Studies on Physiological Effects of Polymethoxyflavone on Photoaging and Its Molecular Mechanism

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Abstract

Sun light causes the hyper-pigmentation and the wrinkle formation on the skin. Therefore, they are collectively referred as “photoaging”. Ultraviolet (UV) B is the lowest wavelength in the sunlight arriving at the surface of earth and mainly contributes to the photoaging. Of the photoaging, the hyper-pigmentation results from over–production of melanin in the skin, which is produced by melanocytes of epidermis. On the other hands, the wrinkle is formed by degradation of extracellular matrix (ECM) of the dermis. In these studies, I have examined the effects of polymethoxyflavone (PMF), extracted from orange peels, against the UVB-induced melanogenesis and ECM degradation.

I have firstly focused the UVB-induced prostaglandin (PG)E_2 production in the skin. PGE_2 is mainly secreted by UVB-irradiated keratinocytes of the epidermis and takes the central role in the skin inflammation, as the erythema and edema formation. Skin inflammation promotes the invasion of inflammatory cells, as lymphocytes and neutrophils, to the dermis. Inflammatory cells secrete ECM degrading enzymes, as matrix metalloproteinase (MMP)s. Moreover, PGE_2 also promotes the melanin production and melanin transfer to keratinocytes through the dendrite elongation of melanocytes. Therefore, because I have thought that PGE_2 also plays a key role in the UVB-induced melanogenesis and ECM degradation, I have examined the PMF effect against the PGE_2 production of HaCaT, human keratinocyte cell line. Firstly, I have found that PMF significantly suppresses the UVB-induced PGE_2 production on HaCaTs. PGE_2 is synthesized from arachidonic acid by several enzymes. Cyclooxygenase (COX)-2 is the rate-limiting enzyme of this reaction and its expression is induced by UVB. Similar to PGE_2, PMF has also inhibited UVB–induced COX-2 mRNA and

protein. It has been reported that COX-2 expression is suppressed by peroxisome proliferator–activated receptor (PPAR)-γ activation. By reporter gene assays, in PPAR-α, γ and δ, it has revealed that PMF strongly activates only PPAR-γ. Additionally, PPAR-γ antagonist has recovered the inhibitory effect of PMF against COX-2 expression. Therefore, it has been suggested that PMF suppresses the UVB-induced COX-2 expression and PGE_2 production through PPAR-γ activation.

It has been known that UVB also induces ECM degradation, independent on inflammation. In that event, keratinocytes, the major cells in the skin, mainly secretes MMPs. I have focused MMP-1, degrading type 1 collagen which is the most abundant ECM in the dermis. Firstly, I have found that PMF suppresses the UVB-induced MMP-1 expression on HaCaTs. It has been known that MMP-1 expression is activated by NF-κB and activator protein 1(AP-1). Although BAY11-7085, NF-κB inhibitor, has not affected the MMP-1 suppression by PMF, only SP600125, AP-1 inhibitor, has recovered it. It has been reported that AP-1 is activated by phosphorylated c-jun N-terminal kinase (JNK). UVB-induced JNK phosphorylation has been inhibited by PMF treatment. Therefore, it has been suggested that PMF suppresses MMP-1 expression by the inhibition of JNK phosphorylation.

Next, I examined the inhibitory effect of PMF against the melanogenesis. Firstly, I have found that PMF suppresses the melanin production on HM3KO, human-derived melanoma cell line. Melanin is synthesized from tyrosine by several enzymes in the melanosomes of melanocytes. Because tyrosinase is the rate-limiting enzyme of the melanin synthesis, I have examined the effects of PMF against tyrosinase. Although PMF has not affected the enzymatic activity and mRNA expression of tyrosinase, it has decreased tyrosinase protein. Tyrosinase is degraded in proteasomes and lysosomes. Of

them, only lysosome inhibitor has recovered tyrosinase degradation by PMF. However, lysosome inhibitor has not recovered the suppression of the melanogenesis by PMF. It has been reported that melanin synthesis is strongly affected by melanosomal pH. I have revealed that PMF acidifies cellular organelles and its majority is the melanosomes. Moreover, ammonium chloride, neutralizing melanosomes, has recovered the suppression of the melanin production and tyrosinase mis-localization by PMF. Therefore, it has been suggested that PMF suppresses the melanin production and tyrosinase localization to the melanosomes by the melanosomal acidification. It has been thought that mis-localized tyrosinase is degraded by the lysosomes.

From above results, I suggest that PMF suppresses the UVB-induced wrinkle formation and hyper-pigmentation in the skin through the suppressions of the PGE_2 production, JNK phospholyration and melanogenesis., respectively. Therefore, I think that PMF is inclusively the useful agent against the wrinkle formation and hyper-pigmentation, which are the features of photoaging.

Abbreviations

AP	adaptor protein
COX	cyclooxygenase
DAMP	3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine
ECM	extracellular matrix
ET	endothelin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Ig	immunoglobulin
JNK	c-Jun N-terminal kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MSH	melanocyte-stimulating hormone
NF-κB	nuclear factor kappa B
NCKX	Na+/Ca2+/K+ exchanger
NHE	Na^+ /H^+ exchanger
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PG	prostaglandin
PMF	polymethoxyflavone
PPAR	peroxisome proliferator-activated receptor
PVDF	polyvinylidenedifluoride membrane
SCF	stem cell factor
SDS	sodium dodecyl sulfate

SLC	solute carrier
Tyrp	tyrosinase-related protein
UV	ultraviolet
VLDL	very low-density lipoprotein

General Introduction

Skin aging is divided into intrinsic aging and photoaging caused by repeated exposure to sunlight. Whereas intrinsic aging is characterized by smooth, pale and finely wrinkled skin, photoaging has the features of coarse, deep wrinkles, dyspigmentation and telangiectasia (1). It is thought that photoaging affects physical appearance stronger than intrinsic aging. Furthermore, photoaging is also involved in skin cancer pathogenesis.

While sunlight has useful effects, such as the promotion of vitamin D synthesis and regulation of biological clock, ultraviolet (UV) also has harmful effects in the skin. UV is classified into UVA (400-315 nm), UVB (315-280 nm) and UVC (under 280 nm). Because only UVA and UVB pass through the ozone layer, human is irradiated by only them. UVB specially has strong effects in the skin and is thought the main cause of photoaging.

It is necessary for understanding photoaging to recognize the skin structure. Skin is broadly divided into the epidermis and dermis. Epidermis is the boundary between the inside and outside of the body, and serves as the defense of the body against foreign substances and inhibition of moisture loss from the inside of the body. Epidermis is constructed of the twenty layered structures of keratinocytes, and classified into the cornified, granular, spinous and basal layer. With the exception of keratinocytes, Langerhans cells, immune cells and melanocytes, synthesizing melanin, exists in the spinous and basal layer, respectively. Dermis is present under epidermis and possesses rich extracellular matrix (ECM). Dermal ECM is composed of TypeI collagen, the major component, elastin and so on. Dermal ECM is provided by fibroblasts and gives the mechanical strength to the skin. Dyspigmentation results from the overproduction of

melanin by melanocytes in the epidermis (Fig. 1). Wrinkle formation is caused by the degeneration and/or degradation of ECM in the dermis (Fig. 1). However, UVB, the main cause of photoaging, cannot directly affect melanocytes and dermal fibroblasts, because UVB goes through only upper epidermis. Therefore, it is thought that photoaging is caused by liquid factors secreted UVB-irradiated keratinocytes.

It has been found out that melanocytes are promoted the melanin production by α-Melanocyte-stimulating hormone (MSH) (2-4), stem cell factor (SCF) /endothelin (ET)-1 (5-7) and prostaglandin (PG) E_2 (8, 9). UVB-irradiated keratinocytes express α-MSH and ET-1 through p53 activation (10-12) and produce PGE_2 through cyclooxygenase (COX)-2 expression (13). They activated melanocyte inducing transcription factor (MITF) through recognized by the receptors expressing on melanocytes (14, 15). MITF promotes the production of the melanin synthesizing enzymes, tyrosinase and so on (16, 17). Melanin is produced from tyrosine by melanin synthesizing enzymes. Particularly, tyrosinase is the rate-limiting enzyme of the melanogenesis. Actually, it has been reported that tyrosinase mutation results in the type1 albino patients, which rarely synthesizes melanin (18, 19). After tyrosinase mRNA transcription, tyrosinase protein is translated in ribosomes and continuously folded in the endoplasmic reticulums. On type1 albino patients, tyrosinase protein is misfolded and eventually degraded in proteasomes (18, 19). As the secreted proteins, folded tyrosinase is sent to the Golgi bodies. Furthermore, through the endosomes, tyorisnase is transferred to the melanosomes, the specific cell organelles of melanocytes. On the other hands, it has been also reported that, by the defaults of the intracellular trafficking, tyrosinase is not translered to the melanosomes and eventually degraded in the lysosomes (20-24). In the melanosomes, tyrosinase and the other melanin

synthesizing enzymes produce melanin. Melanosomes are divided into stage1-4 by its maturation. Tyrosinase is localized into the stage2 melanosomes. By the melanogenesis, Melanosome is matured to Stage3 and 4. Additionally, the matured melanosome is transported to the cell periphery (25, 26). The peripheral melanosomes, containing melanin, is eventually transferred to the nearby keratinocytes through the dendritic process of melanocytes. PGE_2 also stimulates the dendrite elongation of melanocytes (8, 9, 27, 28).

It has been suggested that UV irradiation forms the wrinkle (29). ECM degradation, the main cause of wrinkle formation, is catalyzed by matrix metalloproteinases (MMPs) (29). Over 20 subtypes of MMPs has been currently known and mainly divided into the secretory type and membrane-bound type. Each MMPs have the different substrate specificities. For example, in the skin, type1 Collagen, the major component of dermal ECM, is degraded by MMP-1, 3, 9 (29). On the other hands, type4 and 7 collagen are disrupted by MMP-2, 9 (30). It is thought that MMPs are mainly secreted by lymphocytes invading dermis by UVB-induced inflammation (31). UVB-induced inflammation is promoted by liquid factors secreted by keratinocytes. Actually, because PGE_2 promotes vascular permeability, it could promote lymphocytes invasion (32). It has been also found out that wrinkle is formed by under minimal erythema dose of UV (29). Therefore, it is thought that UVB also promotes MMPs expression of keratinocytes and/or dermal fibroblasts in the skin, independent on lymphocytes.

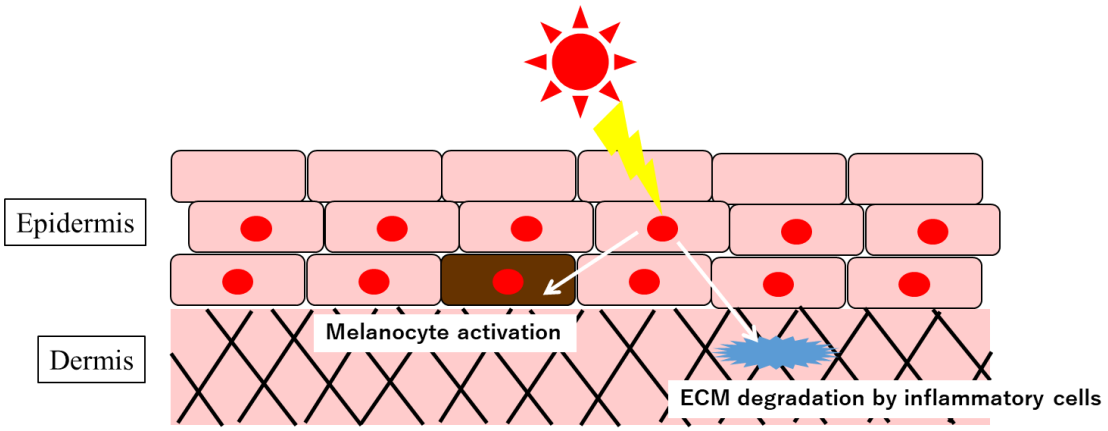
Based on the above photoaging mechanisms, several anti-photoaging agents have been developed. It has been found out that tyrosinase inhibitors, arbutin (33) and kouji acid (34), are useful for the suppression of melanin production. It has been also reported that magnolignan affects the posttranslational modification and stability of tyrosinase

(35), and/or *Chamaemelum Nobile* extract inhibits the melanogenesis activation of ET-1/SCF-1 (36). On the other hand, it has been suggested that all-trans retinoic acid suppresses wrinkle formation through the inhibition of MMPs expression (29). However, it has been reported few studies that single material has spacious effects for anti-photoaging.

Polymethoxyflavone (PMF)s are contained in the citrus peels and is flavonoid that possesses methoxy groups, instead of hydroxyl groups. PMF has been reported to have the anti-inflammatory, anti-atherogenic and antitumor invasion effects (37-40). However, it has been rarely reported whether PMF has the effects against the UVB-induced melanogenesis and/or the degradation of ECM. In these studies, based on above photoaging mechanisms, I have studied whether PMF ameliorates UVB-induced harmful effects against the skin by *in vitro* experiments. Actually, I have researched the effects of PMF against PGE_2, pro-inflammatory and melanocyte activating factor, and MMP production on HaCaTs, human keratinocyte cell line, and the melanin synthesis on HM3KOs, human melanoma cell line, respectively. Furthermore, I have also analyzed the functional mechanisms of PMF's effects.

Figure

Figure 1. The schematic view of photoaging.


Epidermis
Dermis
Melanocyte activation
ECM degradation by inflammatory cells

Chapter 1. Orange peel extract, containing high levels of polymethoxyflavonoid, suppressed UVB-induced COX-2 expression and PGE2 production in HaCaT cells through PPAR-γ activation

1. Introduction

Skin cells maintain intracellular ROS at a low level due to defenses against oxidative stress. However, when the skin is irradiated by UVB excessively, excess ROS generation results in increase of intracellular ROS. ROS causes adverse phenomena such as an inflammatory response, and both formations of photoaged skin and skin cancer (41, 42). UVB-irradiated keratinocytes produce prostaglandin (PG) E_2 due to up-regulation of cyclooxygenase (COX)-2 express (43-45). PGE_2 mediates UVB-induced erythema and edema, and also facilitates the infiltration of neutrophils and lymphocytes into the dermis by enhancement of vascular permeability (32). In the process, lymphocytes invading the dermis, secrete matrix metalloprotease (MMP)s which collapse the dermal matrix structure by degrading collagen and elastin, resulting in wrinkle formation (31). PGE_2 also induces dendritic extension on melanocytes (8, 9, 27, 28), and COX-2 functions in melanogenesis (46). Furthermore, a role of PGE_2 in development of skin cancer has been well recognized (47). Thus, PGE_2 produced by COX-2 involves initiations of various UV-induced adverse phenomena in the skin.

Suppression of PGE_2 production due to UVB-induced up-regulation of COX-2 expression is an important resolution for skin protection against UVB (13). Many materials showing suppression of COX-2 expression and PGE_2 production have been reported. For example, antioxidants such as tocopherol (48) and ascorbic acid (49) derivatives inhibit UVB-induced PGE_2 production, and are used in skin care products. In plants, it has been also reported that extracts of *Polypodium leucotomos* (50) and *Vanda coerulea* stem (51) suppress UVB-induced COX-2 expression and PGE_2 production.

On the other hand, Citrus peel has been used as a traditional medicine for its

beneficial effects on digestion, cough, sputum, and pain. In this study, I evaluated effects of orange peel extract containing over 90% polymethoxyflavonoid (PMF)s against COX-2 expression and PGE_2 production in HaCaT cells. In addition, I also investigated mechanisms of the extract focusing on the peroxisome proliferator-activated receptor (PPAR)-γ.

2. Methods

2-1. Reagents

The PPAR-γ antagonists, GW9662 and T0070907, were purchased from Cayman Chemical (Ann Arbor, MI, USA). The PPAR-α, γ, and δ agonists (WY14643, troglitazone, and GW501516), 1000 and 4500 mg/l glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotic antimycotic solution stabilized (anti-B.M.) and phenylmethanesulfonyl fluoride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS) was purchased from Nissui Pharmaceuticals (Tokyo, Japan). Tangeretin, nobiletin, 3,3',4',5,6,7,8-heptamethoxyflavone and Isogen II were purchased from Wako (Osaka, Japan). One Step SYBR PrimerScript PLUS RT-PCR kit was purchased from Takara Biotechnology (Shiga, Japan). E to Z sample buffer and polyvinylidenedifluoride membrane (PVDF) were purchased from Atto (Tokyo, Japan). Lipofectoamine™ Reagent and Plus™ Reagent were purchased from Life Technologies (Carlsbad, CA, USA). Dual Luciferase Reporter Assay System was purchased from Promega (Fitchburg, WI, USA). First antibodies, COX-2 and β-actin were purchased from Cayman Chemical (Ann Arbor, MI, USA) and BioVision (Milpitas, CA, USA). Goat anti-mouse horseradish peroxidase-conjugated IgG secondary antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

2-2. PMF determination by HPLC

Quantitative analyses of PMF was performed using a high performance liquid chromatography (HPLC) system consisting of a system controller CBM-20A, a solvent delivery unit LC-20AD, an auto-sampler SIL-20ACHT, a column oven CTO-20A, and a

UV-VIS detector SPD-20A (Shimadzu, Kyoto, Japan) equipped with a Column Wakosil II 3C-18HG (2 × 150 mm, Wako, Osaka, Japan). The column oven and the UV detector were set at 45°C and 280 nm, respectively. Orange peel extract was dissolved in methanol (0.5 mg/ml), passed through polytetrafluoroethylene (PTFE) filters (pore size 0.5 μm), and subjected to HPLC. Tangeretin, nobiletin, and 3,3',4',5,6,7,8-heptamethoxyflavone were eluted with 0.1% TFA in water/acetonitrile gradient curve (40%–100%) at a flow rate of 0.5 ml/min and retention times of 6.3, 4.8, and 5.8 min, respectively. Standard curves were prepared using commercial tangeretin, nobiletin, and 3,3',4',5,6,7,8-heptamethoxyflavone.

2-3. Cell culture and UVB irradiation

HaCaT cells are immortalized keratinocytes from a human-derived, cell line (52). HaCaT cells were cultured to 95% confluence in 4500 mg/l glucose DMEM supplemented with 10% FBS and 1% anti-B.M. at 37°C in a 5% CO_2 humidified incubator. HaCaT cells were then serum-starved for 24 hours. Before UVB irradiation, HaCaT cells were washed with phosphate-buffered saline (PBS, Nissui Pharmaceuticals, Tokyo, Japan) and exposed to 30 mJ/cm^2 of UVB (FL-20S・E-30/DMR, TOSHIBA Medical Systems, Tochigi, Japan) in PBS. CV1 cells (African green monkey kidney cell line) were cultured in 1000 mg/l glucose DMEM supplemented with 10% FBS and 1% Anti-B.M. at 37°C in a 5% CO_2 humidified incubator.

2-4. RNA extraction and real-time PCR

HaCaT cells were harvested in Isogen II and mRNA was extracted as directed by Isogen II protocol. Real time PCR amplifications of mRNA were performed with

StepOnePlus (Life Technologies Carlsbad, CA, USA), One Step SYBR PrimerScript PLUS RT-PCR kit, and the following primer pairs (Integrated DNA Technologies, San Diego, CA USA): COX-2 forward 5'- GCTCAGCCATACAGCAAATC-3', COX-2 reverse 5'- TGTGTTTGGAGTGGGTTTCA-3', β-actin forward 5'- AGAAGGATTCCTATGTGGGCG-3', β-actin reverse 5'- CATGTCGTCCCAGTTGGTGAC-3'.

2-5. Protein extraction and western blotting

HaCaT cells were washed with ice-cold PBS and harvested in PBS containing 100 mM phenylmethanesulfonyl fluoride. The lysate was mixed with E to Z sample buffer, loaded onto 5% to 20% SDS-PAGE gels and transferred onto PVDF membranes. Primary antibody reactions were performed with COX-2 (1:1000 dilution, Cayman Chemical) and β-actin (1:10000 dilution). Continuously, bound antibodies were detected by goat anti-mouse horseradish peroxidase-conjugated IgG secondary antibody (1:10000 dilution). Signals were detected with Luminata™ Forte Western HRP Substrate (Millipore, Billerica, MA, USA) and visualized with ChemiDoc™ XRS+ (Bio-Rad, Hercules, CA, USA).

2-6. Measurement of PGE_2 production

PGE_2 levels in the supernatants from cultured cells were determined by ELISA using a prostaglandin E2 Express EIA kit obtained from Cayman Chemical (Ann Arbor, MI, USA).

2-7. Luciferase assay

CV1 cells were simultaneously transfected vector containing PPAR-α/γ/δ gene sequences and reporter vector by use of Lipofectoamine™ Reagent and Plus™ Reagent. Transfected cells were incubated for 44 hours in serum-free DMEM containing orange peel extract or PPAR agonists. Luciferase activities were detected by a Dual Luciferase Reporter Assay System. Luminescence was measured by POWERSCAN MX (DS Pharma Biomedical, Osaka, Japan).

2-8. Statistical analysis

All experiments were performed at least twice, and I obtained similar results, respectively. Data were reported as mean ± SD followed by statistical significance (Student's *t*-test for unpaired experiments). *P*-values < 0.05 were regarded as significant.

3. Results

3-1. PMF formulation of orange peel extracts

Orange peel contains PMFs as major ingredients. PMFs have effects on anti-inflammation (37, 39) and insulin sensitivity improvement (38). The total PMF content of the orange peel extract was more than 90% (Fig. 2). I identified tangeretin, nobiletin, and 3,3',4',5,6,7,8-heptamethoxyflavone as PMFs and their contents were 7.9%, 37.3%, and 46.9% in the extract, respectively.

3-2. The suppression of UVB-induced COX-2 expression by orange peel extract

It has been reported that UVB-irradiated HaCaT cells express COX-2 mRNA and protein (45). In UVB-irradiated HaCaT cells, COX-2 mRNA expression was about 20 times higher than that in non-irradiated cells (Fig. 3a). UVB-induced COX-2 mRNA expression was suppressed dependenting on the orange peel extract concentration. The suppression was significant at more than 10 μg/ml concentration (Fig. 3a). Similarly, UVB-induced COX-2 protein was also inhibited by orange peel extract (Fig. 3b).

3-3. The suppression of UVB-induced PGE_2 production by orange peel extract

COX-2 is a rate-limiting enzyme in the arachidonic acid cascade. It has been reported that COX-2 expression enhances PGE_2 production in UVB-irradiated HaCaT cells (45). In my study, UVB-induced PGE_2 production was 3 times higher in irradiated cells and was significantly inhibited by orange peel extract (Fig. 3c).

3-4. The PPAR-γ activation by orange peel extract

PPAR-γ enhances adipose cell differentiation and glucose uptake, and is a target for

diabetic treatment. It has been reported that naringenin (a flavonoid) is a PPAR-γ agonist (53). I researched PPARs activation of orange peel extract containing high levels of PMF by luciferase assays (Fig. 4a-c). This extract did not significantly activate PPAR-α and δ (Fig. 4a,c). However, it was observed that orange peel extract significantly increased (8-fold) the luciferase activity compared to control cells as measured by a PPAR-γ reporter assay. The results were similar to the positive control, troglitazone (Fig. 4b). Therefore, it was shown that orange peel extract selectively activates PPAR-γ.

3-5. The inhibitory effects of PPAR-γ antagonists against COX-2 suppression by orange peel extract

Recently, it has been reported that PPAR-γ has anti-inflammatory effects (54, 55). I used PPAR-γ antagonists to investigate the relationship between the UVB-induced COX-2 suppression and PPAR-γ activation by orange peel extract (Fig. 5). The PPAR-γ antagonists GW 9662 and T0070907 significantly inhibited COX-2 suppression by orange peel extract as measured by mRNA levels (Fig. 5a). In addition, it was also observed that the decrease in COX-2 protein induced by orange peel extract was inhibited by PPAR-γ antagonists (Fig. 5b). Therefore, it was suggested that orange peel extract suppressed UVB-induced COX-2 expression through PPAR-γ.

4. Discussion

Nobiletin has been reported to have anti-inflammatory effects and suppress UVB-induced COX-2 expression and PGE_2 production (37, 39, 56, 57). Flavonoids have anti-oxidant activities and some have been shown to reduce the adverse effects on cells induced by UVB irradiation. It has been reported that UVB stimulates intracellular ROS and increases COX-2 expression by p38 MAPK activation (45). Hence, it is possible that flavonoids suppress UVB-induced COX-2 expression by ROS elimination. Alternatively, for instance, kaempferol (58) and luteolin (59) have been shown to suppress UVB-induced COX-2 expression by competitive inhibition of the ATP binding domains of Src kinase and protein kinase C. In particular, a computational modeling study revealed that the –OH and C=O of kaempferol could bind amino acid residues of Src kinase ATP binding domain through hydrogen bonding (58). However, PMFs fail to exert effects as proton donors due to displacement of the phenolic-OH group with –OMe group. In addition, it can be assumed that PMFs would be difficult to interact with amino acid residues through hydrogen bonding as kaempferol and luteolin. Although they have limited anti-oxidant activity and interaction with amino acid residues, I found that they both suppressed COX-2 expression and PGE_2 production after UVB irradiation (Fig.3). Therefore it is not likely that orange peel extract suppresses UVB-induced COX-2 expression by ROS elimination and the inhibition of Src kinase activity.

PPAR-γ agonists have anti-inflammatory effects (54, 55). Several flavonoids have been reported to act as PPAR-γ agonists. Naringenin has been reported to be a PPAR-γ agonist (54). PPAR-γ enhances adipose cell differentiation and glucose uptake and is a target for diabetic treatment. Nobiletin (PMF) attenuates very low-density lipoprotein

(VLDL) overproduction, dyslipidemia, and atherosclerosis in mice with diet-induced insulin resistance (38). In addition, 5, 7-dimethoxyflavone, extracted from *K. parviflora*, suppresses UVB-induced MMP expression on dermal fibroblasts by acting as a PPAR-α/γ agonist (60). I investigated the possibility that orange peel extract containing high levels of PMFs selectively activates PPAR-γ. The results of the PPAR-γ reporter assay indicated that the orange peel extract functions as PPAR-γ activator (Fig. 4).

In order to identify the role of the orange peel extract as a PPAR-γ activator, I conducted an experiment using PPAR-γ antagonists. In the experiment, I found that PPAR-γ antagonists abolished the suppression of UVB-induced COX-2 expression induced by orange peel extract (Fig. 5) suggesting that orange peel extract suppresses UVB-induced COX-2 expression through PPAR-γ activation.

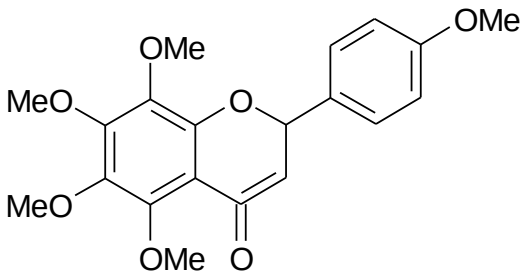
It has been reported that UVB-irradiation induces NF-κB activation (29, 61, 62) and NF-κB increases COX-2 expression (63, 64). PPAR-γ activation has been addressed to suppress NF-κB activity (65-68). PPAR-γ suppresses p65 expression (67), Iκ kinase activity (66), and increases IκBα expression (68), and eventually inhibits nuclear translocation of NF-κB (65, 68). In addition, PPAR-γ agonist interferes with the assembly of NF-κB (69). NF-κB regulates the transcription of many inflammatory factors containing COX-2. Actually, PPAR-γ agonists, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and rosiglitazone, have suppression effects of COX-2 expression and NF-κB activation, inhibited by PPAR-γ antagonist, GW9662 (70). Hence, orange peel extract might also suppress UVB-induced COX-2 expression and PGE_2 production by inhibiting NF-κB activity through PPAR-γ activation.

In conclusion, this study suggests that orange peel extract containing high levels of

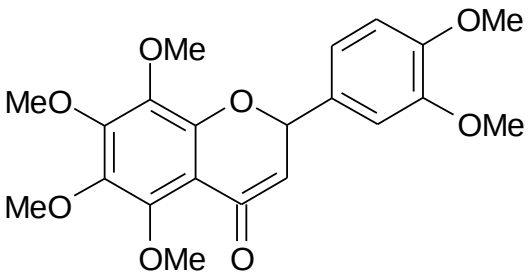
PMF, can suppress UVB-induced COX-2 expression and PGE_2 production through PPAR-γ activation. Orange peel extract can expect to prevent and alleviate inflammatory response, skin pigmentation, wrinkles, and skin cancer caused by UVB exposure.

5. Figures

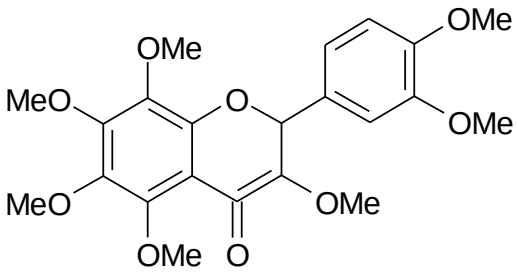
Figure 2. PMF formulation of orange peel extract as measured by HPLC.



Tangeretin : 7.9%



Nobiletin : 37.3%

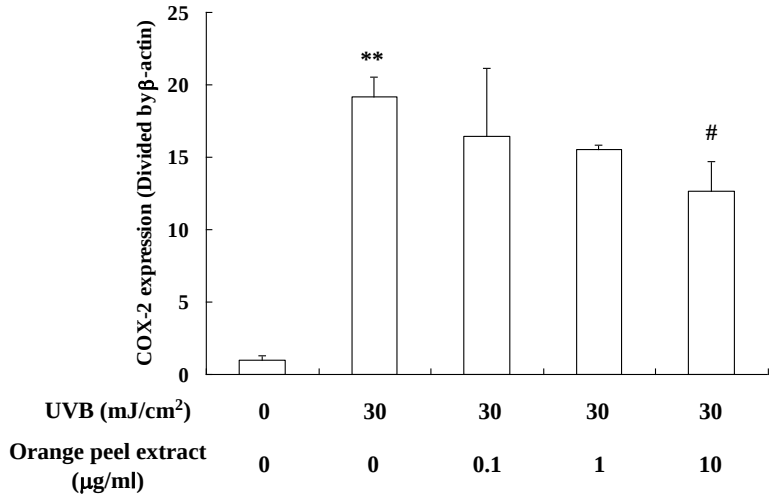


3,3',4',5,6,7,8-heptamethoxyflavone : 46.9%

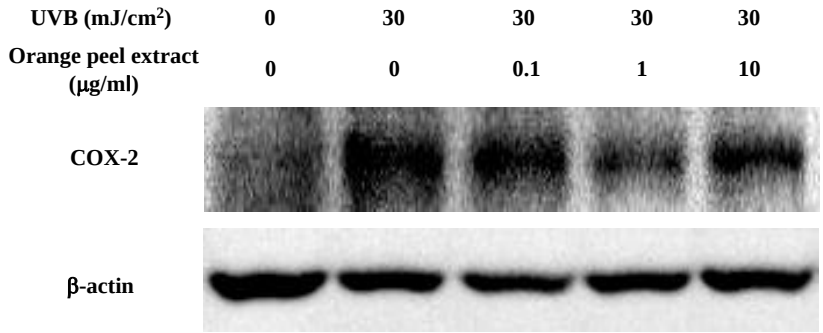
Total : 92.1%

Figure 3. Orange peel extract suppresses UVB-induced COX-2 expression and PGE_2 production in HaCaT cells. (a) HaCaT cells were irradiated by UVB and total RNA was harvested 4 hours later. (b) Total protein was harvested 6 hours after irradiation. (c) The supernatants were harvested for measurement of PGE_2 production 24 hours after irradiation. Data represent the mean ± SD of n = 3. $^{**}P < 0.01$ vs. non-irradiated cells; $^{\#\#}P < 0.01$ vs. irradiated cells.

(a)


COX-2 expression (Divided byβ-actin)
25
20
15
10
5
0
**
#
UVB (mJ/cm2) 0 30 30 30 30
Orange peel extract (μg/ml) 0 0 0.1 1 10

(b)


UVB (mJ/cm2) 0 30 30 30 30
Orange peel extract (μg/ml) 0 0 0.1 1 10
COX-2
β-actin

(c)

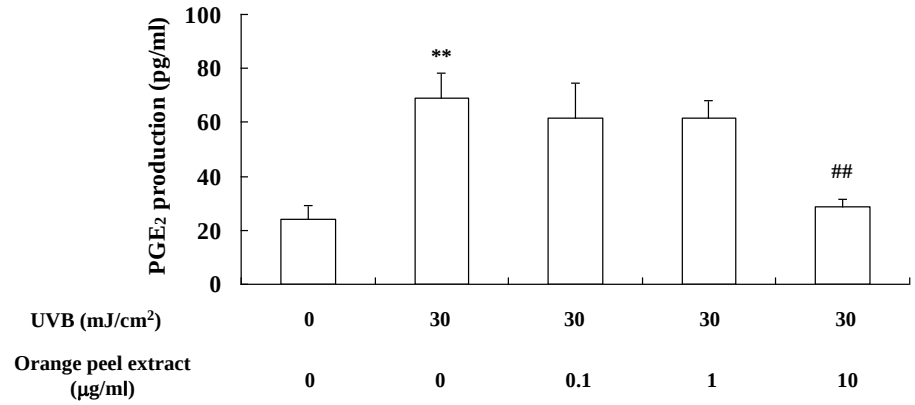

PGE2 production (pg/ml)
100
80
60
40
20
0
**
##
UVB (mJ/cm2) 0 30 30 30 30
Orange peel extract (μg/ml) 0 0 0.1 1 10

Figure 4. PPAR-γ is activated by orange peel extract. PPAR-α (a), γ (b), and δ (c) activation by orange peel extract were analyzed by reporter gene assays. WY14643, troglitazone, or GW501516 were the PPAR-α, γ, or δ agonists, respectively. Data represent the mean ± SD of n = 3. $^{**}P < 0.01$ vs. control.

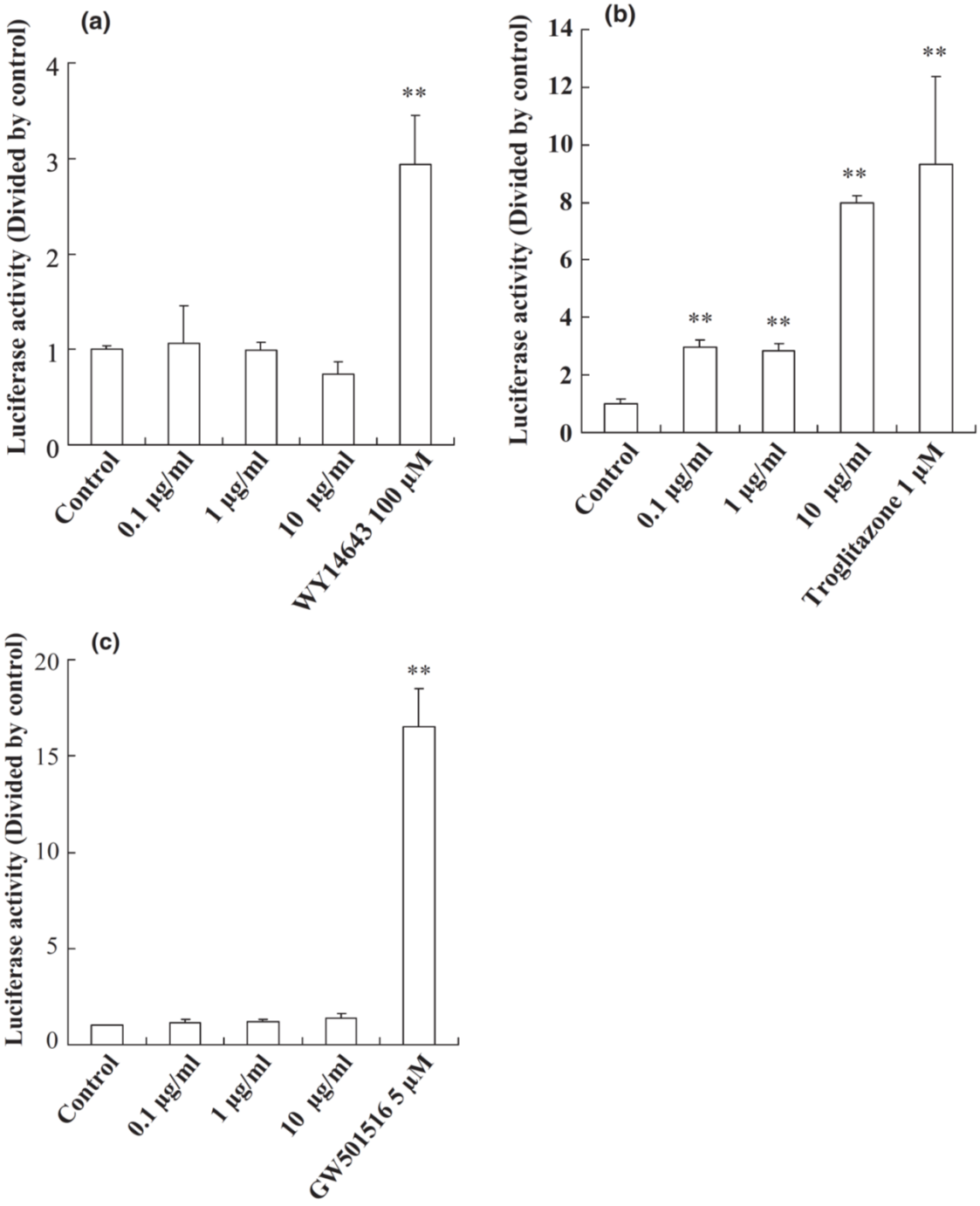
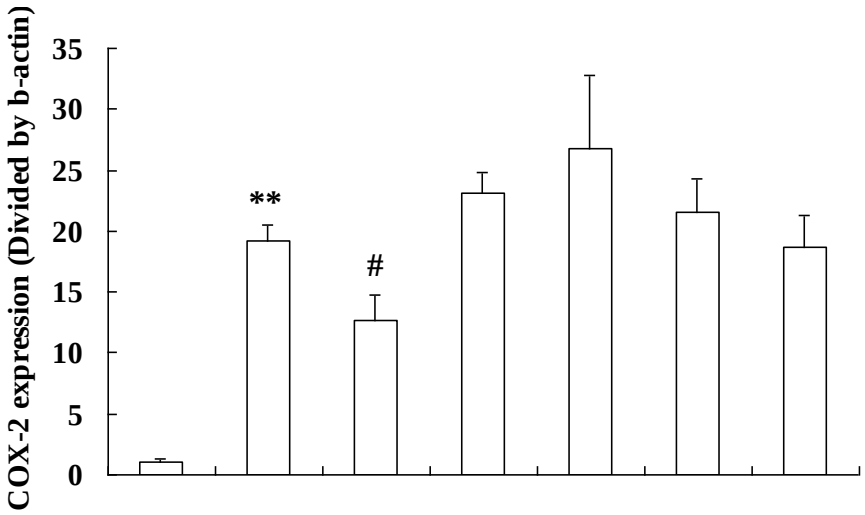

(a)
Luciferase activity (Divided by control)
0
1
2
3
4
**
Control
0.1 μg/ml
1 μg/ml
10 μg/ml
WY14643 100 μM
(b)
Luciferase activity (Divided by control)
0
2
4
6
8
10
12
14
**
**
**
**
Control
0.1 μg/ml
1 μg/ml
10 μg/ml
Troglitazone 1 μM
(c)
Luciferase activity (Divided by control)
0
5
10
15
20
**
Control
0.1 μg/ml
1 μg/ml
10 μg/ml
GW501516 5 μM

Figure 5. The suppression of UVB induced COX-2 expression by orange peel extract is inhibited by PPAR-γ antagonists (GW = GW9662 or T007 = T0070907). Total RNA (a) or protein (b) was harvested from HaCaT cells 4 and 6 hours after UVB irradiation, respectively. Data represent the mean ± SD of n = 3. $^{**}P < 0.01$ vs. non-irradiated cells; $^{\#}P < 0.05$ vs. irradiated cells.

(a)



UVB (mJ/cm^2)	**0**	**30**	**30**	**30**	**30**	**30**	**30**
Orange peel extract (μg/ml)	**0**	**0**	**10**	**0**	**10**	**0**	**10**
Antagonist (10 μM)	**0**	**0**	**0**	**GW**	**GW**	**T007**	**T007**

(b)

UVB (mJ/cm^2)	**0**	**30**	**30**	**30**	**30**
Orange peel extract (μg/ml)	**0**	**0**	**10**	**10**	**10**
Antagonist (10 μM)	**0**	**0**	**0**	**GW**	**T007**

COX-2

β-actin

Chapter 2. A polymethoxyflavone mixture, extracted from orange peels, suppresses the UVB-induced expression of MMP-1

1. Introduction

Alterations of collagen fibers in the dermis are thought to cause the wrinkles observed in intrinsic aging and in photoaging due to an imbalance between collagen synthesis and degradation. Collagen is degraded by matrix metalloproteinases (MMPs). Because UV induces the expression of MMPs in the skin (71), it is considered that the UV-induced expression of MMPs is related to the deep wrinkles seen in photoaged skin.

UV radiation has been reported to induce the expression of MMP-1, MMP-3 and MMP-9 in the skin (29). Following UV irradiation, it is thought that MMP-1 initially disrupts type Ⅰ and III collagens after which MMP3 and/or MMP-9 further degrade the disrupted collagens (29).

The UV-induced expression of MMPs has been observed in the epidermis and dermis, and large amounts of MMPs are produced by the epidermis (72). The cells producing MMPs in the epidermis are mainly keratinocytes (71, 73).

Many agents that can suppress the UV-induced expression of MMPs have been identified. Various flavonoids have been reported to suppress the expression of MMPs. For example, myricetin suppresses MMP-9 and MMP-13 expression induced by UVB (74, 75), and luteolin inhibits UVB-induced MMP-1 expression (76). I focused on polymethoxyflavone (PMF), which is a flavone where the hydroxyl group is replaced with a methoxy group. PMF has been reported to have anti-inflammatory (37), anti-atherogenic (38) and anti-tumor invasion (39, 40) effects. Although it has also been reported that PMF suppresses 12-O-tetradecanoylphorbol 13-acetate (TPA) induced MMP expression (39, 40), to my knowledge, it has not been shown whether PMF prevents UVB from inducing MMP-1 expression.

In the chapter 1, a PMF mixture extracted from orange peels, was shown to suppress

UVB-induced COX-2 expression and PGE_2 production. In the present study, I determined whether the PMF mixture inhibits the expression of MMP-1 by UVB-irradiated immortalized HaCaT human keratinocytes.

2. Methods

2-1. Reagents

I have used the orange peel extract of the chapter 1 (Fig. 2) as the PMF mixture. SP600125, a JNK inhibitor, was purchased from Cayman Chemical (Ann Arbor, MI, USA). Dulbecco's modified Eagle's medium (DMEM) with 4500 mg/l glucose, fetal bovine serum (FBS), antibiotic antimycotic solution stabilized (anti-B.M.), phenylmethanesulfonyl fluoride and a protease inhibitor cocktail were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS) was purchased from Nissui Pharmaceuticals (Tokyo, Japan). BAY11-7085, an NF-κB inhibitor and Isogen II were purchased from Wako (Osaka, Japan). The One Step SYBR PrimerScript PLUS RT-PCR kit was purchased from Takara Biotechnology (Shiga, Japan). E to Z sample buffer and polyvinylidenedifluoride membranes (PVDF) were purchased from Atto (Tokyo, Japan). Primary antibodies to MMP-1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphorylated JNK were purchased from R&D Systems (Minneapolis, MN, USA), GENETEX (Irvine, CA, USA) and Assay Biotechnology Co (Sunnyvale, CA, USA), respectively. Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

2-2. Cell culture and UVB irradiation

HaCaT cells are immortalized human keratinocytes (52). HaCaT cells were cultured to 95% confluence in DMEM containing 4500 mg/l glucose, with 10% FBS and 1% anti-B.M. at 37°C in a 5% CO_2 humidified incubator. HaCaT cells were then serum-starved for 24 hours. Before UVB irradiation, HaCaT cells were washed with PBS and exposed to 20 mJ/cm^2 UVB (FL-20S·E-30/DMR, TOSHIBA Medical Systems,

Tochigi, Japan) in PBS.

2-3. RNA extraction and real-time PCR

HaCaT cells were harvested in Isogen II and mRNAs were extracted according to the Isogen II protocol. Real-time PCR amplifications of mRNAs were performed with a StepOnePlus (Life Technologies Carlsbad, CA, USA), One Step SYBR PrimerScript PLUS RT-PCR kit and the following primer pairs (Integrated DNA Technologies, San Diego, CA USA): MMP-1 forward 5'-ATTCTACTGATATCGGGGCTTTGA-3', MMP-1 reverse 5'-ATGTCCTTGGGGTATCCGTGTAG-3', β-actin forward 5'-AGAAGGATTCCTATGTGGGCG-3', β-actin reverse 5'-CATGTCGTCCCAGTTGGTGAC-3'.

2-4. Protein extraction and western blotting

HaCaT cells were washed twice with ice-cold PBS and then were harvested in RIPA buffer (radio-immunoprecipitation buffer, 50 mM Tris-HCl, pH8.0, 150 mM sodium chloride, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1.0% NP-40) containing 100 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail. The lysates and culture supernatants were mixed with E to Z sample buffer, loaded on 5% to 20% SDS-PAGE gels and transferred to PVDF membranes. Primary antibody reactions were performed with antibodies to MMP (1:1000 dilution), phosphorylated JNK (1:1000 dilution) or GAPDH (1:10000 dilution). Bound antibodies were detected with a secondary antibody (1:10000 dilution). Signals were detected with Luminata™ Forte Western HRP Substrate (Millipore, Billerica, MA, USA) or Western Lightening Ultra (Perkin Elmer, Wellesley, MA, USA) and were visualized with ChemiDoc™ XRS+

(Bio-Rad, Hercules, CA, USA).

2-5. Statistical analysis

All experiments were performed at least twice with similar results. Data were reported as mean ± SD followed by statistical significance (Tukey-Kramer test). *P*-values < 0.05 are regarded as significant.

3. Results

3-1. The PMF mixture suppresses the UVB-induced expression of MMP-1

I first examined whether the PMF mixture suppresses the UVB-induced expression of MMP-1. At the mRNA level, UVB irradiation increased MMP-1 expression more than 3 times after 48 hours, but that increase was suppressed by the PMF mixture (Fig. 6). Furthermore, at the protein level, the PMF mixture also decreased the amount of MMP-1 protein in UVB-irradiated cells and culture supernatants (Fig. 7a, b). Therefore, these results suggest that the PMF mixture suppresses MMP-1 transcription and decreases MMP-1 protein levels in the UVB-irradiated cells and culture supernatants.

3-2. The PMF mixture suppresses MMP-1 expression through the inhibition of JNK activity

It has been reported that UV activates NF-κB (77, 78) and that NF-κB promotes MMP-1 expression (79, 80). Therefore, the effect on MMP-1 suppression was compared between the PMF mixture and BAY11-7085, an NF-κB inhibitor. In contrast to the PMF mixture, BAY11-7085 did not inhibit the UVB-induced expression of MMP-1 (Fig. 8a). Furthermore, BAY11-7085 did not affect the MMP-1 suppression elicited by the PMF mixture. These results suggest that NF-κB is not directly related to the MMP-1 suppression elicited by the PMF mixture.

On the other hand, JNK was also suggested to be related to the UVB-induced expression of MMP-1 (81). Following cellular stresses containing UV irradiation, JNK is phosphorylated and activates c-jun, a component of the adaptor protein-1 (AP-1) heterodimer (82). AP-1 activation was also suggested to be related to the UVB-induced expression of MMP-1 (83). Therefore, the effect on MMP-1 suppression was compared

between the PMF mixture and SP600125, a JNK inhibitor. In contrast to BAY11-7085, SP600125 suppressed MMP-1 expression to a similar extent as the PMF mixture (Fig. 8b). Furthermore, SP600125 did not have a synergistic effect with the PMF mixture (Fig. 8b). From those results, JNK seems to be related to the MMP-1 suppression elicited by the PMF mixture.

3-3. The PMF mixture inhibits JNK phosphorylation induced by UVB irradiation

It has been suggested that UVB irradiation promotes JNK phosphorylation and activation (59). Therefore, I determined whether the PMF mixture affects the JNK phosphorylation induced by UVB irradiation. Similar to the previous study, UVB irradiation promoted JNK phosphorylation, but the PMF mixture inhibited that phosphorylation (Fig. 9). Therefore, the PMF mixture inhibits JNK activation through the suppression of JNK phosphorylation.

4. Discussion

I reported that the PMF mixture suppressed the UVB-induced expression of MMP-1 at the mRNA and protein levels. Furthermore, the PMF mixture had a similar effect on c-jun N-terminal kinase (JNK) against MMP-1 expression and the combination of the PMF mixture and a JNK inhibitor was comparable after only a single application. Additionally, the PMF mixture prevented JNK from being phosphorylated. Therefore, the PMF mixture is thought to suppress the UVB-induced expression of MMP-1 through the inhibition of JNK phosphorylation.

Nobiletin, a component of PMF, had been reported to suppress the TPA-induced expression of MMP-1 and MMP-9 (38, 40). Nobiletin suppresses the TPA-induced expression of MMP-1 and MMP-9 in human fibrosarcoma HT-1080 cells by inhibiting mitogen-activated protein/extracellular signal-regulated kinase (MEK)1/2 phosphorylation and activity (40, 84) and by increasing JNK phosphorylation via PKCβ Ⅱ/ε (40). MEK1/2 phosphorylates extracellular signal-regulated kinase (ERK)1/2 (85), but I did not observe that the PMF mixture inhibits UVB-induced ERK phosphorylation (data not shown). I also demonstrated that the PMF mixture inhibits UVB-induced JNK phosphorylation (Fig. 9). Why did the PMF mixture have different effects from the previous studies? In my study, I experimented by using HaCaT cells, but, in the previous studies, the authors used human fibrosarcoma HT-1080 cells (40, 84). Furthernore, although, in my study, I inuduced MMP-1 expression by UVB irradiation, in the previous studies, MMP expression was induced by TPA treatment (39, 40). The difference between both studies might depend on the different of cell strains and/or stimulations for MMP expression. Actually, in the previous study, whereas nobiletin increased TPA-induced JNK phosphorylation, it did not affect JNK phosphorylation in

cells without TPA treatment (40). In my study, the PMF mixture gave suppression of JNK phosphorylation induced by UVB. Therefore, this suggests that the increase of JNK phosphorylation by nobiletin is the specific response in TPA treatment, but not UVB.

To my knowledge, PMF has not been previously reported to suppress the UVB-induced expression of MMP-1. However, flavonoid has been shown to inhibit the UVB-induced expression of MMP-1. For example, luteolin prevents UVB irradiation from inducing MMP-1 expression (76), and it also suppresses UVB-induced JNK phosphorylation (59). It has been suggested that luteolin binds the ATP-binding sites of PKCε and c-Src, upstream kinases of JNK phosphorylation (59). Therefore, PMF might also bind them. However, it was suggested that the hydroxyl group of flavonoids plays an important role in the kinase inhibition. For example, the hydroxyl group of the flavonoid B ring plays an important role in the inhibition of phosphatidylinositol 3-kinase (86). The hydroxyl group of kaempferol could also have an important function for binding to the ATP binding site of c-Src (58). Therefore, PMF not only has a higher cell permeability than flavonoid (37), but PMF might also suppress JNK phosphorylation through different target(s) from flavonoid.

In the chapter 1, I had reported that the PMF mixture suppresses UVB-induced COX-2 expression through PPAR-γ. On the other hand, a PPAR-γ antagonist could not prevent the PMF mixture from inhibiting the UVB-induced expression of MMP-1 (data not shown). Therefore, in contrast to COX-2 expression, the suppression of MMP-1 expression by the PMF mixture could be independent of PPAR-γ.

My results suggest that the PMF mixture suppresses UVB-induced MMP-1 expression through the inhibition of JNK phosphorylation. Therefore, a PMF mixture could be

useful as an anti-photoaging agent. It is interesting that target(s) of the PMF mixture is (are) now being identified. New functions may be uncovered by the determination of other PMF mixture targets and if the PMF mixture has different target(s) from flavonoids, it may produce synergistic effects with flavonoids.

5. Figures

Figure 6. Effect of the PMF mixture on the UVB-induced expression of MMP-1 mRNA. HaCaT cells were serum-starved for 24 hours before the UV irradiation and then 10 μg/ml PMF mixture was added. HaCaT cells were then irradiated with UVB 20 mJ/cm^2 and 10 μg/ml PMF mixture. mRNAs were harvested after 6, 24 and 48 hours and were detected by real-time PCR. MMP-1 mRNA expression was normalized by β-actin mRNA expression and further by non-irradiated expression at each harvest time. Data represent mean ± SD of n = 3. **P < 0.01 versus non-irradiated cells; ##P < 0.01 versus irradiated cells.

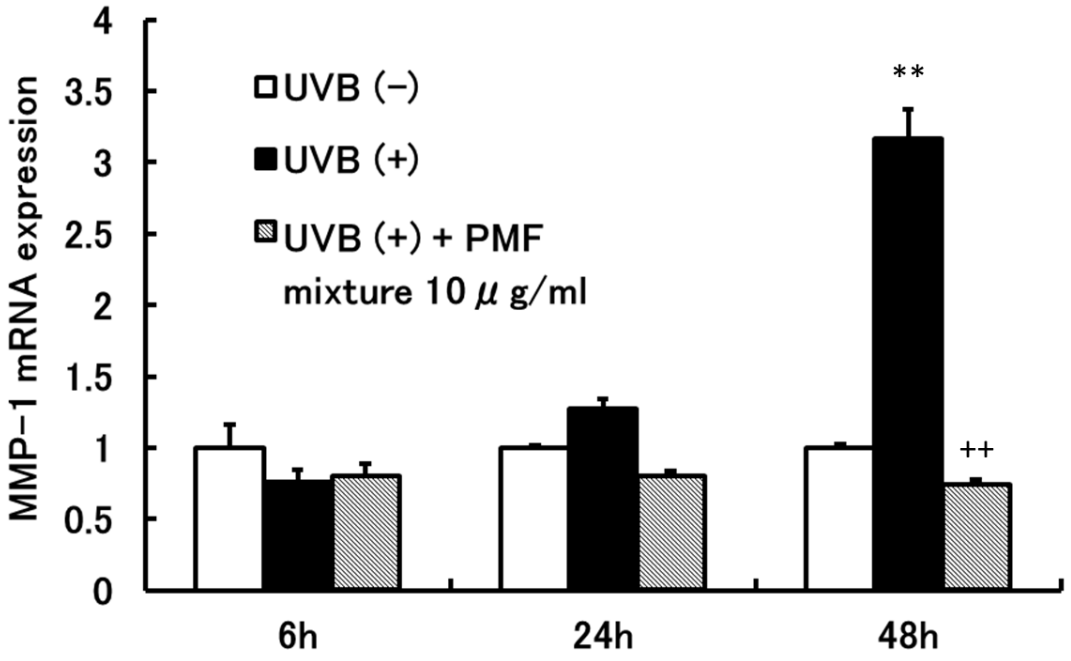
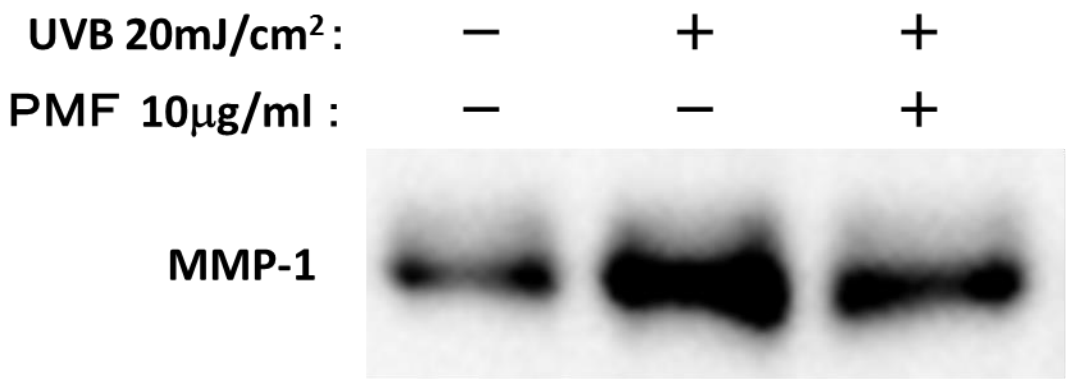

MMP-1 mRNA expression
4
3.5
3
2.5
2
1.5
1
0.5
0
UVB (−)
UVB (+)
UVB (+) + PMF
mixture 10 μg/ml
**
++
6h
24h
48h

Figure 7. Effect of the PMF mixture on UVB-induced MMP-1 protein expression. HaCaT cells were treated as described for Fig. 6. Culture supernatants (a) and cell lysates (b) were harvested 48 hours after UVB irradiation.

(a)


UVB 20mJ/cm2 : − + +
PMF 10μg/ml : − − +
MMP-1

(b)

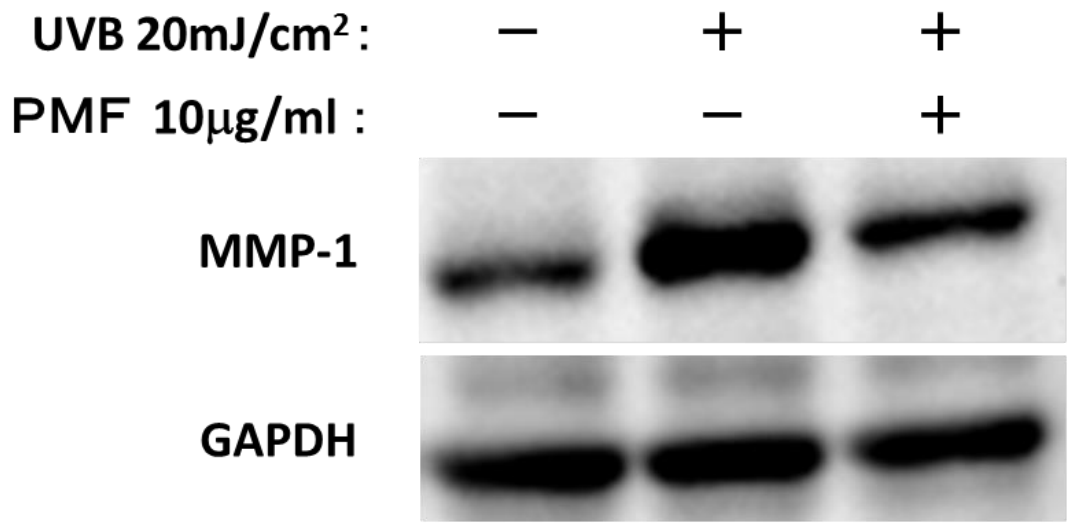
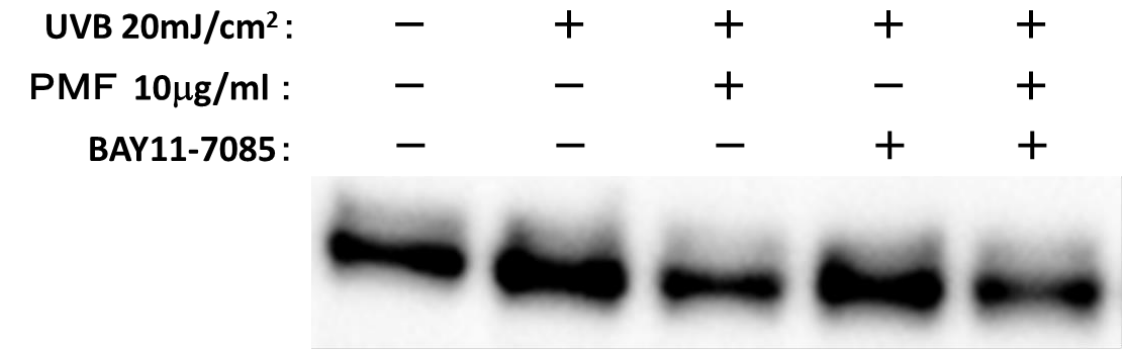

UVB 20mJ/cm2 : − + +
PMF 10μg/ml : − − +
MMP-1
GAPDH

Figure 8. Effects of inhibitors and the PMF mixture on UVB-induced MMP-1 protein expression. HaCaT cells were treated as described for Fig. 6. The NF-κB inhibitor, BAY11-7085 (a), or the JNK inhibitor, SP600125 (b), was simultaneously added with the PMF mixture. Culture supernatants were harvested 48 hours after UVB irradiation.

(a)


UVB 20mJ/cm2 : − + + + +
PMF 10μg/ml : − − + − +
BAY11-7085 : − − − + +

(b)

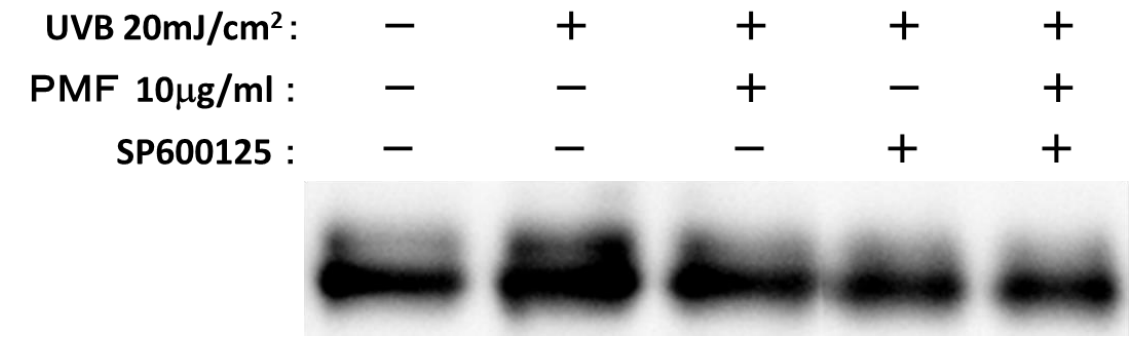
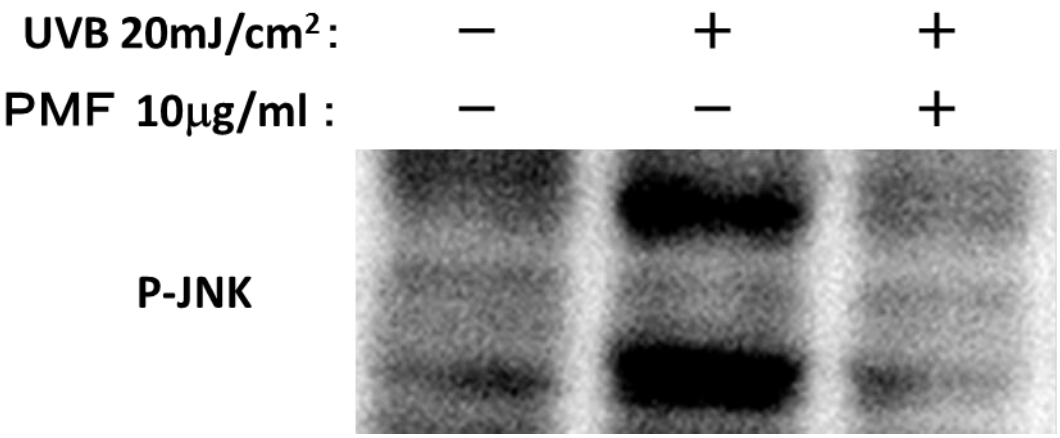

UVB 20mJ/cm2 : − + + + +
PMF 10μg/ml : − − + − +
SP600125 : − − − + +

Figure 9. Effect of the PMF mixture on JNK phosphorylation induced by UVB irradiation. HaCaT cells were treated as described for Fig. 6. Cell lysates were harvested after 6 hours of UVB irradiation.


UVB 20mJ/cm2 : − + +
PMF 10μg/ml : − − +
P-JNK

Chapter 3. A polymethoxyflavone mixture extracted from orange peels, mainly containing nobiletin, 3,3’,4’,5,6,7,8-heptamethoxyflavone and tangeretin, suppresses melanogenesis through the acidification of cell organelles, including melanosomes

1. Introduction

The color of the skin, eyes and hair is determined by melanin produced in melanocytes (87 – 90). Whereas melanin protects the skin from ultraviolet (UV)-induced damage, its over-production causes various types of hyperpigmentation, such as melasma, post-inflammatory melanoderma and solar lentigines. Therefore, the development of whitening agents that can suppress melanin production is desirable.

Melanin is produced in melanosomes, which are melanocyte-specific organelles, by several melanogenic enzymes such as tyrosinase, tyrosinase-related protein (Tyrp)1 and Tyrp2. Melanosomal pH significantly affects melanogenesis as follows: Melanosomal pH in Caucasian melanocytes is lower than in Black melanocytes (91). The difference in the potential for melanin production between both types of skin could be based on differences of melanosomal pH, since the neutralization of melanosomal pH in Caucasian melanocytes increases melanogenesis (92) and a Na^+ /H^+ exchanger (NHE) inhibitor that suppresses proton excretion from melanosomes, decreases tyrosinase activity (93). In B16 mouse melanoma cells, α-melanocyte-stimulating hormone (MSH) and forskolin, which stimulate melanogenesis, neutralize melanosomal pH (94). However, no whitening agents that acidify melanosomes have been reported.

Polymethoxyflavone (PMF) is a flavone where the hydroxyl group(s) is replaced with a methoxy group(s). PMF has been reported to reduce tumor invasion (39, 40) and to have anti-inflammatory (37, 56) and anti-arteriosclerosis (38) effects. In the Chapter 1, I have reported that a PMF mixture extracted from orange peels suppresses UVB-induced prostaglandin E_2 production. In this chapter, I have examined whether the PMF mixture, which is composed of tangeretin, nobiletin and 3,3',4',5,6,7,8-heptamethoxyflavone, suppresses melanogenesis in HM3KO human melanoma cells. Although the PMF

mixture suppresses melanogenesis, it does not inhibit tyrosinase mRNA expression, but does decrease tyrosinase protein levels in HM3KO cells. Lysosome protease inhibitors, as distinct from proteasome inhibitors, prevented the PMF mixture from degrading tyrosinase. Therefore, the PMF mixture promotes tyrosinase degradation in lysosomes. Furthermore, it was found that the PMF mixture and its components acidify cell organelle(s) including melanosomes, and inhibits the localization of tyrosinase into melanosomes. Further, the suppression of melanogenesis and the mislocalization of tyrosinase to melanosomes by the PMF mixture were restored in the presence of ammonium chloride, which is a neutralizing agent. In light of these results, I conclude that the PMF mixture suppresses melanogenesis by disrupting the localization of tyrosinase from melanosomes to lysosomes causing it to be degraded.

2. Methods

2.1. Reagents

I have used the orange peel extract of the chapter 1 (Fig. 2) as the PMF mixture. Fetal bovine serum (FBS) and antibiotic antimycotic solution stabilized (anti-B.M.) were obtained from Biological Industries (Kibbutz Beit-Haemek, Israel) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Dulbecco's modified Eagle's medium (DMEM) with 1000 mg/l glucose and phosphate-buffered saline (PBS) were obtained from Nissui Pharmaceuticals (Tokyo, Japan). Tangeretin, nobiletin, 3,3',4',5,6,7,8-heptamethoxyflavone (heptamethoxyflavone), ammonium chloride and 4% paraformaldehyde were obtained from Wako (Osaka, Japan). The proteasome inhibitor, MG132, and lysosome protease inhibitors, leupeptin and pepstatinA, were purchased from Cayman Chemical (Ann Arbor, MI, USA) and the Peptide Institute, Inc. (Osaka, Japan), respectively. The BCA assay kit was obtained from Takara Bio Inc. (Shiga, Japan). Alamar blue and Lysotracker® red DND-99 were purchased from Life Technologies (Carlsbad, CA, USA). Primary antibodies used were as follows: mouse monoclonal antibodies against tyrosinase, T311 (Novus Biologicals, Littleton, CO, USA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 1A10 (Genetex, Irvine, CA, USA) and PMEL17, HMB45 (Lab Vision Corp., Fremont, CA, USA). Secondary antibodies used were as follows: goat anti-mouse horseradish peroxidase-conjugated IgG (Jackson ImmunoResearch Laboratories West Grove, PA, USA), rabbit anti-mouse IgG2a (γ2a chain) dyLight™ 488 conjugated IgG, rabbit anti-mouse IgG1 (γ1 chain) dyLight™ 649 conjugated IgG and donkey anti-goat IgG (H&L) dyLight™ 488 conjugated IgG (Rockland Immunochemicals Inc., Gilbertsville, PA, USA).

2.2. Cell culture

HM3KO cells are a human-derived melanoma cell line (95). I am gifted HM3KO cells from Prof. Ichihashi, kindly. Cells were cultured in 1000 mg/l glucose DMEM supplemented with 10% FBS and 1% anti-B.M. at 37°C in a 5% CO_2 humidified incubator. Cells were incubated with the PMF mixture（10 mg/ml solution in dimethyl sulfoxide) for 5 days and were then harvested. Inhibitors or ammonium chloride were treated for 2 days prior to harvesting.

2.3. Melanin assay

HM3KO cells were lysed in 2 M NaOH for 20 min at 80ºC, and were centrifuged at 15,000 *g* for 10 min. Protein amounts were quantified using the BCA assay, and melanin contents of supernatants were measured as absorbance at 410 nm.

2.4. In vitro cytotoxicity assay

HM3KO cells were cultured with the PMF mixture, tangeretin, nobiletin or 3,3',4',5,6,7,8-heptamethoxyflavone for 5 days in 96 well plates and were treated with DMEM containing 10% Alamar blue for 2 h and fluorescence intensities were measured at Ex. 530 nm / Em. 590 nm.

2.5. Tyrosinase activity assay

To harvest tyrosinase from HM3KO cells, cells were lysed in 1% Triton X-100 for 10 min. The lysate was dispensed in 96 well plate and added the PMF mixture, tangeretin, nobiletin or 3,3',4',5,6,7,8-heptamethoxyflavone. Furthermore, the lysate was treated

0.04% L-3,4-dihydroxyphenylalanine for 2 h at 37ºC and tyrosinase activity were measured as absorbance at 410 nm.

2.6. RNA extraction and real-time PCR

RNA extraction and real-time PCR were performed as described the chapter 1. HM3KO cells were harvested in Isogen II and mRNAs were extracted according to the Isogen II protocol. Real-time PCR amplifications of mRNAs were performed with a StepOnePlus (Life Technologies Carlsbad, CA, USA), One Step SYBR PrimerScript PLUS RT-PCR kit and the following primer pairs (Integrated DNA Technologies, San Diego, CA USA): Tyrosinase forward 5'- CCATTTCCCTAGAGCCTGTGT -3', Tyrosinase reverse 5'- TGGACAGCATTCCTTCTCCAT -3', β-actin forward 5'-AGAAGGATTCCTATGTGGGCG-3', β-actin reverse 5'-CATGTCGTCCCAGTTGGTGAC-3'.

2.7. Protein extraction and Western blotting

Protein extraction and Western blotting were performed as described the chapter 1. HM3KO cells were washed twice with ice-cold PBS and then were harvested in RIPA buffer (radio-immunoprecipitation buffer, 50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1.0% NP-40) containing 100 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich). The lysates were mixed with sample buffer, loaded on 5% to 20% SDS-PAGE gels and transferred to PVDF membranes. Primary antibody reactions were performed with T311 (1:1000 dilution) or an antibody to GAPDH (1:10000 dilution). Bound antibodies were detected with a secondary antibody (1:10000 dilution). Signals

were detected with Luminata™ Forte Western HRP Substrate (Millipore, Billerica, MA, USA) or Western Lightening Ultra (Perkin Elmer, Wellesley, MA, USA) and were visualized with ChemiDoc™ XRS+ (Bio-Rad, Hercules, CA, USA).

2.8. Immunofluorescence microscopy

HM3KO cells were grown in Nunc™ Lab-Tek™ II Chamber Slides™ (Thermo Scientific, Waltham, MA, USA), fixed with 4% paraformaldehyde for 20 min, then washed with PBS and permeabilized with 0.1% Tween 20. Cells were blocked by Block Ace (DS Pharma Biomedical, Osaka, Japan) for 1 h and were probed with primary and secondary antibodies. Primary antibodies were T311 (1:100 dilution) and HMB-45 (1:1000 dilution). Secondary antibodies were rabbit anti-mouse IgG2a (γ2a chain) dyLight™ 488 conjugated IgG and/or rabbit anti-mouse IgG1 (γ1 chain) dyLight™ 649 Conjugated IgG (1:1000 dilution). Immunofluorescence microscopy was performed using a BZ-X700 (Keyence, Osaka, Japan).

2.9. Detection of acidified cellular organelles

To determine the quantities of acidified cellular organelles, cells cultured in 96 well plates were treated with Lysotracker® red DND-99 for 1 h, and fluorescence intensities were measured at Ex. 550 nm / Em. 560 nm. For observations by fluorescence microscopy, HM3KO cells were cultured in Nunc™ Lab-Tek™ II Chamber Slides™ and acidified organelles were detected by using an Acidic Granule Kit (Oxford Biomedical Research, Rochester Hills, MI). Briefly, HM3KO cells were fixed with 4% paraformaldehyde for 20 min, then washed with PBS treated with 30 μM 3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine (DAMP) for 30 min. Those

cells were treated with 0.5 M ammonium chloride for 10 min, washed with PBS, permeabilized with 0.1% Tween 20 ,washed with PBS and blocked by Block Ace. Acidified cellular organelles in those cells were detected by anti-dinitrophenyl group antibody and donkey anti-goat IgG (H&L) dyLight™ 488 conjugated IgG and observed using a BZ-X700.

2.10. Statistical analysis

All experiments were performed at least three times with similar results. Data were reported as mean +/- SD followed by statistical significance (Tukey-Kramer test). *P*-values < 0.05 are regarded as significant.

3. Results

3.1. The PMF mixture and its components suppress melanogenesis

I observed melanogenesis in HM3KO cells cultured with the PMF mixture for 5 days. Although dimethyl sulfoxide, solvent of the PMF mixture, did not affect melanogenesis (data not shown), at 1 μg/ml, the PMF mixture suppressed melanogenesis to approximately 60% compared to non-treated cells (Fig. 10a). The PMF mixture consisted mostly (~80%) of tangeretin, nobiletin and 3,3',4',5,6,7,8-heptamethoxyflavone, and at 1 μg/ml, each of those components alone was able to suppress melanogenesis (Fig. 10b). Additionally, nobiletin and 3,3',4',5,6,7,8-heptamethoxyflavone significantly suppressed melanogenesis more than tangeretin (Fig. 10b). Because the PMF mixture and its components had no cell toxicities at 1 μg/ml (Fig. 10c), the suppression of melanogenesis by PMF was considered to be independent of cell toxicity. Melanogenesis is suppressed by tyrosinase inhibition. However, the PMF mixture and its components did not inhibit tyrosinase activity derived from HM3KO cells, directly (Fig. 10d).

3.2. The PMF mixture promotes tyrosinase degradation in lysosomes

Melanin production requires tyrosinase. To examine whether the PMF mixture affected tyrosinase expression, I measured tyrosinase mRNA and protein levels of HM3KO cells treated with the PMF mixture. Although the PMF mixture did not affect tyrosinase mRNA levels (Fig. 11a), it did decrease tyrosinase protein levels in HM3KO cells, compared with no treatment (Fig. 11b). It has been reported that tyrosinase is degraded in proteasomes (18, 19, 96, 97) and/or lysosomes (22, 23). Therefore, I determined whether proteasome and/or lysosome inhibitors could prevent the tyrosinase

decrease caused by the PMF mixture. The proteasome inhibitor, MG132, hardly prevented the tyrosinase decrease caused by the PMF mixture but a cocktail of lysosome protease inhibitors, leupeptin and pepstatin A, prevented the PMF mixture from causing tyrosinase degradation (Fig. 11c). Therefore, the PMF mixture promotes tyrosinase degradation in lysosomes.

3.3. The PMF mixture and its components acidify cell organelle(s)

I examined whether the PMF mixture and its components acidify cell organelles including melanosomes. I measured the acidities of cell organelles in HM3KO cells using Lysotracker® Red DND-99. Lysotracker® Red DND-99 is a fluorescent dye that accumulates in acidified cell organelles. The fluorescence intensities of HM3KO cells, indicating acidified organelles, increased depending on the concentration of the PMF mixture and its components (Fig. 12a). Additionally, similar to the suppression of melanogenesis, nobiletin and 3,3’,4’,5,6,7,8-heptamethoxyflavone significantly induced a higher accumulation of Lysotracker® Red DND-99 in cell organelles compared with tangeretin (Fig. 12b). Therefore, the PMF mixture and its components acidify cell organelles and that acidification could be related to the suppression of melanogenesis.

3.4. The PMF mixture acidifies melanosomes

I used immunofluorescence staining to examine whether the PMF mixture acidifies melanosomes, similar to the situation in Caucasian melanocytes. I detected acidified organelles, which had accumulated 3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine (DAMP), bound by an anti-dinitrophenyl group antibody. Melanosomes were detected by HMB45, which is

specific to PMEL17, which localizes in melanosomes (98, 99). The PMF mixture increased DAMP accumulation, similar to Lysotracker® Red DND-99 (Fig. 13a and d). Furthermore, the colocalization of DAMP and HMB45 was observed only in HM3KO cells treated with the PMF mixture (Fig. 13c and f). These results indicate that at least some organelles acidified by the PMF mixture are melanosomes.

3.5. The PMF mixture inhibits the localization of tyrosinase into melanosomes by the acidification of cell organelles

Tyrosinase synthesizes melanin in melanosomes. I examined whether the PMF mixture affects the localization of tyrosinase into melanosomes. In control cells, colocalization between tyrosinase and melanosomes was observed (Fig. 14a, b and c). On the other hand, that colocalization was diminished in HM3KO cells treated with the PMF mixture (Fig. 14d, e and f).

Furthermore, I examined whether ammonium chloride, which neutralized acidic organelles including melanosomes, could abolish the inhibitory effect of the PMF mixture on the localization of tyrosinase into melanosomes. The results showed that ammonium chloride prevented the PMF mixture from suppressing the colocalization between tyrosinase and melanosomes (Fig. 14g, h and i). Therefore, the PMF mixture can suppress the localization of tyrosinase into melanosomes through the acidification of cell organelles, including melanosomes.

3.6. Ammonium chloride restores melanogenesis suppressed by the PMF mixture

I examined whether ammonium chloride could prevent the PMF mixture from suppressing melanogenesis. HM3KO cells treated with 5 mM ammonium chloride for 2

days recovered from the suppression of melanogenesis elicited by the PMF mixture (Fig. 15). Therefore, the PMF mixture suppresses melanogenesis through the acidification of cell organelles, including melanosomes.

4. Discussion

In this study, I demonstrated that the PMF mixture suppressed melanogenesis of human melanoma cell line, HM3KO (Fig. 10). To investigate the underlying mechanisms, I examined the dynamics of tyrosinase in cells affected by the PMF mixture. The PMF mixture reduced protein amounts of tyrosinase without the down regulation of the mRNA (Fig. 11). In general, there are two system of intracellular protein degradation such as ubiquitin-proteasome system and autophagy by lysosome. Although MG132, a proteasome inhibitor, failed to restore the protein level of tyrosinase decreased by the PMF mixture, a cocktail of leupeptin and pepstatin A which were inhibitors of lysosomal proteinases, restored the protein level of tyrosynase. The results indicated that tyrosinase was degraded by lysosome. In the following examination, I found that the PMF mixture acidified intracellular organelles including melanosomes (Fig. 12 and 13), and suppressed the translocation of tyrosinase in melanosomes (Fig. 14). Then, in order to identify the relationship between acidification of intracellular organelles and the translocation of tyrosinase, I examined the effect of ammonium chloride to neutralize pH of intracellular organelles. Ammonium chloride abolished the effects of the PMF mixture on tyrosinase translocation and melanogenesis (Fig. 14 and 15). These results indicate a possibility that acidification effects of the PMF mixture in intracellular organelles contribute to reduction of melanin synthesis of HM3KO cells through tyrosinase degradation in lysosomes.

The translocation of tyrosinase to melanosomes proceeds through the endoplasmic reticulum and trans-Golgi network (TGN). It has been reported that mistransfer of tyrosinase in lysosomes is caused by mutation of the membrane trafficking related genes of Hermansky-Pudlak Syndrome at the post-TGN (20, 21) and inulavosin which is a

flavonid posseing methyl and hydroxyl groups, reduced melanin synthesis promoting the mistranslocation of tyrosinase in lysosomes in the post-TGN (22, 24). Regarding chemical structure, the PMF mixture could similarly induce the mistranlocation of tyrosinase to lysosomes in the post-TGN fashion and reduced melanin synthesis.

It has been suggested that pH changes in organelles other than melanosomes could regulate melanogenesis. GM-95 cells which is B16 mouse melanoma cells having a neutralized TGN, endosomes and lysosomes (100) due to defective glycosphingolipid production, have decreased levels of melanogenesis (101, 102). Furthermore, the acidification of cell organelles also suppresses melanogenesis (103, 104), especially the acidification of melanosomes (23, 91 – 94). This study indicated that the acidification of cell organelle(s) by the PMF mixture could inhibit tyrosinase translocation and melanogenesis. Although I have shown that the PMF mixture acidifies melanosomes, it is left a possibility that acidifications of other organelles such as TGN, lysosomes and/or endosomes contribute to suppression of melanogenesis by the PMF mixture. In HM3KO cells treated with the PMF mixture, acidified cell organelles were mainly concentrated at the cell periphery (Fig. 13), whereas the TGN, lysosomes and endosomes are mainly localized in the peri-nuclear region and in the cytoplasm. On the other hand, melanosomes have been reported to be transported to the cell periphery and anchored to the plasma membrane (25, 26). Therefore, it is considered a possibility that the PMF mixture suppresses melanogenesis through the acidification of melanosomes.

It has been reported that melanosomal pH is regulated by proton influx through a vacuolar-type H^+-adenosine triphosphatase (v-ATPase) and excretion through a Na^+/H^+ exchanger (NHE) and $Na^+/Ca^{2+}/K^+$ exchanger (NCKX). (105). Then, melanosomal pH is determined by the balance of v-ATPase, NHE and NCKX. I observed that the PMF

mixture does not affect the mRNA expression of v-ATPase components and solute carrier family (*SLC*)*45A2* and *SLC24A5*, which encode NHE and NCKX of melanosomes, respectively (data not shown). Recently, it has been reported that, in mice, cystinosin, a cystine/H^+ symporter, is related to the regulation of melanosomal pH (23). The PMF mixture might affect the expression of cystinosin and/or other undiscovered proteins that regulate melanosomal pH. Alternatively, it is considered that the PMF mixture might directly affect the activities of those proteins.

It has been authorized that transfer of melanosomal proteins from the TGN to melanosomes is carried out through early endosomes by vesicle trafficking (106). Intra-vesicle pH also plays an important role in vesicle trafficking (107). It has been suggested that v-ATPase is not only a proton pump, but also plays an important role for vesicle trafficking by working as a pH sensor of intra-vesicles (108 – 112). Therefore, the PMF mixture might affect pH detection of v-ATPase due to melanosomal acidification and, result in inhibition of tyrosinase transfer into melanosomes. To resolve the mechanism of melanosomal acidification by the PMF mixture, it will be necessary to characterize the regulatory mechanisms of melanosomal pH in more detail.

On the other hand, the inhibitory effects of the PMF mixture on melanogenesis might be discussed at the point of tyrosinase activity in melanosome. Since tyrosinase activity is maximal at or near neutral pH and is lost at an acidic pH (91), melanosomal pH generally affects tyrosinase activity. It has been reported that tyrosinase activity is decreased due to the loss of Cu^{2+} from the active center at low pH (113, 114) The PMF mixture was not able to inhibit tyrosinase activity directly (Fig. 10d). However, it is possible that the PMF mixture inhibits tyrosinase activity through melanosome acidification. Therefore, I am not able to eliminate that the PMF mixture suppresses

melanogenesis by the inhibition of tyrosinase activity through melanosome acidification, in addition to tyrosinase degradation and mislocalization. To identify the effect of melanosome acidification by the PMF mixture against tyrosinase activity, it will be necessary to study in more detail.

In Fig. 14e, not only tyrosinase but also HMB45 staining intensity reduced by the PMF mixture treatment. HMB45 specifically reacts with the fibrillar matrix constructed by PMEL17 in Stage II-IV melanosomes, but not Stage I melanosomes (8, 99, 115, 116) Therefore, the PMF mixture might also affect melanosome formation and/or maturation.

In conclusion, I have demonstrated that the PMF mixture inhibits melanogenesis and the localization of tyrosinase into melanosomes through the acidification of cell organelles, including melanosomes. In this study, propose that the PMF mixture can be useful agent for regulation of skin pigmentation.

5. Figures

Figure 10. PMF suppresses melanogenesis. HM3KO cells were incubated in the presence of the PMF mixture, tangeretin, nobiletin or 3,3',4',5,6,7,8-heptamethoxyflavone (heptamethoxyflavone) for 5 days. (a) melanin production in HM3KO cells treated with the PMF mixture. (b) melanin production in HM3KO cells treated with 1 μg/ml PMF mixture, tangeretin, nobiletin or heptamethoxyflavone. (c) cell viabilities of HM3KO cells treated with the PMF mixture, tangeretin, nobiletin or heptamethoxyflavone for 5 days. (d) Activities of tyrosinase treated with the PMF mixture, tangeretin, nobiletin or heptamethoxyflavone. Data were normalized by non-treated cells. Data represent mean +/- SD of n = 3. *P <0.05, **P < 0.01 versus non-treated cells. ##P < 0.01 versus tangeretin-treated cells.

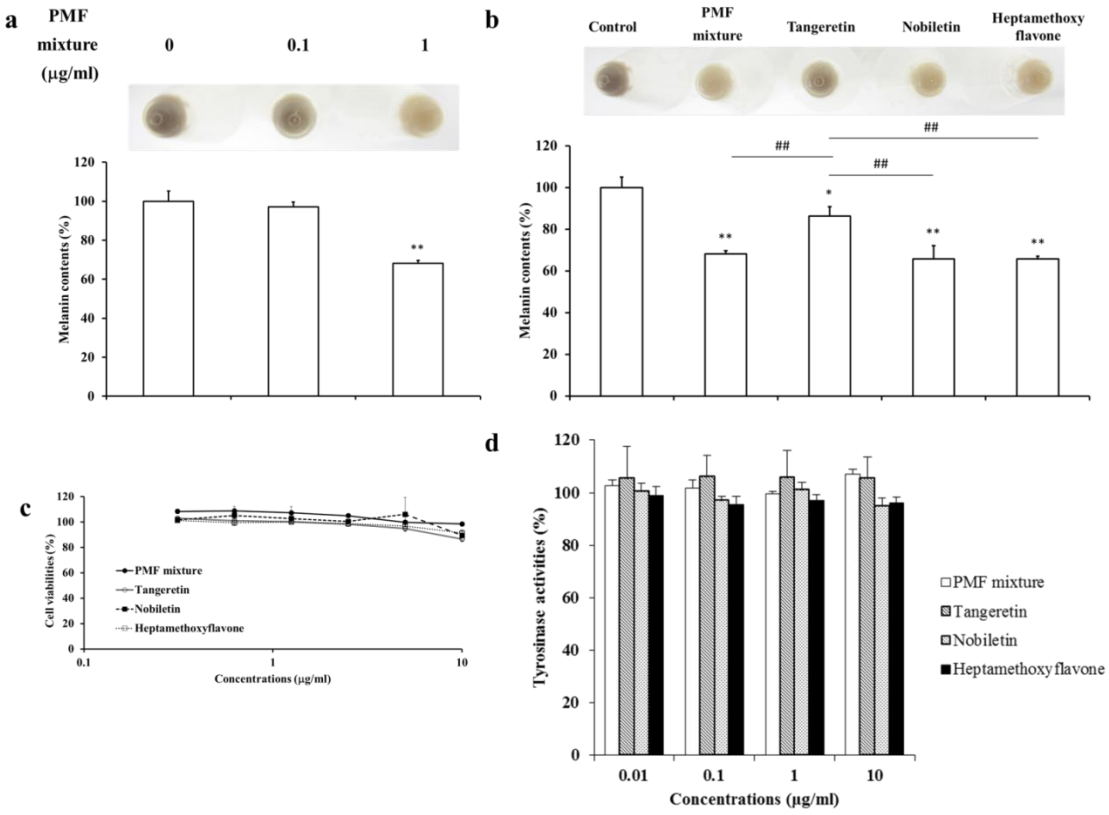
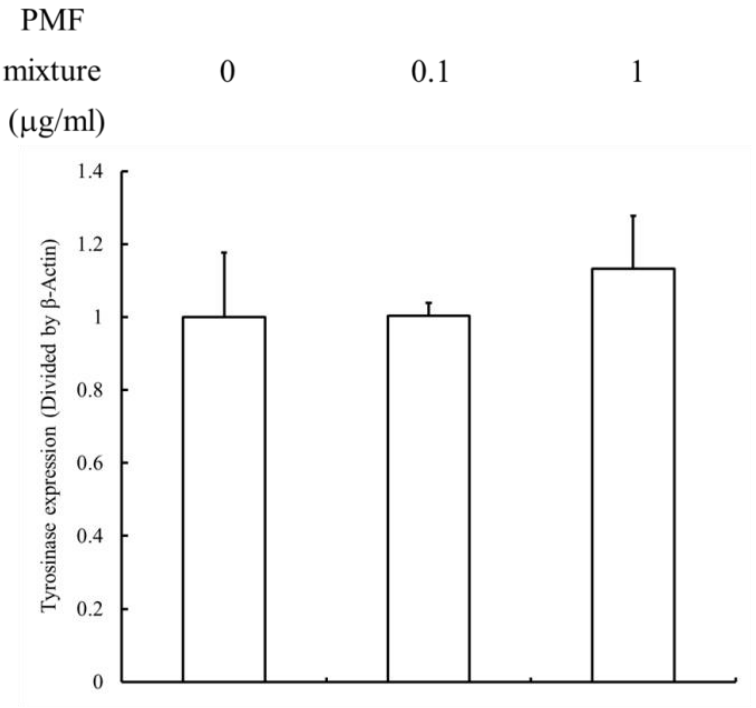
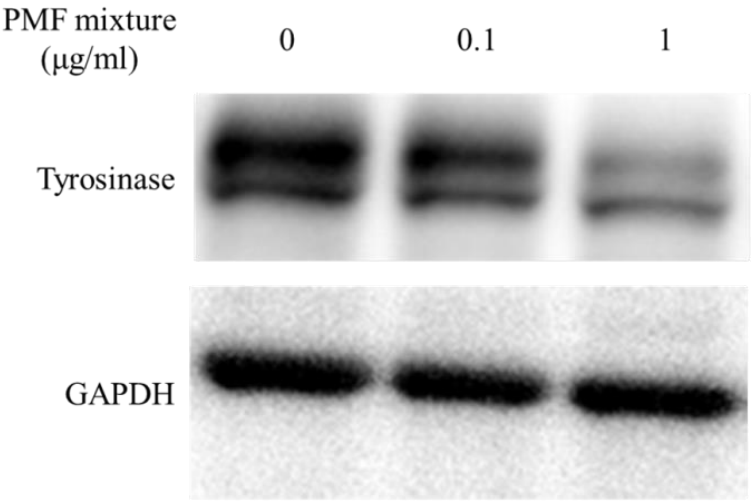

a
PMF mixture (μg/ml)
0
0.1
1
Melanin contents (%)
b
Control
PMF mixture
Tangeretin
Nobiletin
Heptamethoxy flavone
Melanin contents (%)
c
Cell viabilities (%)
PMF mixture
Tangeretin
Nobiletin
Heptamethoxyflavone
Concentrations (μg/ml)
d
Tyrosinase activities (%)
PMF mixture
Tangeretin
Nobiletin
Heptamethoxyflavone
Concentrations (μg/ml)

Figure 11. The PMF mixture does not suppress tyrosinase mRNA expression, but promotes tyrosinase degradation in lysosomes. HM3KO cells were cultured with the PMF mixture for 5 days and tyrosinase mRNA (a) or protein (b) were detected by real-time PCR or Western blotting, respectively. (c) HM3KO cells cultured with 1 μg/ml PMF mixture for 3 days were additionally treated with 1 μg/ml PMF mixture + inhibitors (proteasome inhibitor or the cocktail of lysosome inhibitors) for 2 days and proteins were detected by Western blotting. Inhibitor concentrations were as follows. MG132; 100 nM, the cocktail of leupeptin and pepstatinA; 10 μM, respectively. Data were normalized by non-treated cells. Data represent mean +/- SD of n = 3.

a


PMF mixture (μg/ml)
0
0.1
1
Tyrosinase expression (Divided by β-Actin)
1.4
1.2
1
0.8
0.6
0.4
0.2
0

b


PMF mixture (μg/ml)
0
0.1
1
Tyrosinase
GAPDH

c

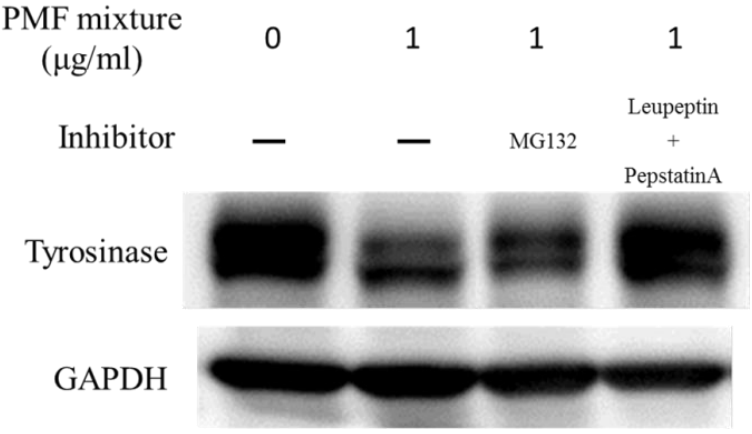
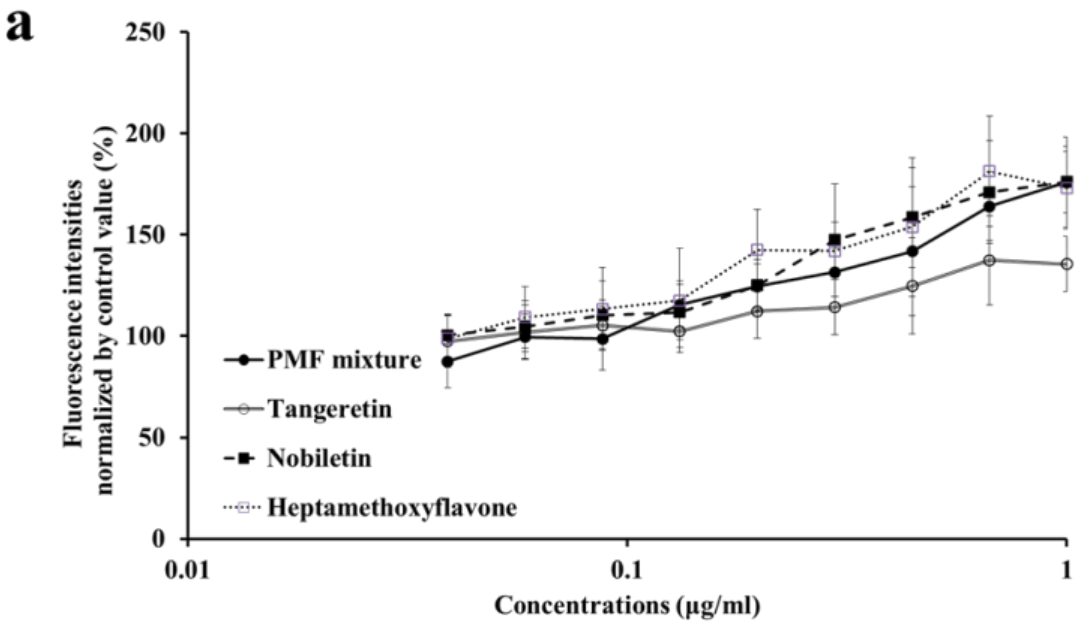

PMF mixture (μg/ml)
0
1
1
1
Inhibitor
—
—
MG132
Leupeptin + PepstatinA
Tyrosinase
GAPDH

Figure 12. PMF acidifies cell organelles. HM3KO cells were incubated in the presence of the PMF mixture, tangeretin, nobiletin or 3,3',4',5,6,7,8-heptamethoxyflavone (heptamethoxyflavone) for 5 days. HM3KO cells were treated with Lysotracker® Red DND-99 for 1 hr and then were measured for fluorescence intensities (Ex. 550 nm / Em. 560 nm). (a) increase of fluorescence intensity at each PMF concentration. (b) fluorescence intensity of HM3KO cells treated with 1 μg/ml of each type of PMF. Data were normalized by non-treated cells. Data represent mean +/- SD of n = 8. **P < 0.01 versus non-treated cells. ##P < 0.01 versus tangeretin-treated cells.


a
250
200
150
100
50
0
Fluorescence intensities normalized by control value (%)
PMF mixture
Tangeretin
Nobiletin
Heptamethoxyflavone
0.01
0.1
1
Concentrations (μg/ml)

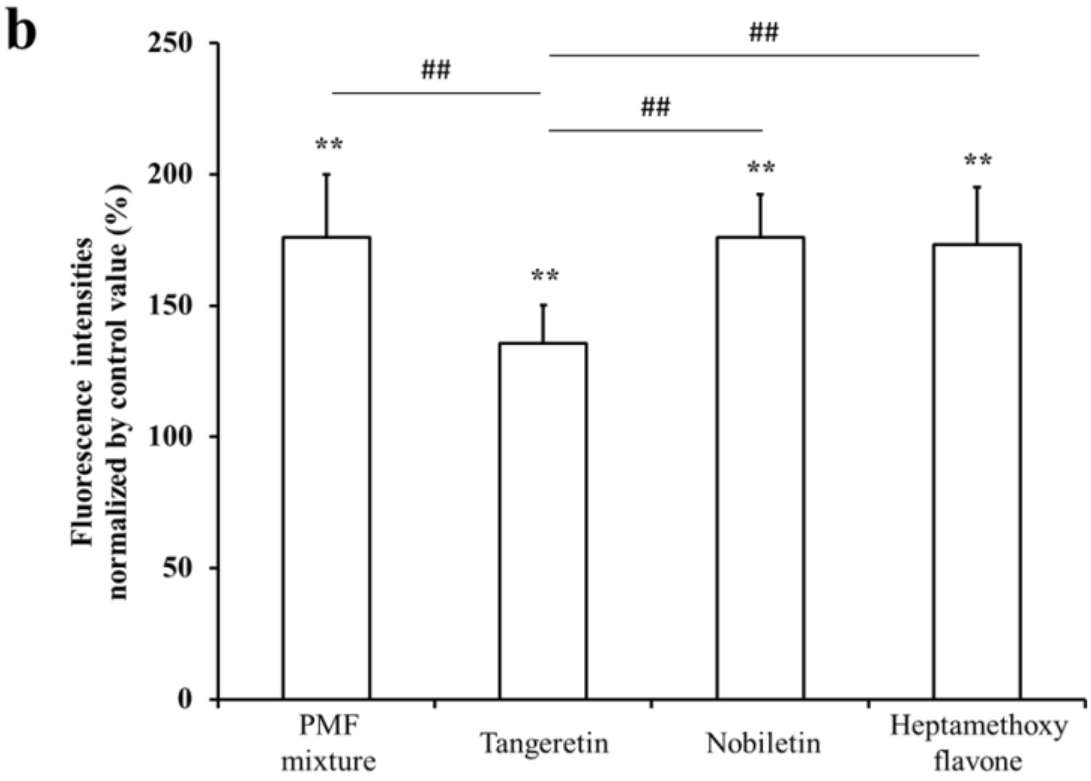

b
250
200
150
100
50
0
Fluorescence intensities normalized by control value (%)
##
##
##
**
**
**
**
PMF mixture
Tangeretin
Nobiletin
Heptamethoxy flavone

Figure 13. The PMF mixture acidifies melanosomes. HM3KO cells were cultured in 1 μg/ml PMF mixture for 5 days and were then treated with 3-(2,4-dinitroanilino)-3'-amino-N- methyldipropylamine (DAMP). DAMP and PMEL17 were detected by anti-DNP antibodies (a, d, green) and by HMB45 (b, e, red), respectively. (c) and (f) are merged images of DAMP and HMB45; nuclei were detected by DAPI (blue). (a)-(c) and (d)-(f) indicate non-treated and 1 μg/ml PMF mixture-treated cells, respectively. Bar = 20 μm.

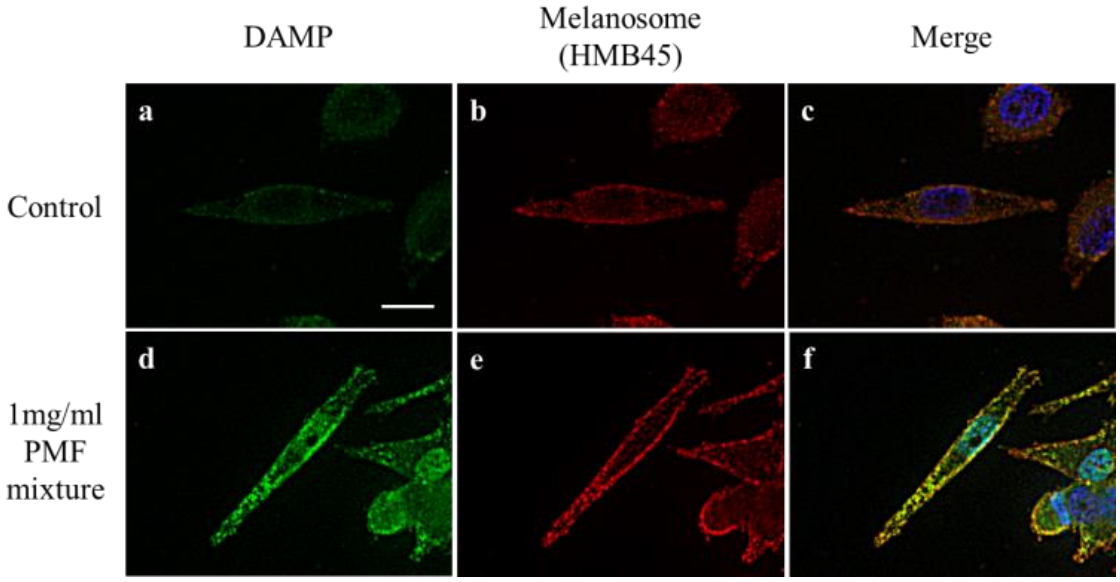

DAMP
Melanosome
(HMB45)
Merge
Control
1mg/ml
PMF
mixture
a
b
c
d
e
f

Figure 14. The PMF mixture suppresses tyrosinase localization into melanosomes. HM3KO cells cultured with 1 μg/ml PMF mixture for 3 days were additionally treated with 1 μg/ml PMF mixture + 5 mM ammonium chloride for 2 days. Tyrosinase and PMEL17 were detected by T311 (a, d, g, green) and HMB45 (b, e, h, red), respectively. (c), (f) and (i) merged images of T311 and HMB45; nuclei were detected by DAPI (blue). (a)-(c), (d)-(f) and (g)-(i) indicate non-treated, 1 μg/ml PMF mixture-treated and 1 μg/ml PMF mixture + 5 mM ammonium chloride-treated cells, respectively. Bar = 20 μm.

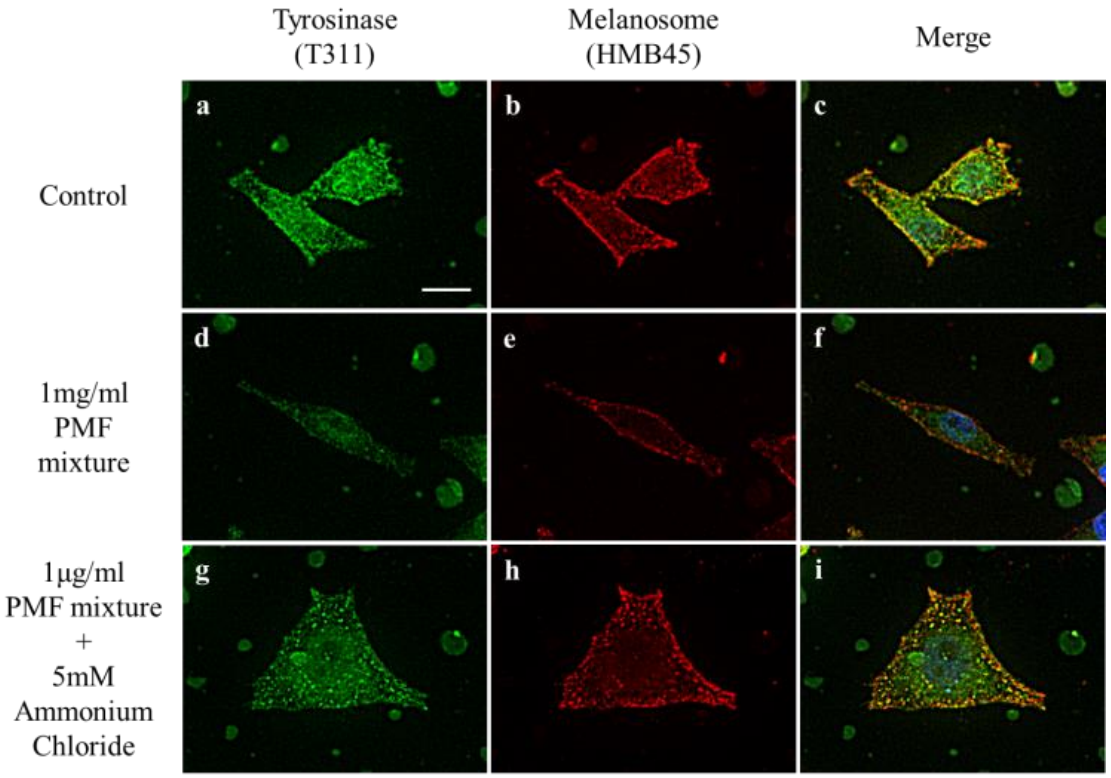
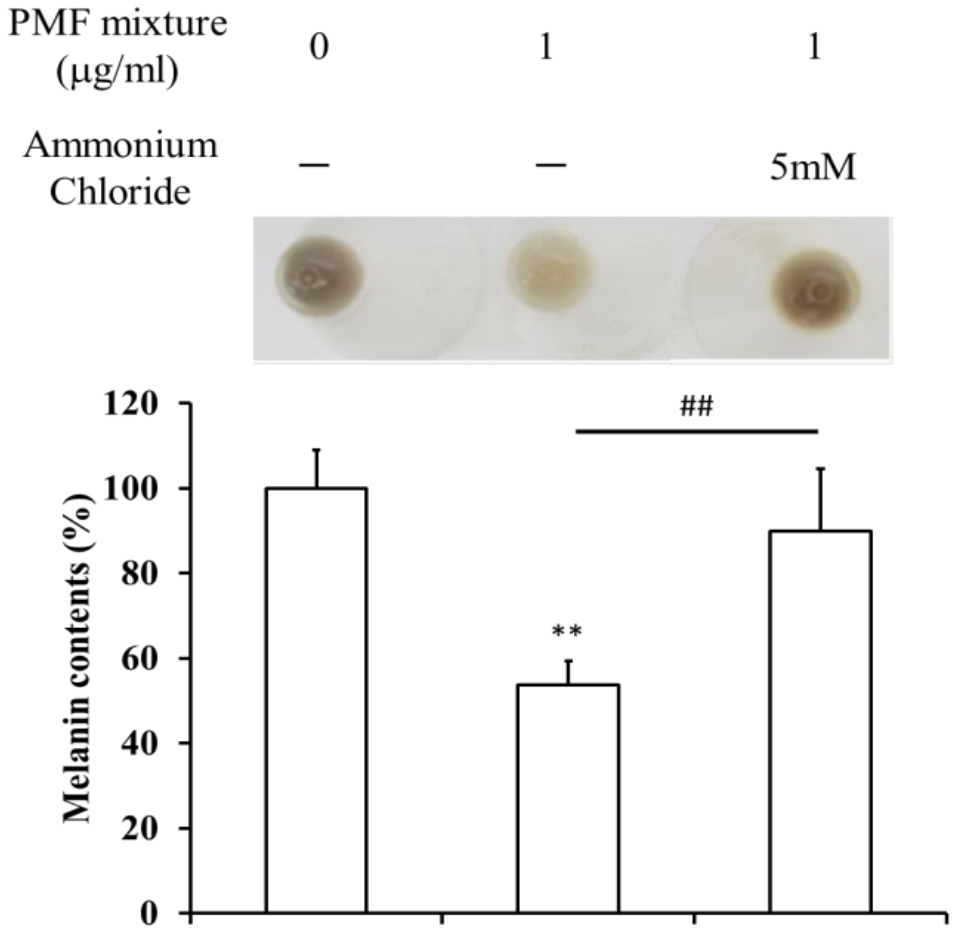

Tyrosinase
(T311)
Melanosome
(HMB45)
Merge
Control
1mg/ml
PMF
mixture
1μg/ml
PMF mixture
+
5mM
Ammonium
Chloride
a
b
c
d
e
f
g
h
i

Figure 15. Ammonium chloride prevents the PMF mixture from suppressing melanogenesis. HM3KO cells cultured with 1 μg/ml PMF mixture for 3 days were additionally treated with 1 μg/ml PMF mixture + 5 mM ammonium chloride for 2 days and melanin production was measured. Data were normalized by non-treated cells. Data represent mean +/- SD of n = 3. **P < 0.01 versus non-treated cells. ##P < 0.01 versus PMF mixture-treated cells.


PMF mixture (μg/ml)
0
1
1
Ammonium Chloride
—
—
5mM
Melanin contents (%)
120
100
80
60
40
20
0
**
##

General Discussion

In general discussion, I mainly argue the PMF effects in the skin. In these studies, I have revealed that PMF inhibits the melanogenesis and PGE_2 production. Because arbutin directly ameliorates the hyperpigmentation through the inhibition of the melanogenesis (33), PMF could also have similar effect (Fig. 16). Furthermore, because PGE_2 activates the melanogenesis and dendritic formation of melanocytes (8, 9), PMF could also suppress the UVB-induced hyperpigmentation through the inhibition of the PGE_2 production (Fig. 16). Actually, it has been reported that COX-2 inhibition also alleviates the hyperpigmentation through the inhibition of the PGE_2 production (46).

Other than PGE_2, α-MSH, ET-1 and SCF have been also known as the melanocyte activating factors. In these studies, it is given below the reason that I have focused on PGE_2. UVB induces α-MSH, ET-1 and SCF expression through p53 activation, Whereas, PGE_2 is produced through COX-2 expression. It has been known that p53 is the tumor-suppressor gene, while PGE_2 and COX-2 could be the targets of cancer therapies. Therefore, I have researched the effect of PMF against PGE_2 production and COX-2 expression.

In these studies, I have suggested that PMF could prevent and/or ameliorate the UVB-induced ECM degradation in the dermis through the suppression of the PGE_2 production and MMP-1 expression (Fig. 16). UVB induces edema and erythema in the skin, and facilitates the infiltration of neutrophils and lymphocytes into the dermis by the enhancement of the vascular permeability. Invading lymphocytes secrete MMPs, and promote the ECM degradation of the dermis. Actually, it has been reported that the delay of lymphocytes elimination from the skin leads to the exacerbation of the skin damages (31). It has been thought that PGE_2 plays a key role in the UVB-induced

edema and erythema, because COX-2 inhibitor effectively suppresses them (117). Because PGE_2 promotes the vascular permeability and infiltration of lymphocytes (32), it is possible that the suppression of PGE_2 production by PMF alleviates the UVB-induced ECM degradation in the dermis. Additionally, it has been reported that UVB below minimal erythema dose also induces ECM degradation and MMPs expression in the skin (29). Because this reaction is independent on inflammation, it has been thought that MMPs and/or the other proteinases are expressed by the skin resident cells, such as keratinocytes and/or dermal fibroblasts. It has been reported that keratinocytes are induced MMP-1, 3, 10, 12 and 13 expressions by UVB irradiation (118). On the other hands, UVB can not penetrate the dermis. Therefore, it has been suggested that dermal fibroblasts are activated by cytokines secreted from keratinocytes, and, accordingly, express proteinase (119). It has been reported that, on UVB irradiation, keratinocytes express more MMPs, than dermal fibroblasts (72). Because, in these studies, PMF has suppressed MMP-1 expression of HaCaTs, it could also inhibit the inflammation-independent ECM degradation.

In these studies, I have also researched the mechanisms which PMF suppresses PGE_2 production and MMP-1 expression. In consequence, it has been suggested that PMF inhibited the PGE_2 production and COX-2 expression through PPAR-γ activation and MMP-1 expression through AP-1 deactivation, respectively. It has been known that COX-2 expression is regulated by NF-κB and PPAR-γ deactivates NF-κB. Therefore, PMF could simultaneously inhibit NF-κB and AP-1. They are the transcription factors which are activated by the inflammatory stimulations, and it has been suggested that there is the crosstalk between their activities. Actually, it has been reported that NF-κB activation by TNF-α inhibits AP-1 activation through JNK deactivation, and the NF-κB

inhibition promotes JNK activation (120). If UVB response of keratinocytes is similar to TNF-α response, inhibiting only NF-κB might promote MMP-1 expression through AP-1 activation. Therefore, inhibiting both NF-κB and AP-1 might be necessary for the suppression of MMP-1 expression. In these studies, it has been suggested that PMF suppresses both NF-κB and AP-1 activation.

PMF has the lower anti-oxidative activity than flavonoid, because PMF has the methoxy groups, instead of the hydroxyl groups. On the other hands, PMF has the unique functions, which flavonoid does not possess. For instance, PMF suppresses the melanogenesis on the lower concentration (about 1 μg/ml), than flavonoid. Furthermore, PMF also has the higher compatibility to the cellular membrane, than flavonoid. Therefore, it has been suggested that PMF is easy to penetrate into the cells (37). In addition, PMF is been thought to infiltrate in the skin, because the stratum corneum is lipids rich. Therefore, in the skin, PMF could be easier to function the physiological activities than flavonoid, because PMF is easy to penetrate in the skin and/or skin cells.

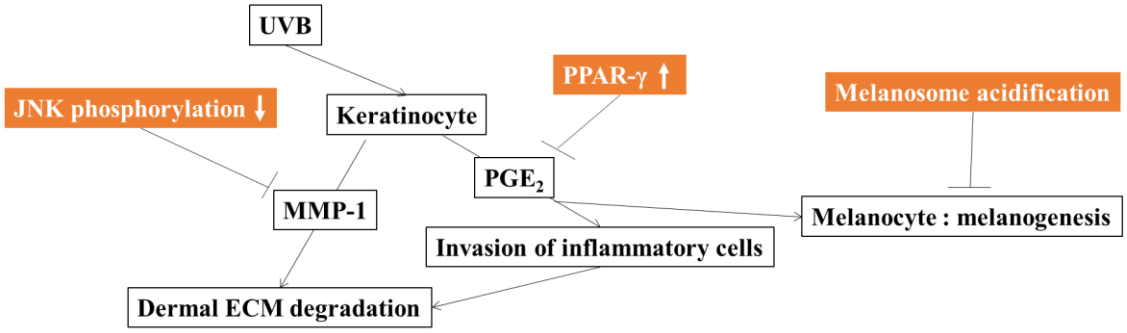
As noted above, PMF strongly suppresses the melanogenesis. Rhododenol, inhibiting the melanin production, has the adverse effect as the vitiligo. Its mechanism has been suggested as below. Rhododenol binds the catalytic active site of tyrosinase, in competition with tyrosine, and, accordingly, suppresses the melanogenesis. On the other hands, it has been revealed that Rhododenol is converted to cytotoxic substance(s), which could result in the vitiligo (121). It is guessed that PMF can not bind the catalytic active site of tyrosinase, because, as shown by these studies, it has not directly inhibited tyrosinase activity. Moreover, in these studies, it has been suggested that PMF suppresses the melanogenesis by the melanosome acidification. It has been revealed that melansomal pH of Caucasian is lower than African American and its melanosome pH

could cause the difference of the melanogenesis between races (91, 92). Therefore, the melanogenesis suppression by PMF might be similar to Caucasian.

In these studies, I have indicated that PMF could inclusively suppress the UVB-induced hyperpigmentation and/or dermal ECM degradation. Furthermore, I have also suggested the mechanisms how PMF functions against them. These results could be beneficial for studying the synergistic effect(s) with the other agent(s), hereafter. Therefore, I suggest that PMF is the newly useful agent against the UVB-induced wrinkles and dyspigmentation, which is the features of photoaging.

Figure

Figure 16. Schematic view of PMF effects against photoaging. PMF suppresses UVB-induced PGE_2 production and MMP-1 expression on keratinocyte through activating PPAR-γ and inhibiting JNK phosphorylation, respectively. Also, PMF suppresses melanogenesis through acidifying melanosome. Therefore, I suggest that PMF is the newly useful agent against the UVB-induced wrinkles and dyspigmentation, which is the features of photoaging.


UVB
JNK phosphorylation ↓
Keratinocyte
PPAR-γ ↑
Melanosome acidification
PGE2
MMP-1
Melanocyte : melanogenesis
Invasion of inflammatory cells
Dermal ECM degradation

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