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Novel anti-inflammatory actions of nobiletin, a citrus polymethoxy flavonoid, on human synovial fibroblasts and mouse macrophages

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Abstract

We previously reported that nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone), a citrus polymethoxy flavonoid, effectively interferes with the production of promatrix metalloproteinase (proMMP)-9/progelatinase B in rabbit synovial fibroblasts [J. Rheumatol. 27 (2000) 20]. In this paper, we further examine the effects of nobiletin on the production of cyclooxygenases (COXs), prostaglandin (PG) E_2, and proinflammatory cytokines in human synovial fibroblasts and the mouse macrophage J774A.1 cell line. Nobiletin suppressed the interleukin (IL)-1-induced production of PGE_2 in human synovial cells in a dose-dependent manner (<64 μM). Additionally, it selectively downregulated COX-2, but not COX-1 mRNA expression. Nobiletin also interfered with the lipopolysaccharide-induced production of PGE_2 and the gene expression of proinflammatory cytokines including IL-1α, IL-1β, TNF-α and IL-6 in mouse J774A.1 macrophages. In addition, nobiletin downregulated the IL-1-induced gene expression and production of proMMP-1/procollagenase-1 and proMMP-3/prostromelysin-1 in human synovial fibroblasts. In contrast, production of the endogenous MMP inhibitor, TIMP-1, was augmented by nobiletin. These anti-inflammatory actions of nobiletin are very similar to those of anti-inflammatory steroids such as dexamethasone, and the upregulation of TIMP-1 production is a unique action of nobiletin. Therefore, these results further support the notion that nobiletin is likely to be a candidate for characterization as a novel immunomodulatory and anti-inflammatory drug.

Keywords: Nobiletin; Polymethoxy flavonoid; Cyclooxygenase; Matrix metalloproteinase-1; Matrix metalloproteinase-3; Proinflammatory cytokine; Human synovial fibroblasts

1. Introduction

MMPs are considered to play a crucial role in the destruction of matrix components in connective tissue under pathological conditions such as rheumatoid arthritis and osteoarthritis (for review, see [1]). For example, high levels of MMP-1/collagenase-1, MMP-3/stromelysin-1, and MMP-9/gelatinase B have been found in synovial tissues and fluid from patients with rheumatoid arthritis [2–4]. Recently, the localization of membrane type (MT)-MMPs in rheumatoid synovium also has been reported [5]. It generally is accepted that proinflammatory cytokines such as IL-1, TNF-α and IL-6 are key mediators that greatly enhance the biosynthesis and secretion of precursors of MMPs (proMMPs) and PGE_2 from mesenchymal cells at inflammatory sites [6].

Recently, we reported that nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone), a citrus polymethoxy flavonoid, effectively suppresses the production of proMMP-9 and decreases the steady-state level of its mRNA in rabbit

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Abbreviations: PG, prostaglandin; COX, cyclooxygenase; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LPS, lipopolysaccharide; LAH, lactalbumin hydrolysate; IL, interleukin; TNF, tumor necrosis factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcriptase-polymerase chain reaction.

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synovial fibroblasts and articular chondrocytes [7]. However, the other anti-inflammatory actions of nobiletin and its effectiveness in other cell types, including human cells, have not been clarified. In this paper, we report that in human synovial fibroblasts, nobiletin interferes with the production of PGE_2 by selectively downregulating *COX-2* gene expression and protein. In addition, the production of IL-1α, IL-1β, IL-6, and TNF-α proteins and their mRNAs was suppressed in mouse macrophages exposed to nobiletin. Furthermore, nobiletin suppressed the production of proMMPs-1 and -3, whereas production of the endogenous MMP inhibitor, TIMP-1, was upregulated in human synovial cells. Therefore, nobiletin may be a candidate for characterization as a novel anti-inflammatory and immunomodulatory drug.

2. Materials and methods

2.1. Materials

Nobiletin was isolated from the juice of *Citrus depressa* Hayata (*Rutaceae*), as described previously [7]. The following reagents were obtained commercially: DMEM and RPMI 1640 from the Invitogen Co.; FBS from Whittaker Bioproducts Inc.; *Escherichia coli* (O55:B5) LPS, LAH, nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and alkaline phosphatase conjugated donkey anti-(sheep IgG) IgG from the Sigma Chemical Co.; trypsin from DIFCO Laboratories; human GAPDH cDNA probe from Clontech Laboratories, and monoclonal human COX-1 and -2 antibodies from the Cayman Chemical Co. Recombinant human IL-1α (rhIL-1α; 2×10^7 units/mg) was supplied by the Dainippon Pharmaceutical Co. Sheep anti-(human proMMP-1), sheep anti-(human MMP-3), and sheep anti-(human TIMP-1) antisera were provided by Dr. Hideaki Nagase of the Kennedy Institute of Rheumatology, Imperial College. Human synovial fibroblasts were purchased from the Cell Systems Co. The mouse macrophage J774A.1 cell line was purchased from the Health Science Research Resources Bank. Other reagents used were the same as in a previous paper [8].

2.2. Culture of human synovial cells and mouse macrophages

Human synovial fibroblasts and the mouse macrophage J774A.1 cell line were maintained in 10% FBS/DMEM/antibiotics and 5% FBS/RPMI 1640/antibiotics, respectively, until confluence. In our experiments, synovial cells at up to the 10th passage and macrophages were plated in 24-multiwell plates or 100-mm diameter culture dishes. After confluence, the culture medium was changed to DMEM/0.2% LAH for human synovial cells or RPMI 1640/0.2% LAH for J774A.1 cells, and nobiletin was added to the culture medium as a DMSO solution. The final DMSO concentration was 0.05% in all cultures, and the same amount of vehicle was added to the control cultures. The harvested culture media were stored at −20° until use. All experiments were conducted at least in duplicate and a typical set of data was shown.

2.3. Quantification of intracellular DNA

The cells fixed with EtOH for 10 min were incubated with 200 μL of 3,5-diaminobenzoic acid dihydrochloride (Sigma) (400 mg/mL) at 60° for 45 min according to the method of Johnson-Wint and Hollis [9]. After the reaction and addition of 1 M hydrochloric acid, fluorescence intensity was measured at an excitation wavelength of 365 nm and an emission wavelength of 530 nm. The content of intracellular DNA was calculated with a standard curve using authentic salmon sperm DNA (6.25–100 μg/mL).

2.4. Western blot analysis of proMMPs, and COXs-1 and -2

The levels of proMMPs in the culture media were monitored by western blot analysis and the relative amounts were quantified by densitometric scanning as described previously [8]. To determine the production of COXs-1 and -2, cells were lysed in a lysis buffer (50 mM Tris–HCl pH 7.5/2% SDS/10% glycerol/5% 2-mercaptoethanol) and then subjected to enhanced chemiluminescence-western blot analysis (Amersham Pharmacia Biotech) using antibodies against the respective human antigens.

2.5. Determination of PGE_2 and cytokines in the culture media

PGE_2 was determined using a commercial enzyme-immunoassay kit (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. The sensitivity of the assay system was <40 pg/mL. Mouse IL-1β was measured in the culture medium using a sandwich enzyme-linked immunosorbent assay kit (BioSource International Inc.) according to the instructions of the manufacturer. The IL-1β (<7 pg/mL) level was determined in the linear range of the concentration curve.

2.6. Semiquantification of mRNA levels by RT-PCR

Total RNA was isolated from cells in 100-mm dishes using Isogen reagents (Nippon Gene Co.). The RNA (3 μg) was subjected to RT-PCR for the measurement of COX-1 and -2, proMMP-1, and GAPDH mRNAs in human synovial fibroblasts, and for the quantification of IL-1α, IL-1β, IL-6, TNF-α and GAPDH mRNAs in J774A.1 cells as described previously [8]. PCR primers used with human synovial fibroblast cDNA were as follows: human COX-1, 5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′ (sense) (516–539 bp)

and 5′-GTGCATCAACACAGGCGCCTCTTC-3′ (antisense) (796–819 bp) [10]; human COX-2, 5′-TTCAAATGAGATTGTGGGAAAAT-3′ (sense) (574–596 bp) and 5′-AGATCATCTCTGCCTGAGTATCTT-3′ (antisense) (855–878 bp) [11]; human proMMP-1, 5′-GGTGATGAAGCAGCCCAG-3′ (sense) (323–340 bp) and 5′-CAGTAGAATGGGAGAGTC-3′ (antisense) (759–742 bp) [12] and human GAPDH, 5′-CCACCCATGGCAAATTCCATGGCA-3′ (sense) (213–235 bp) and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (antisense) (786–809 bp) [13]. PCR primers used for the amplification of mouse IL-1α, IL-1β, IL-6, TNF-α and GAPDH cDNA were from Clontech Laboratories Inc. Amplifications were performed at 92° for 40 s, at 54–60° for 40 s and 72° for 60 s with 20–35 cycles. The amplified PCR products were analyzed on ethidium bromide-stained 1% agarose gels. We confirmed that amplification of COX-1, COX-2, proMMP-1, and human GAPDH was linear for 25–30 cycles; 26–30 cycles for IL-1α and IL-1β; 17–22 cycles for TNF-α and IL-6; and 30–35 cycles for mouse GAPDH. Gene expression was quantified by densitometric scanning using an Image Analyzer LAS-1000 Plus (Fuji Film). The signal intensities of the specific mRNAs were normalized by comparison with that of GAPDH and were calculated as relative amounts.

2.7. Statistical analysis

A one-way ANOVA was used for data statistical analysis. A Dunnet test was applied when multiple comparisons were performed.

3. Results

3.1. Effects of nobiletin on the production of PGE_2 and COX-2 protein and mRNA expression in human synovial fibroblasts

When confluent human synovial fibroblasts were treated with IL-1α (1 ng/mL), the production of PGE_2 was augmented about 10 times as compared with the untreated cells (Fig. 1, lane 1 vs. lane 2). Nobiletin effectively suppressed the IL-1α-mediated production of PGE_2 in a dose-dependent manner (4–64 μM); more than 65% of IL-1α-induced PGE_2 was suppressed by 4 μM nobiletin ($P < 0.001$). Next, we examined whether the inhibition by nobiletin of PGE_2 production was due to the suppression of COX-1 and COX-2 transcripts. As shown in Fig. 2A, western blot analysis indicated that in human synovial cells, nobiletin interfered predominantly with the production of COX-2 but not with COX-1 in a concentration-dependent manner; 64 μM nobiletin inhibited approximately 50% of IL-1α-induced COX-2 ($P < 0.001$). The selective suppression by nobiletin of COX-2 production was supported further by RT-PCR, which showed that nobiletin selectively downregulated expression of the

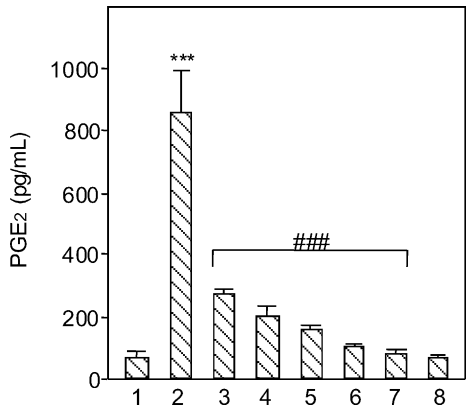

Fig. 1. Suppression of PGE_2 production by nobiletin in human synovial fibroblasts. Confluent synovial cells, at passage 10, were treated in 24-multiwell plates with rhIL-1α (1 ng/mL) and/or nobiletin in 1.0 mL of DMEM/0.2% LAH for 24 hr. The amount of PGE_2 released in the culture medium was determined by enzyme-immunoassay. Treatments: l, control; 2, rhIL-1α; 3–7, rhIL-1α plus nobiletin (4, 8, 16, 32 and 64 μM, respectively); and 8, nobiletin (64 μM). Data are the means ± SD for quadruplicate wells. Key: (***) and (###), significantly different from the control ($P < 0.001$) and the rhIL-1α-treated cells ($P < 0.001$), respectively.

COX-2 transcript as early as 2 hr after treatment (>66% inhibition ($P < 0.001$), Fig. 2B). These suppressive effects of nobiletin were found not to be due to cytotoxicity. When human synovial cells were treated with nobiletin and/or IL-1α, the total cell numbers, as monitored by DNA content, were unaffected (Fig. 3A). Therefore, these results suggest that the inhibitory effect of nobiletin on the production of PGE_2 in human synovial fibroblasts is due

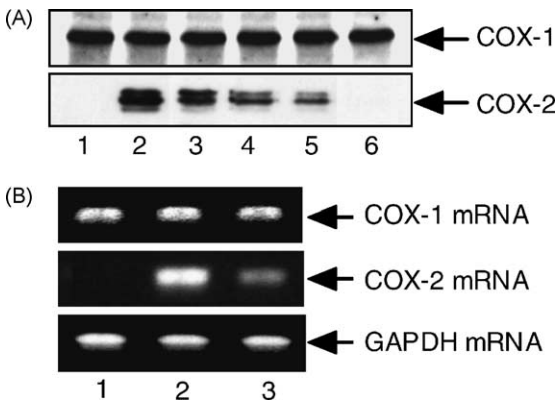

Fig. 2. Selective downregulation by nobiletin of *COX-2* gene expression and COX-2 protein in human synovial fibroblasts. (A) Western blot analysis of COX-1 and COX-2 proteins. Confluent synovial cells, at passage 10, in 100-mm dishes were treated with rhIL-1α (1 ng/mL) and nobiletin in 1.0 mL of DMEM/0.2% LAH for 24 hr. After incubation, the cells were washed once with PBS(−) and lysed. The cell lysate was then subjected to enhanced chemiluminescence-western blot analysis of COX-1 and COX-2 proteins. Treatments: l, control; 2, rhIL-1α; 3–5, rhIL-1α plus nobiletin (16, 32 and 64 μM, respectively); and 6, nobiletin (64 μM). (B) Changes in COX-1 and COX-2 mRNA. Confluent cells at passage 5 were treated, in 100-mm dishes, with rhIL-1α (1 ng/mL) and/or nobiletin (64 μM) for 2 hr, and total RNA was isolated using Isogen as described in the text. Total RNA (3 μg) was subjected to RT-PCR for COX-1 and COX-2. Treatments: l, control; 2, rhIL-1α; and 3, rhIL-1α plus nobiletin (64 μM).

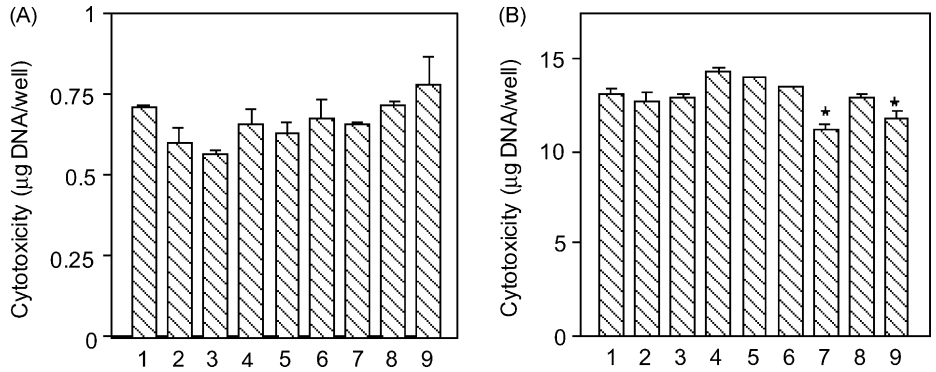

Fig. 3. Effect of nobiletin on the viability of human synovial cells and mouse macrophages. Human synovial fibroblasts (A) at passage 9 and J744A.1 cells (B) cultured in 24-multiwell plates were treated with nobiletin (4–64 μM) in the presence of rhIL-1α (1 ng/mL) or LPS (5 μg/mL), respectively, for 10 hr. The intracellular DNA content was determined to indicate toxicity as described in the text. Data are the means ± SD for quadruplicate wells. Key: (*), significantly different from the control ($P < 0.05$).

to the suppression of *COX-2* gene expression, and subsequent decrease in COX-2 production, and not due to an effect on COX-1.

3.2. Interference in the gene expression of proinflammatory cytokines in J774A.1 mouse macrophages by nobiletin

Macrophages are known to play a role in the production of proinflammatory cytokines at inflammatory sites, such as those that appear in rheumatoid arthritis [14,15]. Therefore, we examined whether nobiletin interfered with the production of proinflammatory cytokines using cultured mouse macrophage J744A.1 cells. When the cells were treated with LPS (5 μg/mL) for 12 hr, the transcripts of IL-1α and IL-1β effectively were augmented (1.8-fold ($P < 0.001$) and 2.9-fold ($P < 0.001$), respectively). However, the transcripts of IL-6 and TNF-α were slightly, but significantly augmented (both 1.1-fold ($P < 0.01$)) as shown in Fig. 4. Nobiletin (32 μM) was found to significantly diminish LPS-mediated *IL-1α* (>50% inhibition ($P < 0.01$)), *IL-1β*, (>48% inhibition ($P < 0.05$)) and *IL-6* (>47% inhibition ($P < 0.01$)) gene expression. In contrast, the suppressive effect of nobiletin was less sensitive toward *TNF-α* gene expression (>30% inhibition ($P < 0.05$)). Thus, it is very likely that the downregulation by nobiletin of IL-1β mRNA correlated with the suppression of LPS-mediated IL-1β production in mouse macrophages; the level of IL-1β in the culture medium of LPS-treated cells was about two times higher than that of nontreated cells (19.62 ± 7.41 pg/mL vs. 37.20 ± 9.93 pg/mL, $P < 0.05$). This LPS-mediated augmentation was almost completely suppressed to control levels (20.33 ± 8.63 pg/mL) by 32 μM nobiletin ($P < 0.05$). Moreover, nobiletin (1–16 μM) was found to effectively interfere with the LPS-induced production of PGE_2 in cultured mouse J774.1 macrophages. (data not shown). It was also found that this suppressive effect of nobiletin (1–32 μM) was not due to cytotoxicity, since the DNA content in these macrophages was not affected, as shown in Fig. 3B.

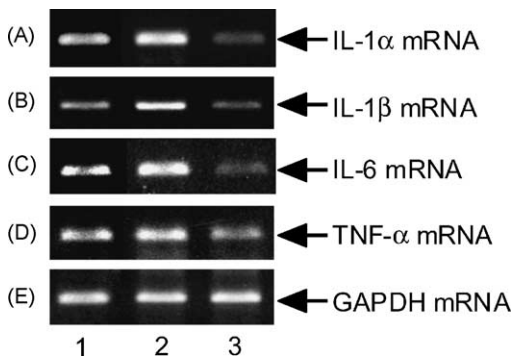

Fig. 4. Effect of nobiletin on *IL-1α, IL-1β, IL-6*, and *TNF-α* gene expression in mouse J774A.1 macrophages. Adherent mouse macrophages, in 60-mm dishes, were treated with LPS (5 μg/mL) and/or nobiletin (32 μM) in 3.0 mL of RPMI 1640/0.2% LAH for 12 hr. Total RNA (3 μg) was subjected to RT-PCR for the amplification of (A) IL-1α, (B) IL-1β, (C) IL-6, (D) TNF-α, and (E) GAPDH mRNA as described in the text. Treatments: l, control; 2, LPS; and 3, LPS plus nobiletin (32 μM).

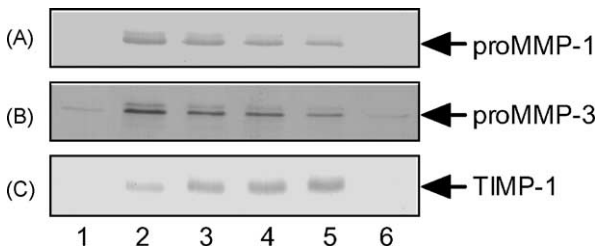

Fig. 5. Effect of nobiletin on the production of proMMPs-1 and -3, and TIMP-1 in human synovial fibroblasts. Confluent synovial cells at passage 9 were treated, in 24-multiwell dishes, with rhIL-1α (1 ng/mL) and/or various concentrations of nobiletin in 1.0 mL of DMEM/0.2% LAH for 24 hr. The harvested culture medium from triplicate wells was subjected to western blot analysis for (A) proMMP-1, (B) proMMP-3, and (C) TIMP-1 as described in the text. Relative amounts of each protein were determined by densitometric analysis. Treatments: l, control; 2, rhIL-1α; 3–5, rhIL-1α plus nobiletin (16, 32 and 64 μM, respectively); and 6, nobiletin (64 μM).

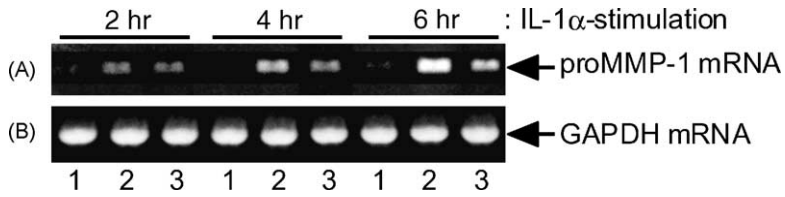

Fig. 6. Time-dependent downregulation by nobiletin of the expression of IL-1α-induced proMMP-1 mRNA in human synovial cells. Confluent synovial cells at passage 7 were treated with rhIL-1α (1 ng/mL) and/or nobiletin (64 μM) for the indicated periods, and then total RNA was extracted using Isogen. Total RNA (3 μg) was subjected to RT-PCR for the amplification of (A) proMMP-1 and (B) GAPDH mRNA. Treatments: l, control; 2, rhIL-1α; and 3, rhIL-1α plus nobiletin (64 μM).

3.3. Downregulation by nobiletin of the production of proMMPs-1 and -3 and upregulation of TIMP-1 production

We recently have reported that nobiletin inhibits the production of proMMP-9 in rabbit synovial fibroblasts and articular chondrocytes [7]. Therefore, we examined the effects of nobiletin on the production of proMMPs and their endogenous inhibitor, TIMP-1, in human synovial cells. In this series of experiments, IL-1α-induced the production of proMMPs-1 and -3, but not proMMP-9. As shown in Fig. 5A and B, nobiletin suppressed the IL-1α-induced production of proMMPs-1 and -3 in a concentration-dependent manner (16–64 μM); more than 40% of the IL-1α-induced production of proMMPs-1 and -3 was suppressed by 64 μM nobiletin ($P < 0.01$). In addition, the effect of nobiletin on the proMMP-1 transcript was examined. IL-1α increased the steady-state level of proMMP-1 mRNA in a time-dependent manner (Fig. 6); the increase in the transcript already was observed as early as 2 hr after the IL-1α treatment. Nobiletin (64 μM) downregulated the expression of proMMP-1 mRNA in human synovial cells; at 6 hr after treatment the IL-1α-induced proMMP-1 transcript was diminished by more than 50%. These results suggested that the suppression of the production of proMMP-1 by nobiletin coincided with the decrease in its transcript. In contrast, nobiletin concentration-dependently upregulated the IL-1α-mediated production of TIMP-1 in human synovial cells (1.3-fold by 64 μM nobiletin ($P < 0.05$), Fig. 5C), indicating that nobiletin might prevent the destruction of connective tissue via the simultaneous upregulation of TIMP-1 and downregulation of proMMPs at inflammatory sites such as those found in rheumatoid arthritis and osteoarthritis.

4. Discussion

Recently, it has been reported that flavonoids exert their anti-inflammatory actions by inhibiting IL-8 [16] and PGE_2 [17] production in fibroblasts and synovial cells. In addition, flavonoids have been found to be cytotoxic to some types of cancer cells *in vitro* [18]. We have reported that a citrus polymethoxy flavonoid, nobiletin, effectively inhibits the production of PGE_2 and proMMP-9 in rabbit synovial fibroblasts [7]. However, neither the action mechanisms of nobiletin nor its effects on the production of proinflammatory cytokines in human cells are fully understood.

PGE_2 is known to play a significant role as an inflammatory mediator in rheumatoid arthritis and osteoarthritis since it increases vascular permeability [19]. COXs are known as key enzymes for the production of PGs including PGE_2, and COX-2, an inducible enzyme, plays a central role in the production of PGE_2 at inflammatory sites [20]. In contrast, COX-1, a constitutively expressed enzyme, is recognized to participate in the production of physiological levels of PGs in tissues, such as in the mucus of the stomach [21]. Therefore, the production of an anti-inflammatory drug that selectively inhibits the production and/or activity of COX-2 is desirable. In this paper, we have demonstrated that nobiletin interferes with the production of PGE_2 in human synovial fibroblasts by selectively downregulating COX-2. A similar observation was reported in interferon-γ/LPS-treated mouse RAW 264.7 macrophages [22]. The inhibition by nobiletin of LPS-induced PGE_2 production in mouse macrophages, as shown in this paper, is likely to interfere selectively with *COX-2* gene expression and the production of COX-2 protein. In two recent reports that describe the effects of flavones on the expression of COX wogonin (5,7-dihydroxy-8-methoxyflavone) was shown to suppress COX-2 expression in mouse skin [23] and mouse RAW 264.7 macrophages [24].

At sites of inflammation associated with rheumatoid arthritis, proinflammatory and immunomodulatory cytokines such as IL-1α, IL-1β, IL-6, and TNF-α are known to exert various actions during the progression of inflammation [14,15]. IL-1α, IL-1β and TNF-α induce and/or enhance the production of PGE_2 as well as proMMPs, including proMMP-1, -3, -9, and -13, in many mesenchymal cell types such as, synovial cells, chondrocytes, and macrophages [1,25–28]. IL-6 also is characterized as an inflammatory factor because it synergistically augments the inflammatory actions of IL-1 in human synovial cells [29]. It is of interest that nobiletin downregulates the production of these cytokines in macrophages. This strongly suggests that nobiletin is bifunctional, i.e. it suppresses the IL-1- and TNF-α-evoked production of PGE_2 and activation of the immune system. These properties of nobiletin are very similar to those of anti-inflammatory steroids including dexamethasone [30–32]. In contrast, it also is a fact that dexamethasone

suppresses the production of TIMP-1, as well as proMMPs in human synovial fibroblasts [8], whereas nobiletin downregulates the production of proMMPs-1 and -3, but upregulates TIMP-1 production. Therefore, it is likely that the novel action of nobiletin towards TIMP-1 is distinct from that of dexamethasone.

It was of concern whether the suppression by nobiletin of COX-2, PGE_2, and proMMP levels in human synoviocytes and the expression of proinflammatory cytokines in mouse macrophages was due to a cytotoxicity. However, nobiletin was not toxic for either synovial cells (<64 μM) or macrophages (<32 μM), as shown in Fig. 3A and B. In addition, it also was found that nobiletin increased the production of TIMP-1 in a concentration-dependent manner (<64 μM), whereas it did not exert any effect on COX-1 expression. These observations further support the notion that nobiletin selectively modulates the production of PGE_2, proinflammatory cytokines, proMMPs, and TIMP-1.

The mechanism by which nobiletin suppresses the production of proMMPs while upregulating TIMP-1 production has not yet been clarified. Since it has been reported that nuclear factor kappa B (NF-κB) plays an important role in the transcriptional activation of IL-1, TNF-α and the proMMPs [33,34], suppression by nobiletin of proMMP and cytokine production is likely to be due to the direct modulation of NF-κB binding to the promoter regions of the genes coding for these proteins. However, we have reported that nobiletin accelerates the production of TIMP-1 while suppressing the production of proMMPs-1 and -9 in human fibrosarcoma HT-1080 cells by interfering with AP-1-, not NF-κB-binding [35]. Thus, further studies are needed to clarify the exact mode of action of nobiletin on the production of proMMPs and cytokines in human synovial fibroblasts.

In conclusion, we have provided novel evidence that nobiletin, a polymethoxy flavonoid, interferes with the production of PGE_2 via the selective downregulation of the *COX-2* gene in human synovial fibroblasts. In addition, we show that nobiletin decreases the expression of IL-1α, IL-1β, TNF-α, and IL-6 mRNAs in mouse macrophages. These properties of nobiletin are very similar to those of dexamethasone. In contrast, it is noteworthy that nobiletin downregulated the production of proMMPs-1 and -3 in human synovial fibroblasts but upregulated the expression of TIMP-1. These observations, therefore, indicate that nobiletin exerts both anti-inflammatory and immunomodulatory actions and is characterized as a novel anti-inflammatory and immunomodulatory drug.

Acknowledgments

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References

[1] Nagase H, Okada Y. Proteinases and matrix degradation. In: Kelley WN, Ruddy S, Harris Jr ED, Sledge CB, editors. Textbook of rheumatology. Philadelphia: W.S. Saunders Co.; 1996. p. 323–41.
[2] Arend WP, Dayer JM. Cytokines and cytokine inhibitors or antagonists in rheumatoid arthritis. Arthritis Rheum 1990;33:305–15.
[3] Walakovits LA, Moore VL, Bhardwaj N, Gallick GS, Lark MW. Detection of stromelysin and collagenase in synovial fluid from patients with rheumatoid arthritis and posttraumatic knee injury. Arthritis Rheum 1992;35:35–42.
[4] Tetlow LC, Lees M, Ogata Y, Nagase H, Woolley DE. Differential expression of gelatinase B (MMP-9) and stromelysin-1 (MMP-3) by rheumatoid synovial cells *in vitro* and *in vivo*. Rheumatol Int 1993;13:53–9.
[5] Yamanaka H, Makino K, Takizawa M, Nakamura H, Fujimoto N, Moriya H, Nemori R, Sato H, Seiki M, Okada Y. Expression and tissue localization of membrane types 1, 2 and 3 matrix metalloproteinases in rheumatoid synovium. Lab Invest 2000;80:677–87.
[6] Woessner Jr JF. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 1991;5:2145–54.
[7] Ishiwa J, Sato T, Mimaki Y, Sashida Y, Yano M, Ito A. A citrus flavonoid nobiletin suppresses production and gene expression of matrix metalloproteinase 9/gelatinase B in rabbit synovial fibroblasts. J Rheumatol 2000;27:20–5.
[8] Lin N, Sato T, Ito A. Triptolide, a novel diterpenoid triepoxide, from *Tripterygium wilfordii* Hook. f. suppresses the production and gene expression of promatrix metalloproteinases-1 and -3 and augments the expression of tissue inhibitors of metalloproteinases-1 and -2 in human synovial fibroblasts. Arthrits Rheum 2001;44:2193–200.
[9] Johnson-Wint B, Hollis S. A rapid *in situ* deoxyribonucleic acid assay for determining cell number in culture and tissue. Anal Biochem 1982;122:338–44.
[10] Funk CD, Funk LB, Kennedy ME, Pong AS, Fitzgerald GA. Human platelet/erythroleukemia cells prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. FASEB J 1991;5:2304–12.
[11] Sato T, Nakajima H, Fujio K, Mori Y. Enhancement of prostaglandin E_2 production by epidermal growth factor requires the coordinate activation of cytosolic phospholipase A_2 and cyclooxygenase-2 in human squamous carcinoma cells. Prostaglandins 1997;53:355–69.
[12] Templeton NS, Brown PD, Levy AT, Marguilies IMK, Liotta LA, Stetler-Stevenson WG. Cloning and characterization of human tumour cell interstitial collagenase. Cancer Res 1990;50:5431–7.
[13] Tokunaga K, Nakamura Y, Sakata K, Fujimori K, Ohkubo M, Sawada K, Sakiyama S. Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. Cancer Res 1987;47:5616–9.
[14] Feldmann M, Brennan FM, Chantry D, Haworth C, Turner M, Abney E, Buchan G, Barrett K, Barkley D, Chu A, Field M, Maini RN. Cytokine production in the rheumatoid joint: implications for treatment. Ann Rheum Dis 1990;49:480–6.
[15] Hirano T, Mat T, Turner M, Miyasaka N, Buchan G, Tang B, Sato K, Maini R, Feldmann M, Kishimoto T. Excessive production of interleukin-6/B cell stimulatory factor-2 in rheumatoid arthritis. Eur J Immunol 1988;18:1797–801.
[16] Sato M, Miyazaki T, Kambe F, Maeda K, Seo H. Quercetin a bioflavonoid, inhibits the production of interleukin-8 and monocyte

chemoattractant protein-1 expression by tumor necrosis factor-α in cultured human synovial cells. J Rheumatol 1997;24:1680–4.
[17] Chung CP, Park JB, Bae KH. Pharmacological effects of methanolic extract from the root of *Scutellaria baicalensis* and its flavonoids on human gingival fibroblasts. Planta Med 1995;61:150–3.
[18] Carroll KK, Guthrie N, So FV, Chambers AF. Anticancer properties of flavonoids, with emphasis on citrus flavonoids. In: Rice-Evans CA, Packer L, editors. Flavonoids in health and disease. New York: Marcel Dekker; 1998. p. 437–46.
[19] Goodwin JS. Are prostaglandins proinflammatory, anti-inflammatory, both or neither? J Rheumatol (Suppl) 1991;28:26–9.
[20] Geng Y, Blanco FJ, Cornelisson M, Lotz M. Regulation of cyclooxygenase-2 expression in normal human articular chondrocytes. J Immunol 1995;155:796–801.
[21] Masferrer JL, Zweifel BS, Manning PT, Hauser SD, Leahy KM, Smith WG, Isakson PC, Seibert K. Selective inhibition of inducible cyclooxygenase-2 *in vivo* is anti-inflammatory and nonulcerogenic. Proc Natl Acad Sci USA 1994;91:3228–32.
[22] Murakami A, Nakamura Y, Torikai K, Tanaka T, Koshiba T, Kuwahara S, Takahashi Y, Ogawa K, Yano M, Tokuda H, Nishino H, Mimaki Y, Sashida Y, Kitanaka S, Ohigashi H. Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation oxidative stress and tumor promotion in mice. Cancer Res 2000;60:5059–66.
[23] Park BK, Heo MY, Park H, Kim HP. Inhibition of TPA-induced cyclooxygenase-2 expression and skin inflammation in mice by wogonin, a plant flavone from *Scutellaria radix*. Eur J Pharmacol 2001;425:153–7.
[24] Chi YS, Cheon BS, Kim HP. Effects of wogonin a plant flavone from *Scutellaria radix*, on the suppression of cyclooxygenase-2 and the induction of inducible nitric oxide synthase in lipopolysaccharide-treated RAW 264.7 cells. Biochem Pharmacol 2001;61:1195–203.
[25] Chang J, Gilman SC, Lewis AJ. Interleukin-1 activates phospholipase A_2 in rabbit chondrocytes: a possible signal for IL-1 action. J Immunol 1986;136:1283–7.
[26] Dayer JM, Goldring SR, Robinson DR, Krane SM. Collagenase and prostaglandin in connective tissue destruction: cell–cell and humoral interactions. Bull Schweiz Akad Med Wiss 1979;35:329–43.
[27] Case JP, Lafyatis R, Kumkumian GK, Remmars EF, Wilder RL. IL-1 regulation of transin/stromelysin transcription in rheumatoid synovial fibroblasts appears to involve two antagonistic transduction pathways, an inhibitory, prostaglandin-dependent pathway mediated by cAMP, and a stimulatory, protein kinase C-dependent pathway. J Immunol 1990;145:3755–61.
[28] Ito A, Nose T, Takahashi S, Mori Y. Cyclooxygenase inhibitors augment the production of promatrix metalloproteinase 9 (progelatinase B) in rabbit articular chondrocytes. FEBS Lett 1995;360:75–9.
[29] Ito A, Itoh Y, Sasaguri Y, Morimatsu M, Mori Y. Effects of interleukin-6 on the metabolism of connective tissue components in rheumatoid synovial fibroblasts. Arthritis Rheum 1992;35:1197–201.
[30] Lanza L, Scudeletti M, Monaco E, Monetti M, Puppo F, Filaci G, Indiveri F. Possible differences in the mechanism(s) of action of different glucocorticoid hormone compounds. Ann NY Acad Sci 1999;876:193–7.
[31] Petrovsky N, McNair P, Harrison LC. Diurnal rhythms of proinflammatory cytokines: regulation by plasma cortisol and therapeutic implications. Cytokine 1998;10:307–12.
[32] Morita M, Kasahara T, Mukaida N, Matsushima K, Nishizawa M, Yoshida M. Induction and regulation of IL-8 and MCAF production in human brain tumor cell lines and brain tumor tissues. Eur Cytokine Netw 1993;4:351–8.
[33] Jue DM, Jeon KI, Jeong JY. Nuclear factor kappaB (NF-κB) pathway as a therapeutic target in rheumatoid arthritis. J Korean Med Sci 1999;14:231–8.
[34] Han Z, Boyle DL, Manning AM, Firestein GS. AP-1 and NF-kappaB regulation in rheumatoid arthritis and murine collagen-induced arthritis. Autoimmunity 1998;28:197–208.
[35] Sato T, Koike L, Miyata Y, Hirata M, Mimaki Y, Sashida Y, Yano M, Ito A. Inhibition of activator protein-1-binding activity and phosphatidylinositol 3-kinase pathway by nobiletin, a polymethoxy flavonoid, results in augmentation of tissue inhibitor of metalloproteinases-1 production and suppression of production of matrix metalloproteinases-1 and -9 in human fibrosarcoma HT-1080 cells. Cancer Res 2002;62:1025–9.