ± standard deviations.

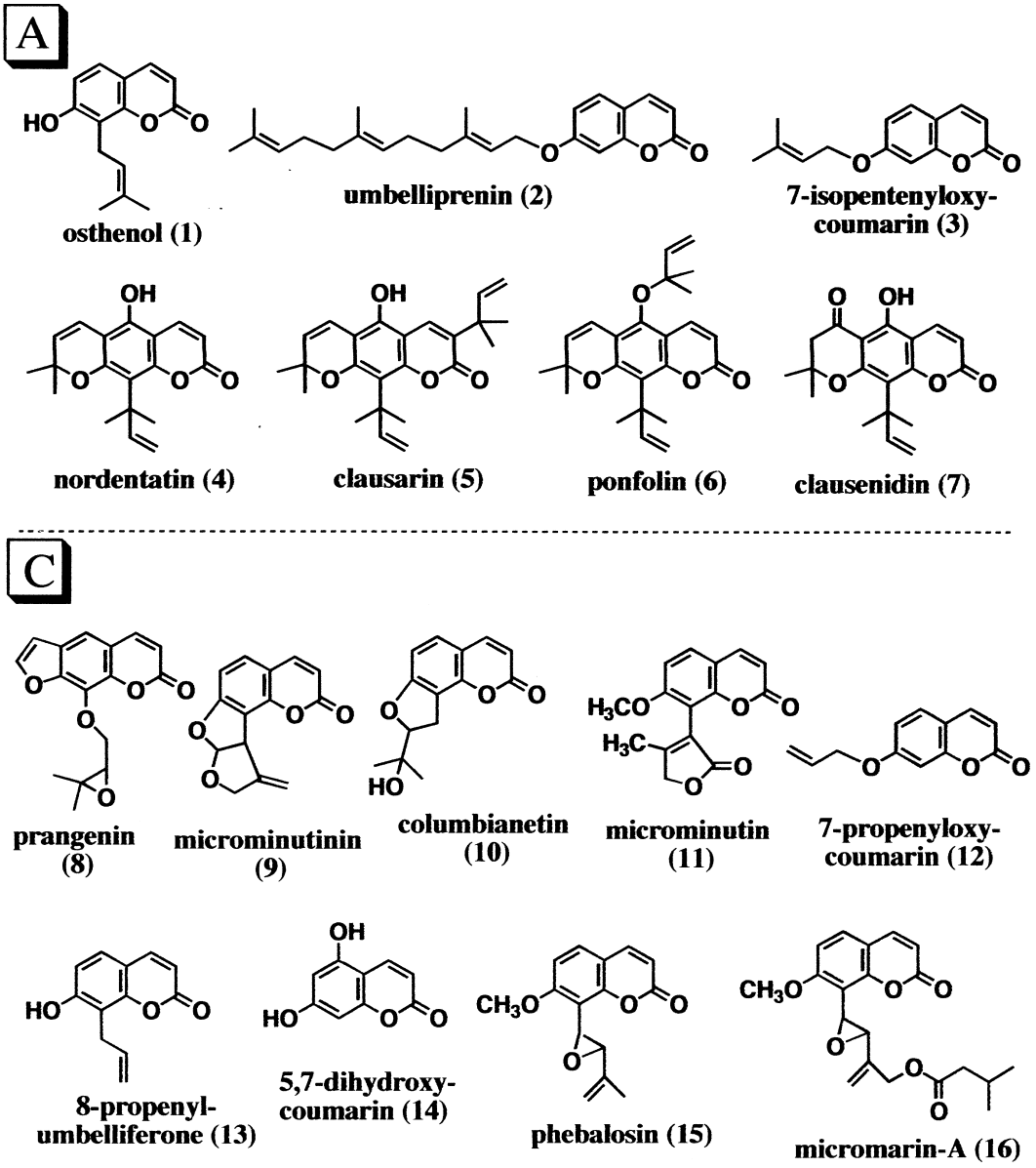

Figure 1. Structures of naturally-ocurring and synthetic coumarins in groups A and C.

Results and Discussion

Sixteen coumarin-related compounds had been isolated or synthesized as previously reported.[5–17] We examined the inhibitory effects of these coumarins on both LPS- and IFN-γ-induced NO generation in RAW 264.7 cells, at concentrations of 50 and 10 μM. NO generation was measured by monitoring the levels of NO_2^-, a stable NO metabolite in the media. Cytotoxicity was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. No notable cytotoxicity was observed in any of the experiments (data not shown). The NO_2^- concentration in the media from LPS/IFN-γ-stimulated cells was 40.3 ± 7.3 μM.

We had previously classified the natural coumarins into groups A–C, according to their activities:[4] (A) highly active group containing prenyl unit(s); (B) moderately active group bearing a prenyl unit cyclized to the coumarin ring; and (C) completely inactive group bearing a prenyl unit(s) modified with hydroxyl group(s) and/or having other functional groups, except for the prenyl unit. Based on the structures, the 16 coumarins in the present study could be divided into two groups, A and C (Fig. 1). (−)-Epigallocatechin gallate (EGCG), a green tea polyphenol, was used as a positive control, since it has been reported to inhibit NO generation.[8]

As shown in Figure 2, 7 coumarins belonging to group-A showed significant inhibitory activity at a 50 μM concentration while most of those to group-C were inactive, as predicted from the above categorization, although prangenin (**8**) and micromarin-A (**16**) inhibited NO generation by 36.9 and 86.1%, respectively. The marked suppressive potency of **16** may be attributable to the hydrophobicity of its high side chain regulating cellular uptake, which has been suggested to be an increasing factor for NO generation suppression.[4] When tested at a concentration of 10 μM, four coumarins in group-A; nordentatin (**4**), clausarin (**5**), ponfolin (**6**), and clausenidin (**7**), suppressed NO generation by 63.6–91.2%, respectively. Their inhibitory potencies were evaluated as comparable to or higher than EGCG. Thus, those coumarins which carry 1,1-dimethylallyl group(s) (**4**–**7**) were recognized as the most suppressive coumarins tested in the present and previous[4] studies.

Figure 3 shows the suppressive efficacy of clausenidin (**7**), at concentrations of 10 and 50 μM, toward LPS-and IFN-γ-induced iNOS protein expression. The activities were comparable to EGCG. While EGCG has been reported to suppress NO generation by inhibiting the transcriptional activity of nuclear factor kappa-B, thereby attenuating iNOS protein expression,[18] the action mechanisms of **7** remain to be addressed. In addition, we have not confirmed whether **7** inhibits iNOS activity nor perturbs NO_2^-/NO_3^- conversion.

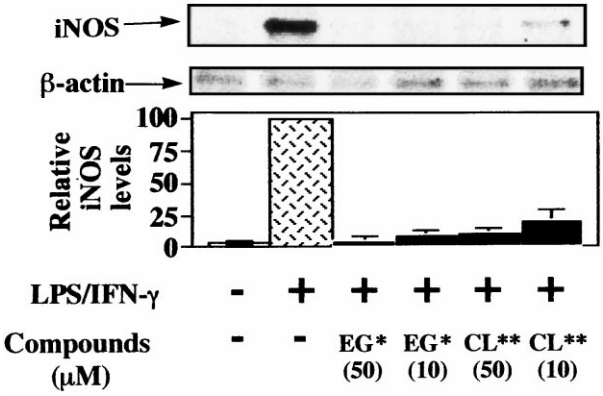

Figure 3. Suppressive effects of ECGC and clausenidin on LPS/IFN-γ-induced iNOS protein expression in RAW 264.7 cells. Confluent RAW 264.7 cells on a 6-well plate were stimulated and incubated, as described in Materials and Methods. Ten micrograms of protein was separated on a 10% polyacrylamide gel and electrophoretically transferred onto a polyvinylidene difluoride membrane. The blots were developed using ECL detection. iNOS band levels were corrected by those of β-actin as an internal standard. Each experiment was done in triplicate, and the data are shown as mean ± standard deviations. One of the representative pictures is shown. *EGCG, **Clausenidin (**7**).

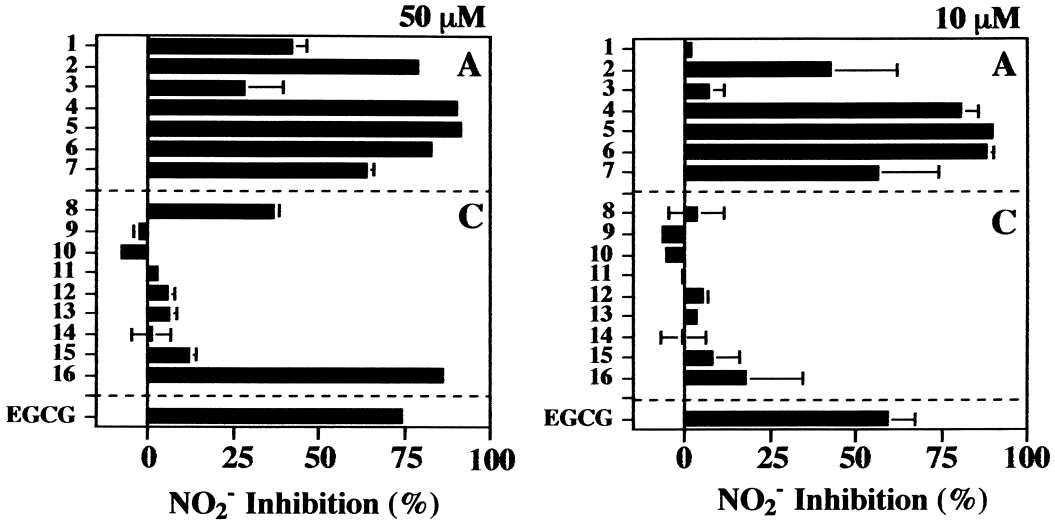

Figure 2. Inhibitory effects of coumarin-related compounds on LPS/IFN-γ-induced NO generation in RAW 264.7 cells. RAW 264.7 cells, grown on a 24-well plate, were treated with LPS (100 ng/mL), BH_4 (10 mg/mL), IFN-γ (100 U/mL), L-arginine (2 mM), and a test compound. After 24 h, the levels of nitrite (NO_2^-) were measured by Griess assay. Each experiment was done in triplicate, and the data are shown as mean ± standard deviations.

In conclusion, while the suppressive effects of coumarins on NO generation have been recently presented,[4,19] this is the first known report demonstrating that a natural coumarin-related compound is able to suppress iNOS protein expression in a cell culture system although a synthetic coumarin, cloricromene, has been reported to be an inhibitor of iNOS induction.[20] Further, the coumarin categorization proposed in the previous[4] and present study may be useful to predict their suppressive activities toward NO generation, since they are distributed ubiquitously throughout the plant kingdom.

Acknowledgements

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