Oral supplementation with cocoa extract reduces UVB-induced wrinkles in hairless mouse skin

Jong-Eun Kim, Dasom Song, Junil Kim, Jina Choi, Jong Rhan Kim, Hyun-Sun Yoon, Jung-Soo Bae, Mira Han, Sein Lee, Ji Sun Hong, Dayoung Song, Seong-Jin Kim, Myoung-Jin Son, Sang-Woon Choi, Jin Ho Chung, Tae-Aug Kim, Ki Won Lee

PII: S0022-202X(16)00457-7
DOI: 10.1016/j.jid.2015.11.032
Reference: JID 171

To appear in: The Journal of Investigative Dermatology

Received Date: 15 July 2015
Revised Date: 26 November 2015
Accepted Date: 30 November 2015


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Oral supplementation with cocoa extract reduces UVB-induced wrinkles in hairless mouse skin

Jong-Eun Kim¹,²,†, Dasom Song¹,²,†, Junil Kim³,†, Jina Choi³,⁴, Jong Rhan Kim¹,², Hyun-Sun Yoon⁵,⁶,⁷, Jung-Soo Bae⁵,⁶, Mira Han⁵,⁶, Sein Lee¹,², Ji Sun Hong¹,², Dayoung Song³,⁴, Seong-Jin Kim³, Myoung-Jin Son⁸, Sang-Woon Choi⁹, Jin Ho Chung⁵,⁶,¹⁰, Tae-Aug Kim³,⁴,*, and Ki Won Lee¹,²,¹⁰,*

¹WCU Biomodulation Major, Center for Food and Bioconvergence, Department of Agricultural Biotechnology, Seoul National University, Seoul, Republic of Korea; ²Advanced Institutes of Convergence Technology, Seoul National University, Suwon, Republic of Korea; ³CHA Cancer Institute and ⁴Department of Biochemistry, School of Medicine, CHA University, Seongnam, Republic of Korea; ⁵Department of Dermatology, Seoul National University College of Medicine, Seoul, Republic of Korea; ⁶Institute of Human-Environment Interface Biology, Seoul National University, Seoul, Republic of Korea; ⁷Department of Dermatology, Seoul National University Boramae Hospital, Seoul, Republic of Korea; ⁸Health Foods Team, Lotte R&D Center, Seoul, Republic of Korea; ⁹CHAUM Life Center, School of Medicine, CHA University, Seoul, Republic of Korea; ¹⁰Institute on Aging, Seoul National University, Seoul, Republic of Korea

† These authors contributed equally to this work

*Correspondence to: Ki Won Lee, kiwon@snu.ac.kr, Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Republic of Korea. Tel.: 82-2-880-4853. Fax: 82-2-873-5095.

Tae-Aug Kim, takim@cha.ac.kr, CHA Cancer Institute, Department of Biochemistry, School of Medicine, CHA University, Seongnam 463-400, Republic of Korea. Tel: +82-31-881-7177, Fax: +82-31-881-7185
Abstract

Cacao beans contain various bioactive phytochemicals that could modify the pathogeneses of certain diseases. Here, we report that oral administration of cacao powder (CP) attenuates UVB-induced skin wrinkling by the regulation of genes involved in dermal matrix production and maintenance. Transcriptome analysis revealed that 788 genes are down- or up-regulated in the CP supplemented group, compared to the UVB-irradiated mouse skin controls. Among the differentially-expressed genes, cathepsin G and serpin B6c play important roles in UVB-induced skin wrinkle formation. Gene regulatory network analysis also identified several candidate regulators responsible for the protective effects of CP supplementation against UVB-induced skin damage. CP also elicited anti-wrinkle effects via inhibition of UVB-induced MMP-1 expression in both the human skin equivalent model and human dermal fibroblasts (HDFs). Inhibition of UVB-induced AP-1 via CP supplementation is likely to affect the expression of MMP-1. CP supplementation also down-regulates the expression of cathepsin G in HDFs. 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, a major in vivo metabolite of CP, showed effects similar to CP supplementation. These results suggest that cacao extract may offer a protective effect against photoaging by inhibiting the breakdown of dermal matrix, which leads to an overall reduction in wrinkle formation.

Keywords: cacao; UVB; skin wrinkle; MMP-1; cathepsin G

Introduction

Many natural products are known to influence the development of skin structures and its biological functions. Cacao beans have the antioxidant capacity higher than the capacity provided by green teas and red wine (Lee et al., 2003; Subhashini et al., 2010). The antioxidant activity of cacao can modify the pathogeneses of a different spectrum of diseases, including the cardiovascular diseases, cancer, and other chronic conditions (Park et al., 2014). Recent studies have demonstrated the beneficial effects of cacao consumption are associated with human health, especially with the improved condition of the skin (Park et al., 2014; Scapagnini et al., 2014). Cacao provides positive effects on the skin structure and the dermal microcirculation (Katz et al.,
2011; Neukam et al., 2007), and its topical preparations are able to protect the skin from the
oxidative damages arising from ultraviolet (UV) radiation (Katz et al., 2011). Besides the
photoprotection against UVB-induced erythema, long-term ingestion of cacao also ameliorates
aberrant skin conditions by increasing the blood flow to the cutaneous and subcutaneous tissues to
increase the skin density and hydration (Heinrich et al., 2006). Our previous studies have shown
that cacao inhibits skin cancer growth and skin inflammation both \textit{in vitro} and \textit{in vivo} (Kang et
al., 2008; Kim et al., 2010; Lee et al., 2006). Although there are accumulating evidences that
cacao consumption can improve the skin health, the molecular mechanisms responsible for these
beneficial effects have not been thoroughly investigated.

Skin wrinkling is a typical characteristic of photoaging that results from chronic exposure
to solar UV radiation. Repeated exposure to UV light decreases procollagen production and
breaks down collagen fibers. The process is partially due to the overexpression of matrix
metalloproteinases (MMP) (Fisher et al., 1997; Ichihashi et al., 2009; Xu and Fisher, 2005). Our
previous studies demonstrated that cathepsin G regulates MMP expression and UVB-induced skin
photoaging (Son et al., 2009; Son et al., 2012). Cathepsins comprise a family of serine proteases
whose members are classified into A, B, C, D, E, G, H, and L groups, according to their substrate
specificities (Son et al., 2009). Cathepsins B, D, K, and G may act as biomarkers in photoaged
human skin (Zheng et al., 2011). Cathepsin G is a single 30-kDa polypeptide released by the
neutrophils and the UVA-irradiated normal human fibroblasts (Son et al., 2009). Inhibitors of
cathepsin G may be useful for the prevention of UVB-induced photoaging since they could
ameliorate the ECM damage and MMP upregulation (Son et al., 2012). Serpin b6 is a member of
the superfamily of serine protease inhibitors known as serpins. Serpins bind with serine proteases
involved in inflammatory processes, coagulation, fibrinolysis, tumorigenesis and apoptosis. The
association of serpin b6 with cathepsin G has been postulated to inhibit cathepsin G activity (Scott
et al., 2007).

In this study, we first examined the protective effect of CP on UVB-induced wrinkle
formation in hairless mice, then we showed gene expression profiles using RNA sequencing
analysis in comparison with several other well-known food materials used to modify skin health
(Cho et al., 2007; Marini et al., 2012). To better investigate the anti-photoaging effects of CP
and the implications for clinical settings, we measured its effects using a human dermal
fibroblasts (HDF) and human skin equivalent (HSE) model.

Results

Oral administration of CP reduces UVB-induced wrinkle formation and prevents UVB-induced collagen degradation

To investigate the effect of CP on wrinkle formation, the dorsal skins of hairless mice were exposed to UVB with low and high concentrations of CP (CL, 39.1 mg/kg, CH, 156.3 mg/kg) and pycnogenol (Pyc, 625 mg/kg) for 8 weeks as described (Fig. 1A). UVB-induced wrinkle formation was markedly reduced in the CP-administered groups (Fig. 1B). Quantification of skin wrinkle severity through the assessment of the area of wrinkling (Fig. 1C) and visual wrinkle grade (Fig. 1D) confirmed a significant decrease in wrinkle formation in the CP groups. We then stained the skin samples of the mice with Masson’s trichrome staining to observe the effect of CP on amorphous collagens of the skin (Fig. 1E). Collagen levels gradually recovered in the CP groups to an extent greater than the UVB-irradiated group (Fig. 1E, Supplement figure 1). The physical aesthetics of the CP groups were similar or superior to those of the pycnogenol-treated group (Fig. 1 C, D and E). Taken together, these results suggest that oral administration of CP reduces UVB-induced wrinkle formation and prevents UVB-induced collagen degradation.

Expression profiling of differentially-expressed genes (DEGs) mediated by CP supplementation and/or UVB-irradiation of mouse skin tissue

To identify genes associated with the UVB-protective effect of CP supplementation in skin, we systematically analyzed the transcriptome from the mice exposed to UVB-irradiation and/or administrated with CP and pycnogenol. The heat map of differentially expressed genes (DEGs) in the UVB-irradiated mice indicated that 788 genes were up- or down-regulated by at least one concentration of CP supplementation (Fig. 1F). Among the 788 DEGs, 156 genes were up-regulated by UVB compared to control and down-regulated by CH compared to UVB-IR (Fig. 1G); and 199 genes were down-regulated by UVB-IR compared to the controls and up-regulated by CH compared to the UVB irradiated group (Fig. 1H). Supplementation with CH
elicited transcriptomic recovery on the up- and down-regulated genes post-UVB-IR (Fig. 1G and 1H). Furthermore, CP administration shows more impact on transcriptomic recovery than the recovery induced by Pycnogenol (Fig. 1G and 1H), suggesting that CP may be a more potent anti-photoaging agent than Pycnogenol.

**Expression patterns of genes associated with anti-photoaging**

To further characterize gene expression patterns, we identified the genes associated with anti-photoaging effects using the related gene ontology (GO) terms including Extracellular Matrix Disassembly (Fig. 2A), Cell Adhesion (Fig. 2B), Lipid Metabolic Process (Fig. 2C), and Proteinaceous Extracellular Matrix (Fig. 2D and Supplementary Table 1). The gel-like extracellular matrix (ECM) is the largest component of the dermal skin layer and is comprised of a variety of fibrous structural proteins, including collagens, elastin, laminin, and proteoglycans such as dermatan sulfate and hyaluronan (Bradley et al., 2015). Differentially expressed genes in ECM disassembly indicated that CP-fed mice had markedly inverted changes in their UVB-mediated transcriptomes (Fig. 2A). CP significantly diminished UVB-induced cathepsin G (Ctsg) expression. Interestingly, the effect of CP on these expression patterns was more significant than that of pycnogenol (Fig. 3A). Among the various serpin b6 genes, CP supplementation specifically enhanced the expression of serpin b6c (Fig. 3B). These findings suggest that both inhibition of cathepsin G and induction of serpin B6c by CP supplementation may contribute to a protective effect against UVB-induced wrinkle formation. To identify potential mediators of the changes in transcriptome expression patterns, we constructed a Gene Regulatory Network (GRN) analysis composed of the DEGs in Figure 3C, and significantly enriched transcription factors (TFs) obtained from the TF-target relationships derived from the Encyclopedia of DNA Element (ENCODE) Data (Consortium, 2012; Gerstein et al., 2012) and SignaLink database (Fazekas et al., 2013) (Fig. 3D and 3E). Thus, GRN analysis identifies the mediators involved in the anti-photoaging effects of CP.

**CP prevents UVB-induced MMP-1 upregulation in HDF and in HSE layers**
To better understand whether the anti-wrinkling effects of CP in mice could be relevant for clinical settings, we examined the effect of CP on collagenase (MMP-1) in HDF and HSE layers. CP treatment elicited a decrease in MMP-1 protein expression in a concentration-dependent manner and significantly suppressed the mRNA levels of UVB-induced MMP-1 (Fig. 4A and 4B). Furthermore, CP inhibited UVB-induced AP-1 transactivation (Fig. 4C). These inhibitory effects arose within a concentration range that did not significantly affect cell viability in the presence of UVB irradiation (Fig. 4D). These results suggest that CP may downregulate both UVB-induced MMP-1 protein and gene expression through the suppression of UVB-induced AP-1 transcriptional activity in HDF. To confirm the effects of CP on cathepsin G expression, we measured the expression of cathepsin G in HDF. Cathepsin G expression is decreased by CP treatment in HDF (Fig. 4E). Next, to verify whether the anti-wrinkle effect of CP in mice and in vitro could be applied to humans, we examined the effect of CP on collagenase (MMP-1) in HSE as described in supplement Fig 2. Immunohistochemical staining showed that CP markedly inhibited UVB-induced MMP-1 levels with increasing levels of CP (Fig. 4F).

DHPV significantly decreased UVB-induced MMP-1 protein expression, gene transcription and AP-1 transactivation in HDF.

To investigate the metabolite effect of CP on UVB-induced wrinkle formation, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (DHPV) (Fig. 5A), a major metabolite form of CP in the body, was used as shown in the previous study (Urpi-Sarda et al., 2009). The effect of DHPV on UVB-induced MMP-1 protein and gene expression in vitro have been measured (Figure 5B and 5C). DHPV decreased MMP-1 protein expression (Fig. 5B) and significantly suppressed UVB-induced MMP-1 mRNA level (Fig. 5C), compared with those of the UVB-irradiated cells. We also examined the effect of DHPV on AP-1 transcriptional activity induced by UVB irradiation, showing that DHPV suppressed UVB-induced AP-1 transactivation in HDF (Fig. 5D). DHPV inhibits Capthesin G expression similar to that of CP (Fig. 5E). The concentrations of DHPV used in this experiment were not toxic to proliferation of human dermal fibroblast in the presence of UVB irradiation (Fig. 5F). These results indicated that CP metabolite DHPV may act as a driver...
to inhibit UVB-induced wrinkle formation by suppressing MMP-1 protein expression and gene transcription by inhibiting AP-1 activity in HDF.

Discussion

UVB is the major etiological factor of skin photoaging and carcinogenesis. In our previous studies, we measured the minimal UVB dose on the dorsal skin of the mice as the minimal edema dose (MEDD), setting MEDD as 100 mJ/cm². According to study of Bernerd et al., daily dose of UV on earth is 100~200 J/cm² and average ratio of UVA/UVB is 27.3 (Marionnet et al., 2015). Based on the calculation of physiological UVB dose, 200 mJ/cm² of UVB was used in this study: Average UVB dose of about 2 h activity in outside [37.75 J/cm² (UV dose of New York)/28.3(UVB/UV)/14 (day time) = 95 mJ/h cm²].

CP-supplemented diets have been suggested to elicit many beneficial effects, particularly for skin health (Park et al., 2014). In the present study, we investigated the anti-wrinkle effects of CP in vivo. Oral administration of CP reduced UVB-induced wrinkle formation and prevented UVB-induced collagen degradation in hairless mice. We also used a human equivalent skin model and primary human skin fibroblasts, and found that CP inhibits UVB-induced MMP-1 expression in both models. In addition, our clinical study shows that CP (4g/day for 24 weeks) significantly reduces wrinkle formation without side effects (data not shown). To investigate the anti-wrinkling mechanisms of CP, we performed an RNA SEQ array. 788 genes were found to be up- or down-regulated by CP treatment in UVB-irradiated skin tissues. Such significant changes in transcriptome may imply the existence of signature molecules to regulate UV-induced skin aging.

Of particular note, cathepsin G was significantly inhibited whereas serpin b6c was upregulated in the presence of CP. Cathepsin G is known to induce fibronectin fragmentation (Son et al., 2009). It has previously been reported that serpin b6 is a potent inhibitor of cathepsin G (Scott et al., 1999). Although the detailed molecular relationship between skin wrinkling and cathepsin G activity has not been clearly elucidated, cathepsin G activity has in the past been linked to skin wrinkling (Son et al., 2009), and is known to regulate MMP-1 mRNA expression (Son et al., 2012). Our in vivo study shows that CP-supplementation inhibits UVB-induced skin wrinkling...
concurrent with the inhibition of cathepsin G and upregulation of serpin b6c. Cathepsin G expression was similarly inhibited by CP treatment in HDFs.

Intriguingly, we found that the CP-mediated skin response GRN included critical regulators of photoaging in skin such as NFE2L2 (Kawachi et al., 2008; Tian et al., 2011), peroxisome proliferator-activated receptor (PPAR) \( \gamma \) and TP53 (El-Domyati et al., 2013; Lee et al., 2012) (Fig. 3C). NFE2L2 is known as NF-E2-related factor2 (Nrf2) and a transcription activator that binds to antioxidant response (ARE) elements and Maf recognition elements in the promoter regions of target genes. Nrf2 is also important for the coordinated responses to oxidative stress (Itoh et al., 2010; Sykiotis and Bohmann, 2010). UVA strongly induces Nrf2 expression in human skin fibroblasts but is weakly induced in skin keratinocytes. Knockdown of Nrf2 has been shown to markedly increase cell damage by UVA irradiation in skin keratinocytes, suggesting that Nrf2 may protect human skin keratinocytes from UVA radiation-induced damage (Tian et al., 2011). Furthermore, UVB-induced sunburn reactions and oxidative DNA damage have been observed to be more prominent in \( nrf2^{-/-} \) mice (Kawachi et al., 2008). The photoprotective effect of Nrf2 is closely related to the inhibition of ECM degradation and inflammation (Saw et al., 2014). Enhanced Nrf2 activity in keratinocytes has also been associated with epidermal barrier function and antioxidant defense (Schafer et al., 2012). PPARs are a family of nuclear hormone receptors and play key roles in lipid metabolism and glucose homeostasis (Kota et al., 2005; Kuenzli and Saurat, 2003; Lalloyer et al., 2011; Varga et al., 2011). Evidence suggests that PPAR\( \alpha/\gamma \) regulated gene responses have an effect on age-related inflammatory and photoaging mediators such as cytokines, MMPs, and AP-1, in NF-\( \kappa \)B signaling (Chung et al., 2008; Kim et al., 2012; Michalik and Wahli, 2007). In addition, the tumor suppressor gene p53 plays an important role in protecting cells against DNA damage from sources of extrinsic stress (Nelson and Kastan, 1994). An earlier study has examined the significant impact of UVB on p53 (van Kranen et al., 1997). Moreover, p53 is known to be activated by DNA damage, oxidative stress and inflammation (Ak and Levine, 2010; Han et al., 2008; Nelson and Kastan, 1994; Reuter et al., 2010), and has recently been identified as a UV target gene that associates with \( V_{600E}^{\text{BRAF}} \) to induce melanoma formation (Viros et al., 2014). These genes could be novel candidates responsible for the CP-mediated UVB-protective effects
observed. We also constructed a GRN composed of possible candidate TFs regulating cathepsin G and serpin b6c based on the unfiltered TF-target relationships in the ENCODE data. This GRN analysis suggested that the expression of these genes may be regulated by other transcription factors such as SPI1 and MAFK.

When human beings take in CP orally, it is then metabolized in the body. Namely, CP is changed into its metabolite and interacts with skin cells in form of metabolite. Various metabolites occur at digestion and absorption by CP consumption. According to the previous study (Urpi-Sarda et al., 2009), DHPV is mainly produced in plasma and appears the biggest variation after regular consumption of CP compared with before consumption of CP. Epicatechin and procyanidins which are major constituent in CP are metabolized into DHPV (Urpi-Sarda et al., 2009). We, then, hypothesized that the anti-wrinkle effect of CP may be derived from DHPV. As a result, DHPV suppressed UVB-induced MMP-1 protein expression and gene transcription by inhibiting AP-1 activity as the same as the effect of CP on those in HDF. DHPV, therefore, was considered to be active compound based on these results. Further studies should validate the various metabolites of CP of their effects on skin structure and developments.

Taken together, our studies indicate that CP supplementation contributes to a reduction in wrinkle formation and collagen degradation. Transcriptomic changes in response to UVB-irradiation in CP-supplemented mice provide evidence for an anti-photoaging effect of CP extract. Oral treatment of CP significantly down-regulates cathepsin G while up-regulating serpin b6c, which itself is known to inactivate cathepsin G. Therefore, CP supplementation may prevent breakdown of the dermal matrix. For clinical application, we examined the effects of CP and its major metabolites DHPV on photoaging in HDF. These results underline the potential for CP extracts to be further developed as anti-photoaging agents.

Materials and Methods

Preparation of CP

CP was provided by Barry Callebaut (Lebbeke-Wieze, Belgium). Cacao beans were roasted and ground to make cacao liquor, which was separated with cacao butter, to produce
cacao cakes. CP was produced by grinding the cacao cakes. The flavanol content in the resultant CP was 71.5 mg/g, which was determined independently by the Korea Health Supplement Institute (Gyeonggi-do, Korea). CP was dissolved in 0.5% sodium carboxymethylcellulose for animal treatment and in 50% ethanol for cell treatment.

**Animals and treatments**

Six-week-old female albino hairless mice (Skh-1) were obtained from Bio Genomics, Inc. (Seoul, Korea). All experimental protocols were approved by the Institutional Animal Care and Use Committee (Case Number: 14-0008-S1A0) of the Biomedical Research Institute at Seoul National University Hospital. Groups of 8-10 mice were allocated to receive one of six treatment types. CP or a positive control were administered to the mice according to the following treatments groups: CP Low (39.1 mg/kg of CP), CP High (156.3 mg/kg of CP), and pycnogenol (positive control, 625 mg/kg of pycnogenol). CP and vehicle (0.5% sodium carboxymethylcellulose) were orally administered for 8 weeks, and body weight and food intake were monitored on a weekly basis. A photoaging experiment was also performed, as described previously (Kim et al., 2005). A UVB irradiation device containing TL20W/12RS UV lamps (Philips, Eindhoven, Netherlands) with an emission spectrum between 275 and 380 nm (peak, 310–315 nm) served as the UV source. A Kodacel filter (TA401/407; Eastman Kodak, Rochester, NY) was mounted 2 cm in front of the UV lamp to remove wavelengths of less than 290 nm (UVC) (Seo et al., 2001). Initially, we measured the minimal UVB dose on the dorsal skin of the mice as the MED comparable with a minimal erythema dose in human skin. In contrast to human skin, mouse skin showed peak responses to UVB primarily as edema, manifesting as an increased thickness of dorsal skin at 48 hours post-UVB irradiation (Benavides et al., 2009; Learn et al., 1995). The irradiation doses were increased weekly in increments of 0.5 MED (1 MED = 100 mJ/cm²) up to 2 MED and then maintained at 2 MED thereafter. UVB irradiation was stopped after 8 weeks (Fig. 1A) (Jin et al., 2010; Kim et al., 2013; Park et al., 2014; Yoon et al., 2014).

**RNA sequencing analysis**
For the transcriptome analysis, frozen skin tissue was pulverized in liquid nitrogen, and total RNA was isolated using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The integrity of the RNA was assessed by 1% agarose gel electrophoresis and visualization of the 18S and 28S RNA species after ethidium bromide staining. RNA sequencing was performed by TheragenEtex Inc. DEGs in CL- and CH-fed mice skin irradiated with UVB were identified by comparing with the normal diet-fed mice irradiated with UVB using both 2-fold criteria and P<0.05 value cut-offs obtained from student’s t-test. We then amalgamated the two DEG groups (in CL- and CH-fed mice) into CP-mediated DEGs.

**GRN analysis**

We constructed the CP-mediated skin response GRN by retrieving a reliable human GRN from the study by Gerstein et al. (Gerstein et al., 2012) and SignaLink Version 2 (Fazekas et al., 2013) as follows: The significant TFs for the human homologs of the CP-mediated DEGs for were obtained by performing hypergeometric tests between the DEGs and a target gene group for a TF using both the P< 0.05 criteria and the size of the intersection between the DEG group and a target gene group > 1 criteria. We then amalgamated the significant TFs and their target genes into the CP-mediated skin response GRN. The CP-mediated skin response GRN was composed of two isolated connected components (Fig. 3C and 3D).

**Cell culture and treatments**

Primary human dermal fibroblasts (HDFs) were isolated from the outgrowth of foreskin obtained healthy 12 year-old volunteers with the approval of the Institutional Review Board of Seoul National University Hospital (Approval No. H-1101-116-353) and Seoul National University (No. E1408/001-002). HDFs were cultured in DMEM with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin at 37°C and 5% CO₂. Serum-starved monolayer cultures of HDF were exposed to UVB irradiation and treated with CP dissolved in 50% ethanol. HDFs were exposed to UVB at a dose of 0.02 J/cm² using a UVB source (Bio-Link crosslinker, VilberLourmat, Cedex 1, France) with a spectral peak set at 312 nm.
Preparation of human skin equivalent model

Neoderm®-ED, a human skin equivalent model, was purchased from TEGO Science (Seoul, Korea). Briefly, HDFs were cultured onto a collagen matrix for 1 day, before keratinocytes were seeded on top of the matrix and co-cultured for 4 days. Next, the keratinocytes and HDF block were raised for exposure to the air. CP was treated for 1 h after 12 days of air exposure. The human skin equivalent layer was then irradiated with 0.05 J/cm² UVB twice per day for 8 days. Media was changed every 2 days, and the layer was incubated at 37°C with 5% CO₂.

Assessment of wrinkle formation

To determine the severity of wrinkling, each hairless mouse was anesthetized and their UVB-exposed dorsal skin (wrinkle formation area) was photographed. The severity of wrinkling was measured by four trained graders using the Bissett’s visual wrinkle scale (Bissett et al., 1987). A skin wrinkle replica was made with silicone rubber (Silflo Dental Impression Materials, Potters Bar, UK) from the backs of untreated mice. This was photographed using a coupling charge system video camera. Wrinkle severity was assessed using a photographic scale (0, none; 1, minimal; 2, mild; 3, moderate; 4, severe; and 5, very severe) and analyzed by Skin-Visiometer SV 600 software (CK Electronic GmbH, Köln, Germany). The visiometer consists of a computerized instrument that creates a skin microrelief map from the replica using a light transmission method.

General laboratory experiments such as RT-PCR, Western Blotting Masson’s trichrome staining, immunohistochemistry and luciferase reporter gene assay are described in supplement materials and methods.

Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Duncan’s statistical range test. P values of less than 0.05 were considered statistically significant.

Conflict of Interest
The authors state no conflict of interest

Acknowledgement
This work was supported by the R&D program of MOTIE/KIAT (Establishment of Infrastructure for Anti-aging Industry Support, No.N0000697) in Republic of Korea. We thank Jieun H. Kim for proofreading the manuscript and Dr. Akira Oshima for helping histological analysis.

References


Kota BP, Huang TH, Roufogalis BD. An overview on biological mechanisms of PPARs.


Lee KW, Kim YJ, Lee HJ, Lee CY. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. J Agric Food Chem 2003;51:7292-5.


Scott FL, Hirst CE, Sun J, Bird CH, Bottomley SP, Bird PI. The intracellular serpin proteinase inhibitor 6 is expressed in monocytes and granulocytes and is a potent inhibitor of the azurophilic granule protease, cathepsin G. Blood 1999;93:2089-97.


Varga T, Czimmerer Z, Nagy L. PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. Biochim Biophys Acta 2011;1812:1007-22.


Figure Legends

Figure 1. Effect of cacao powder (CP) on UVB-induced wrinkle formation. (a) A schematic diagram of the animal experiment. (b) Back skins of hairless mice (8-10 mice per each group) were exposed to UVB for 8 weeks as scheduled in (A). Bottom figures are replica from back of mice as developed in materials and methods (c) Skin impressions were analyzed by Skin-Visiometer software after 8 weeks of treatment. (d) The severity of skin wrinkling was visually graded as described in the Materials and Methods after 8 weeks of treatment. Data represent the means ± SEM (n = 8-10). Means with letters (a-c) within a graph are significantly different from each other at p<0.05. (e) Masson's trichrome staining for collagen fibers. Collagen fibers appear blue. Scale bar = 100 µm. (A) The vehicle was 0.5% sodium carboxymethylcellulose. (B) UVB, (C) UVB+ CL (low concentration of CP), (D) UVB+CH (high concentration of CP) and (E) UVB+Pyc (Pycnogenol). (f) Expression profile of differentially expressed genes (DEGs) mediated by CP in mouse skin tissue. Heat map of DEGs mediated by low or high CP concentration. Expression of genes represent log2 ratio of indicated group. (g) Heat map of DEGs up-regulated by UVB and down-regulated by high concentrations of CP. (h) Heat map of DEGs down-regulated by UVB and up-regulated by high concentrations of CP. Heat maps (g and h) are drawn based on Log2 FPKM of each group. Blue color indicates low expression of genes in each group while red color represents high level of gene expression.

Figure 2. Expression of genes involved in dermal matrix formation, following GO terms. Expression profiles of DEGs were classified by GO terms as follows (a) Extracellular Matrix Disassembly, (b) Cell Adhesion, (c) Lipid Metabolic Process, and (d) Proteinaceous Extracellular Matrix. Bar-graph shows the relative log2 mRNA ratio of the indicated targets from RNA sequence data of each group with statically significance (P<0.05).
Figure 3. CP regulates the expression of extracellular matrix (ECM) genes. (a) CP supplementation significantly inhibits expression of cathepsin G in UVB-irradiated skin tissue. (b) The expression of Serpin B6c is markedly enhanced by CP supplementation. The graph is representative of the RNA sequence analyses from five mice. Bars followed by the same letter do not differ significantly (P<0.05). (c) The largest connected component of CP-mediated skin response Gene Regulatory Network (GRN). (d) The second largest connected component of the GRN in response to UVB and CP administration. GRNs were constructed as described in materials and methods. (e) Candidates of transcription factors (TFs) that regulate the specific target genes such as Cathepsin G, Serpin B6c, Collagen 25A1 (COL25A1), and Fibronectin 1 (FN1). Hexagon nodes with green borders denote TFs. Red and blue nodes denote up-regulated and down-regulated genes by high concentrations of CP, respectively.

Figure 4. CP inhibits UVB induced MMP-1 expression in human dermal fibroblast (HDF) and human skin equivalent (HSE). (a) CP inhibits the expression of UVB-induced MMP-1. MMP-2 is used as a loading control. (b) MMP-1 mRNA levels for the CP group are analyzed by real-time quantitative PCR (RT-qPCR). Data (n = 3) represent the means ± SD. (c) Regulation of AP-1 transcriptional activity by CP. A luciferase reporter gene assay was performed in HDF as described in the materials and methods. (d) Cell viability after CP treatment. Cell viability is measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay. (A-D) HDF cells are pretreated with CP at the indicated concentrations for 1 h, and then further treated with 0.02 J/cm² UVB for 48 h at 37 °C. Data (n = 4) represent the means ± SD. Means with letters (a-e) within a graph are significantly different from each other at p<0.05. (e) Cathepsin G expression is inhibited by CP. Treatment of CP and UVB is the same as described in (a-d). (f) CP inhibits UVB-induced MMP-1 protein expression in HSE. HSE was developed as described in Schematic diagram of HSE system (Supplement figure 2). HSE serial sections from the human skin equivalent were mounted onto silane-coated slides and subjected to immunohistochemical staining using anti-MMP-1 antibody as described in the Materials and Methods. Scale bar = 50 µm,
Figure 5. A major metabolite, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (DHPV) inhibits UVB-induced MMP-1 protein expression, gene transcription and AP-1 transactivation in HDF. (a) Chemical structure of DHPV. (b) Expression of MMP-1 was inhibited by DHVP under UVB-IR in HDF. MMP-2 was used as a loading control. (c) Suppression of MMP-1 mRNA expression by DHVP was analyzed by RT-qPCR. Triplicate samples were used and experiments were repeated three times; the mean ± SD. (d) The effect of DHVP on AP-1 transcriptional activity in HDF. AP-1 transactivation ability was measured by a luciferase reporter gene assay. Data (n = 3) represent the mean ± SD. (c-d) Means with letter (a-c) within a graph are significantly different from each other at p<0.05. (e) Inhibition of UVB-induced Cathepsin G expression by DHPV. (f) DHVP did not affect cell viability. Data (n = 8) represent the mean ± SD. Means with letter (a) within a graph are no significantly different from each other at p < 0.05. (b-f) Conditions of treatment of DHPV and UVB in HDF are the same as described in Fig. 4.
Figure 3

(a) Bar graph showing relative mRNA expression levels of Cathepsin G with treatments CL, CH, and Pyc in UVB exposure.

(b) Bar graph showing relative mRNA expression levels of Serpin B6c with treatments CL, CH, and Pyc in UVB exposure.

(c) Network diagram illustrating various proteins and their interactions in the context of UVB exposure.

(d) Zoomed-in network diagram highlighting specific proteins and interactions.

(e) Another zoomed-in network diagram with a different focus on protein interactions.
Figure 5

(a) Molecular structure of DHPV.

(b) Western blot analysis of MMP-1 and MMP-2.

(c) Graph showing relative MMP-1 mRNA levels.

(d) Graph showing relative AP-1 transactivation.

(e) Western blot analysis of DHPV and Cathepsin G.

(f) Graph showing relative cell viability.