**Nobiletin Suppresses MMP-9 Expression through Modulation of p38 MAPK Activity in Human Dermal Fibroblasts**

Jin-Ju Kim, a,# Sovannarith Korm, a, b Won-Seok Kim, b Ok-Seon Kim, a Ji-Seon Lee, a
Hyung-Geun Min, a Young-Won Chin, a, d and Hyuk-Jin Cha*, a

a Department of Life Science, College of Natural Science, Sogang University; Seoul 121–742, Republic of Korea; b Department of Dermatology, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine; Seoul 110–746, Republic of Korea; c Clean up Skin Clinic; Seoul 150–034, Republic of Korea; and d College of Pharmacy, Dongguk University-Seoul; Goyang, Gyeonggi-do 410–820, Republic of Korea.

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We aimed to identify a novel flavonoid from the in-house natural products to suppress matrix metalloproteinases (MMPs), which is responsible for degradation of collagen and other extracellular matrix proteins. Total eight natural products were screened for identification of a novel MMP-9 suppressor using MMP-9 reporter system, where the prompt initial screening with multiple samples is readily examined. Among the extracts used in the present study, one extract (Citrus unshiu) was found active in this assay system. Furthermore, three representative flavonoids in this active extract of Citrus unshiu peel were tested in MMP-9 reporter system. Nobiletin (NB) of the tested flavonoids suppressed MMP-9 expression without cytotoxicity, which was validated by both real-time polymerase chain reaction (PCR) and zymography analyses. Sustained p38 mitogen activated protein kinase (MAPK) activity, closely associated with induction of MMP-9 under stress condition, was markedly reduced by NB treatment, which implies that modulation of p38MAPK by nobiletin is responsible for reduction of MMP9 expression. Hence, nobiletin, identified from MMP-9 reporter system based screening, may be further applied for the purpose of delaying collagen degradation in skin fibroblasts.

**Key words** Citrus unshiu; nobiletin; flavonoid; matrix metalloprotease 9; p38 mitogen activated protein kinase (MAPK)

The integrity of skin connective tissue, which is important for the elasticity of the skin, relies on the levels of dermal extracellular matrix (ECM) proteins, such as collagen type I and III, elastin, and fibronectin. Aging or chronic exposure to ultraviolet (UV) causes loss of ECM and the consequent decreased resilience of skin, which are strongly associated with various phenotypes of aged skin (e.g. wrinkle formation). The integrity of skin connective tissue, which is important for the elasticity of the skin, relies on the levels of dermal extracellular matrix (ECM) proteins, such as collagen type I and III, elastin, and fibronectin. Aging or chronic exposure to ultraviolet (UV) causes loss of ECM and the consequent decreased resilience of skin, which are strongly associated with various phenotypes of aged skin (e.g. wrinkle formation).11,12 Matrix metalloproteinases (MMPs), members of the zinc-dependent endoprotease family, serve as important enzyme in degrading dermal ECM.13 Of note, excess expression of MMPs in keratinocytes and dermal fibroblasts, under a variety of stress conditions such as UV radiation (UVR), and heat-shock,3 which both are closely associated to the aged skin phenotypes,4,5 contributes to the degradation of ECM proteins, leading to wrinkle formation in photo-aged skin.6 Therefore, MMPs, induced by a variety of external stresses and capable of degrading collagen and other ECM proteins, have been widely studied as a target for relieving the appearance of aged skin.7 Among them, gelatinase A (MMP-2) and gelatinase B (MMP-9) were increased by UV radiation in human dermal fibroblasts (hDFs)8,9 and are responsible for degrading collagens, which are components of the epidermal basement membranes.10 Thereby, the increased activity or expression of gelatinases induced by UV radiation to cause disruption of the basement membrane is considered to be a main cause of skin photoaging,11,12 which can provide a rationale to target the gelatinases to prevent UV induced wrinkle formation.17,12 In special, MMP-9 expression is dependent upon activator protein 1 (AP-1) transcriptional activity,13 which is regulated by mitogen activated protein kinases (MAPKs).14 It is why phorbol-12-myristate 13-acetate (PMA) that can serve as a potent activator of MAPK, is widely used for inducing MMP-9 expression.7,15

In line with this, modulation of MAPK activity, especially p38 MAPK by number of small molecules has been suggested to be a possible strategy to suppress the induction of MMP-9 after stress conditions.15,16 During the screening of plant extracts using MMP-9 inhibition assay, the extract of Citrus unshiu (SWINGLE MARCOW) Rutaceae peel was found to inhibit MMP-9 expression. The fruits of C. unshiu, juicy and delicious fruits, are popularly consumed in China, Japan and Korea. The extracts or individual constituents of C. unshiu peel have been reported to possess a variety of biological activities including anti-oxidant, anti-inflammatory activities, and inhibition of hepatitis C virus infection.17,18 In this study, we aimed to investigate whether or not a methylated flavonoid, nobiletin (NB), present in the peel extract, suppress MMP-9 expression under PMA stimulation, through modulation of p38MAPK activity.

**MATERIALS AND METHODS**

**Materials** Extracellular signal-regulated kinase 2 (ERK2) antibody (cat# SC-154) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Primary antibodies against phosphorylated p38 (pp38, Thr180/Tyr182 #4631), phosphorylated c-Jun N-terminal kinase (JNK) 1/2 (pJNK1/2, Thr183/Tyr185 #9251), phosphorylated ERK1/2 (pERK1/2, Thr202/Tyr204 #9106) were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies

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* The authors declare no conflict of interest.
* These authors contributed equally to this work.

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were obtained from Jackson Immuno Research Laboratories (West Grove, PA, U.S.A.). PMA (cat# P1585), Nobiletin (cat# N1538), Hesperidin (cat# H5254) and Naringin (cat# 71162) were obtained from Sigma (St. Louis, MO, U.S.A.).

**Preparation of Extracts** Each plant material of 100 g (leaves of Saururus chinensis, pericarps of Garcinia mangostana, whole plants of Hypericum japonicum, leaves of Thuja orientalis, roots of Scutellaria baicalensis, peels of Citrus unshiu, leaves of Houttuynia cordata, roots of Euphorbia kansui) were extracted with methanol (300 mL) three times in room temperature and then evaporated in vacuo.

**Primary Cell Culture and Treatments** Human dermal fibroblasts (hDFs) were purchased from Korean Cell Bank (Dietto 551) and maintained as described elsewhere. Briefly, human fibroblast cells were maintained in fibroblast medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (FBS). Each cell population was maintained until confluence and was then trypsinized for seeding. After two or three passages, the cells were used in the experiments. hDF were maintained in DMEM medium supplemented with 10% (v/v) FBS with 0.1% gentamicin (Gibco, Cat. 15750-060, Grand Island, NY, U.S.A.). Cells in either medium were kept at 37°C in a humidified incubator with 5% CO₂. Each cell type was seeded at 90% confluence. The cells were starved using 0.1% serum-containing media for 24 h. Natural product's (Ex#1: CN-Ex) extracts (20 µg/mL), nobiletin, naringin, and hesperidin (20 µm) were treated 1 h prior to PMA treatment (up to 200 µM). The cells were then further incubated in 0.1% serum-containing media in the presence of natural extracts, Nobiletin, Naringin and Hesperidin for another 24 h.

**RNA Isolation and Quantitative Real-Time PCR Analysis** Total RNA was extracted from cells using Trizol Reagent (Invitrogen), followed by treatment with DNase I, according to the manufacturer's instructions. Trizol was removed by the addition of chloroform, and mRNA was precipitated by isopropanol. The RNA precipitates were washed with 75% ethanol. The amount and purity of RNA were determined by measuring the optical density at 260 and 280 nm using a UV spectrometer, and the integrity of the RNA was verified by agarose gel electrophoresis. Gene-specific primers were designed to amplify MMP-9 (Forward; 5-CTT CCG GAT TGG GAT-3, Reverse; 5-GGT GCT GGC TGA GTA GAT-3), MMP-2 (Forward; 5-CTT CCG GAA AAG ATG GAT G-3, Reverse; 5-GGT GCT GGG TGA GTG AAT-3), and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Forward; 5-AAG GGT CAT CAT CTC TGCC-3, Reverse; 5-GTT ATG GCA TGG ACT GTG GT-3). All amplifications were performed in a final reaction mixture (20 µL) containing 1× final concentration of SYBR supermix, 500 nM of gene-specific primers, and 2 µL of template, using the following conditions: an initial denaturation at 95°C for 5 min, followed by 45 cycles of 95°C for 45 s, 58°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 5 min. After amplification, the baseline and threshold levels for each reaction were determined using the company’s software package (Light cycler 480II Roche). For validation of PCR, 30 cycle amplified products were separated on 2% agarose gels and visualized by ethidium bromide staining.

**Microporation Transfection** 293T cells were transfected with Mock (empty) and pGL4.14-MMP9-Luc plasmid vector using a Microporator (Neon™, Invitrogen, Carlsbad, CA) as the manufacturer recommended. After 48 h, transfected 293T cells underwent drug-selection by 500 µg/mL of hygromycin B.

**Immunoblotting** Immunoblotting was performed as described elsewhere.

**Gelatin Zymography** The enzymatic activities of MMP-2 and MMP-9 were assayed by gelatin zymography in the absence of serum. Supernatants from PMA treated hDF cells were concentrated 10 times using YM-30 and were then added to non-reducing sample buffer. The samples were electrophoresed on an 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel containing gelatin (10 µg/mL). The gel was incubated for 30 min with washing buffer (50 mM Tris–HCl, pH 7.5, 2.5% Triton X-100), followed by a brief rinsing in washing buffer without Triton X-100, and then was incubated with incubation buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 0.02% NaN₃) at 37°C. After incubation for 20 h, the gel was stained with 0.25% Coomassie Brilliant Blue R250 (Sigma Chemical Co., St. Louis, MO, U.S.A.) and then was destained. MMP activity was indicated by a clear zone of gelatin digestion.

**Statistical Analysis** The graphical data were presented as means±S.D. Statistical significance among three groups and between groups was determined using one-way ANOVA following Bonferroni post-test and Student’s t-test, respectively. Significance was assumed for p<0.05 (*) and p<0.01 (**).

**RESULTS AND DISCUSSION**

In order to search for a novel natural compound to block MMP-9, one of the key proteases degrading skin ECM and a putative target for anti-wrinkle agents among the in-house natural products, we first performed initial screening by determining MMP-9 mRNA expression levels in human dermal fibroblasts (hDFs) after treatment of natural products under PMA stimulation. Among 5 extracts and small molecules including galegine and metformin, whose anti-melanogenic effects were revealed previously, an extract of C. unshiu (Ex#3: CN-Ex) was shown to significantly weakened the MMP-9 expression (Fig. 1A) in a similar rate to metformin (Met, used as positive control in this assay). The effect of CN-Ex on MMP-9 appeared to be specific as CN-Ex failed to alter the MMP-2 expression but showed markedly lowered MMP-9 transcription, determined by semi-quantitative real-time PCR (Fig. 1B). Dose dependent reduction of MMP-9 but not MMP-2 expression by CN-Ex was clearly revealed in independent experiments (Figs. 1C, D). It should be noted that the flavonoid in the CN-Ex (e.g. naringenin (NG)) has been reported to protect skin cells from UV mediated photo-damage, which was also responsible for induction of MMP-9 expression and also wrinkle formation. Additionally, the biological functions of flavonoids in CN-Ex including the anti-invasive effect of cancer by suppressing MMP-2 and 9 expressions were determined. Therefore, we next attempted to identify the main bioactive substance responsible for suppressing MMP-9 expression among the known major active flavonoids in CN-Ex. For this purpose, we first selected three widely studied flavonoids in CN-Ex, hesperidin (HP), naringin (NG) and nobiletin (NB), of which effect toward MMP-9 expression was examined. Among them,
Human dermal fibroblast (hDF) cells were plated to about 90% confluence. After 24 h of seeding, the cells were starved in 0.1% serum-containing media for another 24 h. Three Exs (20 µg/mL) from natural products (Ex#1: Saururus chinensis, Ex#2: Garcinia mangostana, Ex#3: Citrus unshiu), galegine and metformin (Met, 5 mM) were treated for 1 h prior to PMA (20 nM) stimulation and were further incubated for another 24 h. (A) MMP-9 or (B) MMP-2 expression levels in hDF cells were determined by real-time PCR. Relative expression levels are presented as a bar graph. (C) MMP-9 or (D) MMP-2 mRNA expression levels in hDF cells were determined by real-time PCR after treatment with the indicative dose of CN-Ex treatment (5, 10, 20 µg/mL). Active flavonoids of CN-Ex, Naringin (NR, 50 µM), Nobiletin (NB, 50 µM), or Hesperidin (HP, 50 µM) were added 1 h prior to PMA (20 nM) treatment and were further incubated for another 24 h. (E) MMP9 or (F) MMP-2 expression levels in hDF cells were determined by real-time PCR analysis and are presented as a bar graph (NS: not significant). *p < 0.05, **p < 0.01, and ***p < 0.001.
NB appeared to suppress MMP-9 but not MMP-2 expression significantly under PMA stimulation (Figs. 1E, F, Supplementary Fig. S1A).

Next, we generated the cells that stably expressed the MMP-9 promoter linked reporter (pMMP-9-Luc) for a wider level of screening (Fig. 2A, top panel). The MMP-9 reporter...
that NB treatment also inhibits PMA induced MMP-9 expression in hDFs. Thus, the distinct dose dependent suppression of MMP-9 expression by NB was clearly revealed by determining the activity of MMP-9 reporter (Fig. 2C) and the level of MMP-9 protein (Fig. 2D). In vitro zymography assay also confirmed a marked reduction of MMP-9 but not MMP2 gelatinase activity after NB treatment under PMA stimulation (Fig. 2E). Considering unaltered MMP2 expression by NB treatment (Fig. 1F, middle panel), equal gelatin degradation serves an appropriate loading control (Fig. 2E, bottom panel).

Next, in order to determine the cytotoxic or cytostatic effect of NB, the fold change of cell numbers after 6d of incubation with NB was determined. As shown in Fig. 2F, up to 6d of indicative dose of NB treatment (up to 50 \( \mu \text{M} \)), the growth rate remained unaltered (Fig. 2F). The MMP-9 induction by PMA was mostly regulated by AP-1 transcriptional activity,\(^\text{23}^\) which is mediated by MAPK such as p38, JNK and ERK1/2.\(^\text{25}^\) Thus, inhibition of MAPKs was demonstrated to be associated to reduction of MMP-9 transcription.\(^\text{26}^\) Thus, we next determined MAPK activity under PMA with or without NB treatment. The results showed that markedly increased p38, JNK and ERK1/2 by PMA challenge were gradually lowered in time dependent manner, which might have resulted from prompt dephosphorylation of MAPKs by their specific phosphatases after activation.\(^\text{27}^\) However, due to the sustained action of PMA, MAPK activity appeared to be retrieved 4h later (Fig. 3). Nevertheless, CN-Ex (Fig. 3A) or NB (Fig. 3B) treatment remarkably inhibited recovery of p38 activation under PMA, whose activity is critical for MMP-9 expression,\(^\text{15}^\) indicating that inhibition of p38 MAPK by NB would be a key event in the suppression of MMP-9 expression under PMA stimulation. As consistent with previous reports,\(^\text{15,28,29}^\) p38 inhibitor (SB203580: SB) treatment could suppress MMP-9 expression under PMA stimuli as similar as the results of NB treatment (Fig. 3C).

Accordingly, NB of CN-Ex was identified as a novel suppressor of MMP-9 through modulation of p38 MAPK activity. This finding suggests that NB may be utilized to retain collagen in skin fibroblasts.

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