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Research Article

Syringaresinol derived from *Panax ginseng* berry attenuates oxidative stress-induced skin aging via autophagy

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ABSTRACT

Background: In aged skin, reactive oxygen species (ROS) induces degradation of the extracellular matrix (ECM), leading to visible aging signs. Collagens in the ECM are cleaved by matrix metalloproteinases (MMPs). Syringaresinol (SYR), isolated from *Panax ginseng* berry, has various physiological activities, including anti-inflammatory action. However, the anti-aging effects of SYR via antioxidant and autophagy regulation have not been elucidated.

Methods: The preventive effect of SYR on skin aging was investigated in human HaCaT keratinocytes in the presence of H_2O_2, and the keratinocyte cells were treated with SYR (0–200 μg/mL). mRNA and protein levels of MMP-2 and -9 were determined by real-time PCR and Western blotting, respectively. Radical scavenging activity was researched by 2,2 diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays. LC3B level was assessed by Western blotting and confocal microscopy.

Results: SYR significantly reduced gene expression and protein levels of MMP-9 and -2 in both H_2O_2-treated and untreated HaCaT cells. SYR did not show cytotoxicity to HaCaT cells. SYR exhibited DPPH and ABTS radical scavenging activities with an EC_{50} value of 10.77 and 10.35 μg/mL, respectively. SYR elevated total levels of endogenous and exogenous LC3B in H_2O_2-stimulated HaCaT cells. 3-Methyladenine (3-MA), an autophagy inhibitor, counteracted the inhibitory effect of SYR on MMP-2 expression.

Conclusion: SYR showed antioxidant activity and up-regulated autophagy activity in H_2O_2-stimulated HaCaT cells, lowering the expression of MMP-2 and MMP-9 associated with skin aging. Our results suggest that SYR has potential value as a cosmetic additive for prevention of skin aging.

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1. Introduction

Skin consists of two main layers, the epidermis and dermis. During skin aging processes, degenerative changes of the dermis are more pronounced than those of the epidermis. The dermis is composed of keratinocytes and extracellular matrix (ECM) such as collagen, elastin, and proteoglycans [1]. With the aging of skin, the dermis loses ECMs, particularly collagen [2–4]. One of the main mechanisms for reducing ECM levels in the dermis is activation of matrix metalloproteinases (MMPs). MMPs are zinc-dependent endopeptidases composed of four subgroups (collagenases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs), cleaving ECM proteins and are functionally classified into [5]. MMP-2 and -9 belong to the gelatinase group and are involved in the breakdown of collagen and elastic fibers [6,7]. MMPs are increased

Abbreviations: ROS, reactive oxygen species; PCR, polymerase chain reaction; ECM, extracellular matrix; MMP, matrix metalloproteinase; SYR, Syringaresinol; DPPH, 2,2 diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; 3-MA, 3-Methyladenine; UV, ultraviolet; CMA, chaperone-mediated autophagy; FoxO3a, Forkhead box O3; MAPK, mitogen-activated protein kinase; DMEM, Dulbecco's Modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide.

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in both intrinsically aged skin and in photoaged skin caused by prolonged exposure to ultraviolet (UV) light [8,9]. Therefore, lowering MMP levels might be a promising approach for anti-aging.

Autophagy, derived from the Greek word "self-eating," is a set of highly conserved cellular mechanisms by which unnecessary or dysfunctional organelles and cellular components are broken down and recycled in response to nutrient or stress conditions [10]. Macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) are classified types of autophagy process [11]. Macroautophagy forms autophagosomes with double layered-membrane structures that engulf cellular cargo [12,13]. Formation of autophagolysosomes is generated by fusion of autophagosomes with lysosomes [14,15]. Autophagy plays a role in various normal cellular functions, including cellular homeostasis maintenance, differentiation [16], and immune responses [17]. Dysregulation of autophagy can contribute to diverse pathologies, including cancer, neurodegeneration, aging, and heart disease [18]. Defects of autophagy also are associated with skin aging. The role of autophagy in skin aging is understood mainly in terms of regulating proteostasis, but its precise function remains to be elucidated [19]. Nevertheless, attempts to increase autophagy in senescent skin are warranted for anti-aging.

Panax ginseng has numerous therapeutic biochemical and pharmacological effects such as immunomodulatory [20], anti-inflammatory [21,22], antioxidative [23], anti-cancer [24,25], anti-fatigue [23], and anti-diabetic effects [26]. Based on these therapeutic effects, various parts and active pharmacological components of ginseng, including root, berry, and ginsenosides, have been used as functional foods [27]. Syringaresinol (SYR) (Fig. 1A Left panel), a plant lignan, is one of the biologically active ingredients present in ginseng berry [28]. SYR has anti-inflammatory [29], anti-fungal, and anti-cancer activities [30]. In addition, in skin-related studies, SYR has been reported to decrease the activity of Forkhead box O3 (FoxO3a) and mitogen-activated protein kinase (MAPK), which are aging-related factors [31,32]. However, the regulatory roles of SYR in aging process of skin are not fully elucidated. Therefore, in the present study, the beneficial effects of SYR derived from *Panax ginseng* on skin aging and the underlying biological mechanism for these effects, particularly those on antioxidant and autophagy regulation were looked over.

2. Materials and methods

2.1. Materials

HaCaT skin keratinocyte cells (American Type Culture Collection (ATCC) (Rockville, MD, USA) was used. Antibiotics (penicillin-streptomycin solution) was purchased from Hyclone (Logan, UT, USA). 3-Methyladenine (3-MA, Fig. 1A Left panel), 3-(4-5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The cDNA synthesis kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Primers for determining the mRNA levels of *MMP-2* and *-9* were from Bioneer (Seoul, Korea). Antibodies against MMP−2, −9, LC−3B, and β-actin were employed, as reported previously [33]. SYR (Fig. 1A Left panel) was purified from *Panax ginseng berry* as reported previously [34]. The purity of the compound was >80% (Fig. 1A Right panel). A stock solution of SYR was prepared with 100% DMSO.

2.2. Cell culture

Keratinocyte HaCaT cells were cultured in DMEM with 10% FBS and 1% antibiotics (penicillin and streptomycin) in a 5% CO_2 incubator at 37 °C.

2.3. RNA extraction and quantitative polymerase chain reaction

HaCaT cells (2×10^5 cells/ml) plated for 18 h were exposed with H_2O_2 (500 μM) or SYR (100 or 200 μg/mL). After 24 h, total RNA was prepared by general extraction method with TRIzol reagent as carried out before [35]. For synthesizing complementary DNA, a cDNA synthesis kit was used, and quantitative polymerase chain reaction was performed with Pcrbio's qPCRBIO SyGreen mix [36]. Primer sequences of MMP-2 and -9 were summarized in Table 1.

2.4. Preparation of cell lysates and immunoblotting analysis

Total lysates of HaCaT cells were obtained using lysis buffer as reported previously [37]. The prepared whole cell lysates were tested after clarification by centrifugation at 12,000 rpm for 1 min at 4 °C. Immunoblotting analysis was used to detect levels of MMP2, MMP9, LC-3B, and β-actin. Each antibody was treated to the PVDF membrane in 3% BSA in TBST at a ratio of 1:2500 at 4 °C. After 18 h, the second antibodies (anti-mouse or anti-rabbit antibodies) were reacted for 1 h at a ratio of 1:2500 at 20 °C. Immunoreactive bands were detected with enhanced peroxidase detection (EPD) of ELPIS-BIOTECH in Chemidoc of ATTO.

2.5. Cell viability assay

Whether SYR induces death of HaCaT cells was elucidated by a conventional MTT assay [38].

In 2×10^5 of HaCaT cells, SYR (12.5–200 μg/mL) was treated for 24 h.

2.6. DPPH and ABTS colorimetric assays

Either SYR (12.5–200 μg/mL) or ascorbic acid (250 μM) was reacted with 300 μM DPPH or 740 μM ABTS for 15 min at 37 °C. Then, the absorbance of each mixture was observed at 517 nm or 730 nm with spectrophotometer. The radical scavenging activity was presented as percent inhibition using the formula: Radical scavenging activity (%) = [(A0-A1)/A0] x 100, where A0 is the absorbance of DPPH or ABTS alone, and A1 is the absorbance of the sample.

2.7. Confocal microscopy

HaCaT cells (5×10^4 cells/mL) were transfected with 1 μg/mL of a GFP-LC3B plasmid using transfection reagent, lipofectamine. After 24 h incubation, the cells were exposed to H_2O_2 (500 μM) or SYR (200 μg/mL) for 12 h. Then, the cells were fixed with 4% paraformaldehyde (PFA) in PBS for 10 min, followed by treating Triton X-100 (0.5%) in PBS for 10 min for permeabilization of the cell membrane. The cells were continuously treated with PBS containing 1% BSA for 1 h at 20 °C for blocking. DNA staining was carried out by adding PBS with 10 μg/mL Hoechst 33342 for 10 min. After mounting on glass slides, images of the cells were observed using a laser-scanning confocal microscope (Zeiss LSM 710 META, Oberkochen, Germany) with a 63x oil-immersion objective lens.

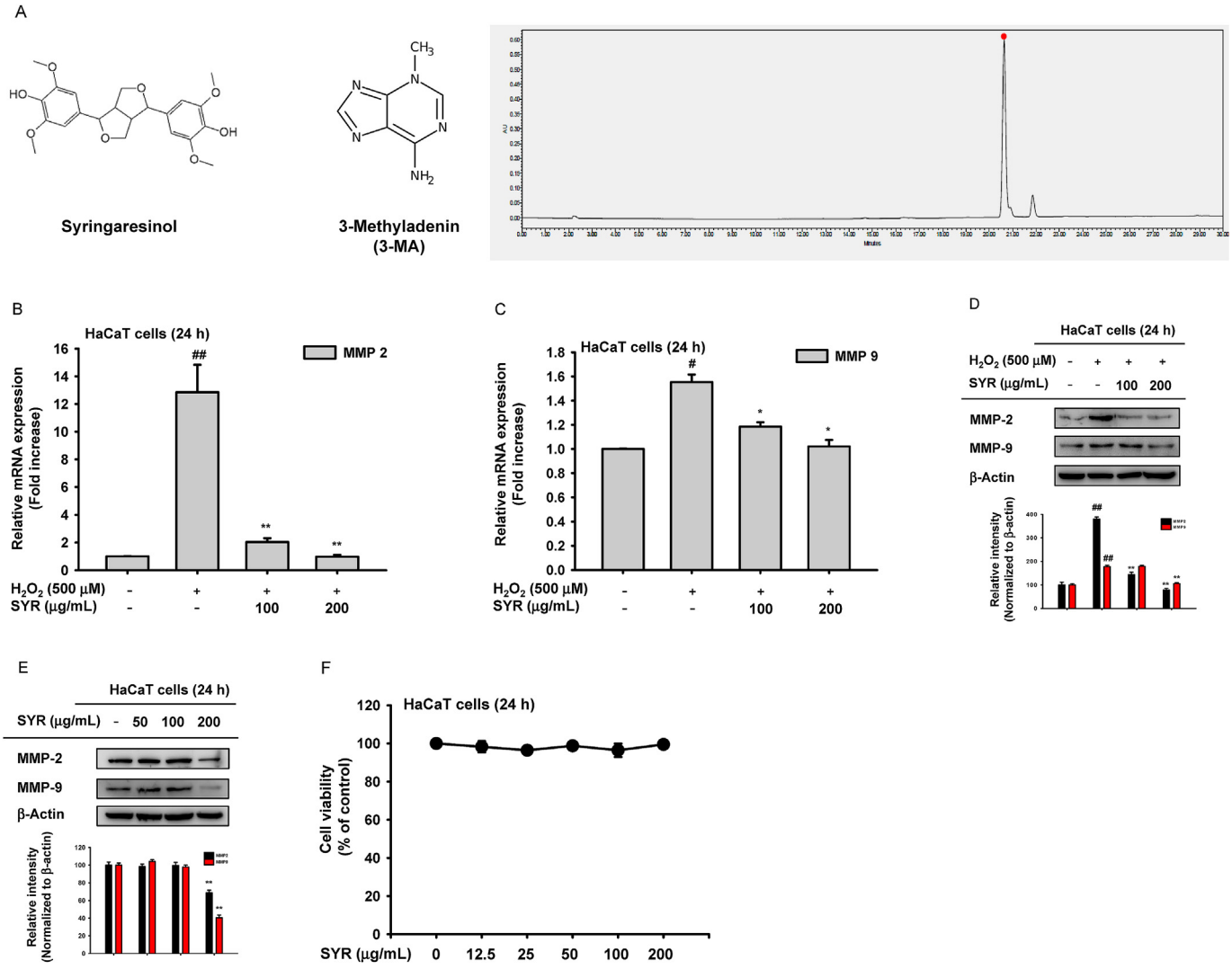

Fig. 1. Anti-aging activity of SYR in HaCaT cells. (A) Chemical structure of syringaresinol and 3-Methyladenin (Left panel) and analysis profile of syringaresinol by HPLC (Right panel). (B, C, and D) Following SYR (100 and 200 μg/mL) treatment, HaCaT cells were incubated with H_2O_2 (500 μM) for 24 h mRNA levels of MMP-2 (B) and MMP-9 (C) were determined by real-time PCR. Protein levels of MMP-2 and MMP-9 were measured by Western blotting (D). (E) HaCaT cells were treated with various concentrations (0–200 μg/mL) of SYR and then incubated for 24 h. Protein levels of MMP-2 and MMP-9 were determined by immunoblotting analysis. (F) Viability of SYR-treated HaCaT cells was measured by MTT assay. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the normal group; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the control group.

Table 1
Primer list used in this study.

Gene	Direction	Sequences
MMP-2	F	CAAGTGGAGAGCAGTTGAGGACATC
	R	TGAGGACATCTCCCACGTCAA
MMP-9	F	GCCACTTGTCGGCGATAAGG
	R	CACTGTCCACCCCTCAGAGC
GAPDH	F	CACTCACGGCAAATTCAACGGCA
	R	GACTCCACGACATACTCAGCAC

F: Forward, R: Reverse.

2.8. Statistical analysis

PCR and Western blot analyses were performed three independent experiments with each experimental group. Band intensity was determined by ImageJ. For MTT, DPPH, and ABTS assays, two separate experiments were carried out. All data in this study are presented as mean ± standard deviation (SD) obtained from each experiment. Mann-Whitney test was used to evaluate significance of each data. Statistical significance was judged with a p value less than 0.05.

3. Results

3.1. Anti-aging activity of SYR in HaCaT cells

Regardless of intrinsic or extrinsic aging, ROS functions a significant role in the skin aging process, and the generated ROS activate MMPs to accelerate skin aging [39–41]. Therefore, anti-aging activity of SYR was investigated by measuring changes of MMP-2 and -9 in hydrogen peroxide (H_2O_2)-treated HaCaT cells. Gene expression and protein levels of MMP-2 were found to be meaningfully enhanced in response to H_2O_2 (Fig. 1B and D). MMP-9 expression also was increased in H_2O_2-stimulated cells, although to a lesser extent than MMP-2 (Fig. 1C and D). SYR (100 and 200 μg/mL) reduced the MMP-2 and MMP-9 mRNA levels that were elevated by H_2O_2 (Fig. 1B and C). Consistently, protein levels of MMP-2 and MMP-9 was downregulated in H_2O_2-treated cells

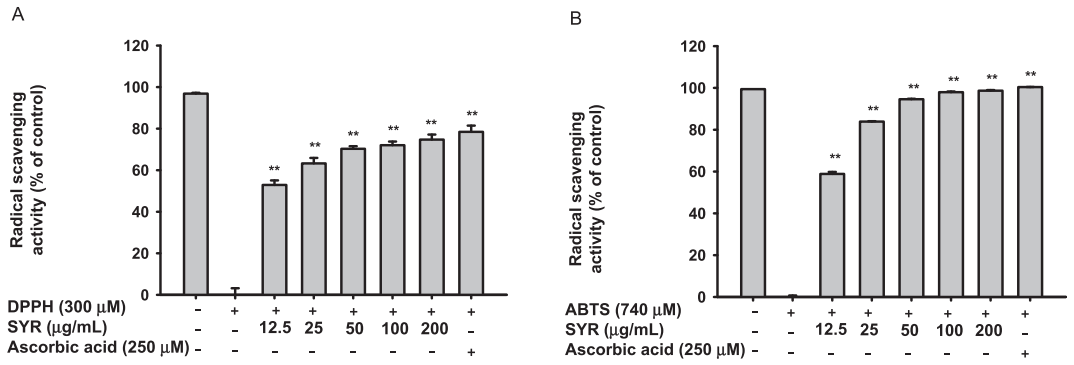

Fig. 2. Antioxidant activity of SYR. (A) DPPH and SYR (0–200 μg/mL) were incubated together at 37 °C for 30 min. Absorbance at 517 nm was measured by spectrophotometry. (B) SYR (0–200 μg/mL) was mixed with ABST in the dark at 37 °C for 30 min. Absorbance at 730 nm was observed by spectrophotometry. *$p < 0.05$ and **$p < 0.01$ compared with the control group.

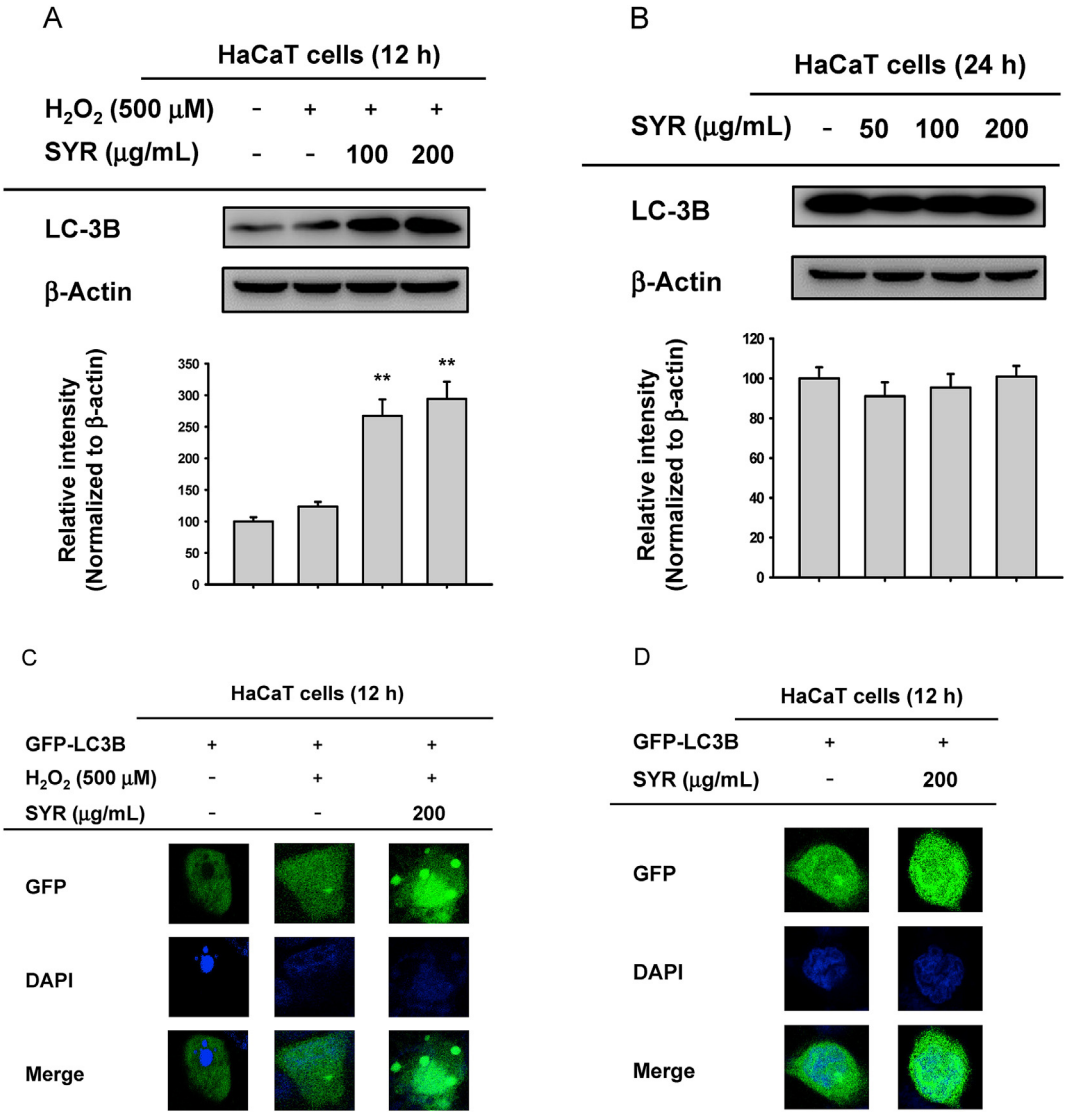

Fig. 3. Effect of SYR on autophagy activation. (A) SYR (100 and 200 μg/mL)-treated HaCaT cells were incubated with H_2O_2 for 12 h. Then, the total level of LC3B was determined by Western blotting. β-Actin was used as a loading control. (B) HaCaT cells were treated with SYR (50, 100, and 200 μg/mL) for 24 h, and then the total levels of LC3B and β -actin were determined by immunoblotting analysis. (C and D) HaCaT cells were transfected with a plasmid expressing GFP-LC3B and incubated with 200 μg/mL SYR and H_2O_2 for 12 h (C). HaCaT cells were transfected with plasmid GFP-LC3B and incubated with 200 μg/mL SYR for 24 h (D). Confocal microscopy images were observed using a laser-scanning confocal microscope (Zeiss LSM 710 META). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the normal group; *$p < 0.05$ and **$p < 0.01$ compared with the control group.

(Fig. 1D). SYR at a concentration of 200 μg/mL reduced MMP-2 and MMP-9 expression even in the absence of H_2O_2 stimulation (Fig. 1E). Cell viability was not affected by SYR (Fig. 1F), indicating that inhibition of MMP-2 and MMP-9 was not due to cytotoxicity.

3.2. In vitro antioxidant activity of SYR

Antioxidant activity of SYR was analyzed by DPPH and ABTS assays, which are broadly used methods for determining antioxidant activity of a wide range of plant components. The DPPH radical scavenging activity was dose-dependently enhanced with treatment of SYR compared with the blank group, and the lowest half-maximal effective concentration (EC_{50}) was 10.77 μg/mL (Fig. 2A). SYR also significantly increased the ABTS radical scavenging activity, and the EC_{50} against ABTS radicals was 10.35 μg/mL (Fig. 2B). In addition, SYR had a radical scavenging activity similar to that of ascorbic acid (250 μM), a strong antioxidant, indicating that SYR has potent antioxidant activity.

3.3. Activation of autophagy by SYR in HaCaT cells

To investigate whether SYR is participated in autophagy regulation, we detected he autophagy marker LC3B using Western blotting and confocal microscopy. SYR (100 and 200 μg/mL) significantly increased LC3B expression in H_2O_2-treated HaCaT cells, and SYR (50, 100, and 200 μg/mL) did not affect LC3B expression in HaCaT cells without H_2O_2 (Fig. 3A and B). In parallel, exogenous LC3B expression was upregulated by SYR (200 μg/mL) in H_2O_2-stimulated HaCaT cells but not in H_2O_2-free HaCaT cells (Fig. 3C and D).

3.4. The association between anti-aging activity of SYR and autophagy activation

The relevance of autophagy activation to anti-aging was assessed in recovery experiments with 3-MA, an autophagy inhibitor. SYR (200 μg/mL) inhibited the MMP-2 expression elevated by H_2O_2, but the decreased MMP-2 level was restored in the 3-MA-treated group (Fig. 4A). However, the decreased MMP-2 expression by SYR was not affected by 3-MA in HaCaT cells without H_2O_2 (Fig. 4B).

4. Discussion

As the amount of UV light increases due to the recent deterioration of the ecological environment, methods to prevent or delay skin aging are receiving increased attention [42]. In addition, there is growing demand for natural, herbal cosmetics because of their weaker or absent side effects [43]. In this study, we investigated the feasibility of using SYR as an anti-aging material. Since degradation of the epidermal and dermal extracellular matrix during skin aging promotes visible signs such as wrinkle formation, the anti-aging activity of SYR was assessed in terms of MMP-2 and MMP-9 regulation. SYR (200 μg/mL) inhibited the expression of MMP-2 and -9 at mRNA and protein levels regardless of the presence of H_2O_2 in human keratinocyte HaCaT cells (Fig. 1B–E), which indicates SYR has skin anti-aging activity.

Antioxidants such as ascorbic acid, tocopherols, and polyphenols increase resistance to oxidative stress, preventing and improving skin aging [44]. Most plant compounds have antioxidant activity as part of their physiological adaptation to a highly oxidized environment [45], and these compounds exert anti-aging activity via antioxidant properties. SYR also exhibited strong antioxidant activity similar in potency to that of ascorbic acid (Fig. 2A and B). This suggests the antioxidant activity of SYR is an effective mechanism for preventing aging.

In addition, SYR significantly activated autophagy in H_2O_2-treated HaCaT cells (Fig. 3A and C). While the relevance of autophagy to the aging process is not yet fully understood, we directly identified the relationship between the anti-aging activity of SYR and autophagy. The inhibitory activity of SYR on MMP-2 was reduced under conditions in which autophagy activity was

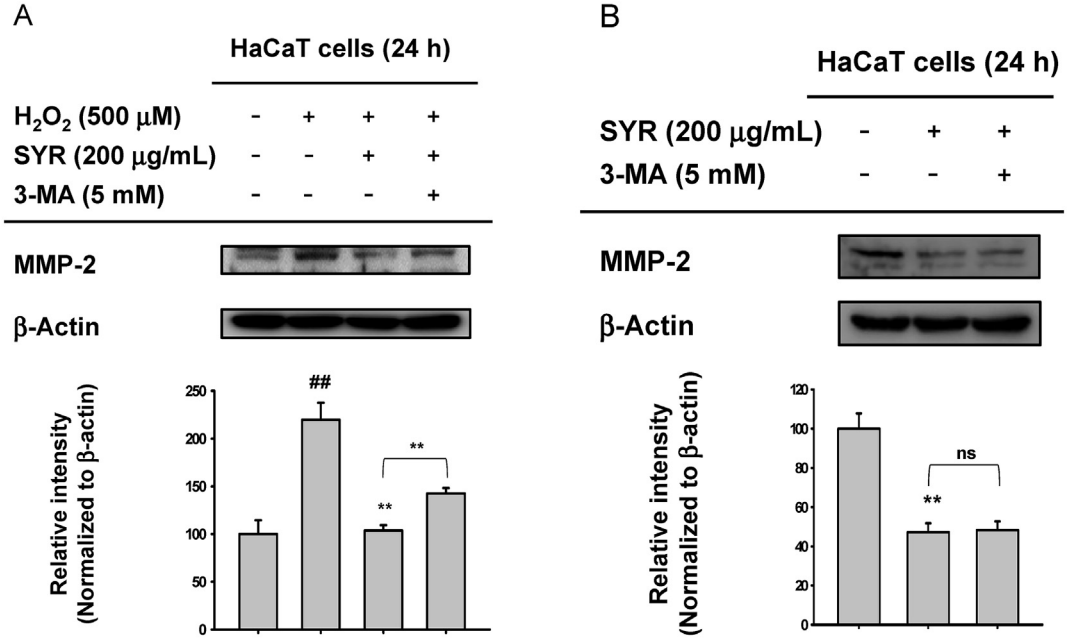

Fig. 4. Relevance of autophagy activation to the anti-aging activity of SYR. (A and B) HaCaT cells were incubated with H_2O_2 (500 μM) in the presence or absence of SYR (200 μg/mL) or 3-MA (5 mM) for 24 h (A). HaCaT cells were treated with SYR (200 μg/mL) in the presence or absence of 3-MA (5 mM) for 24 h (B). The expression of MMP-2 and β-actin was determined by Western blotting. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the normal group; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the control group; ns, not significant.

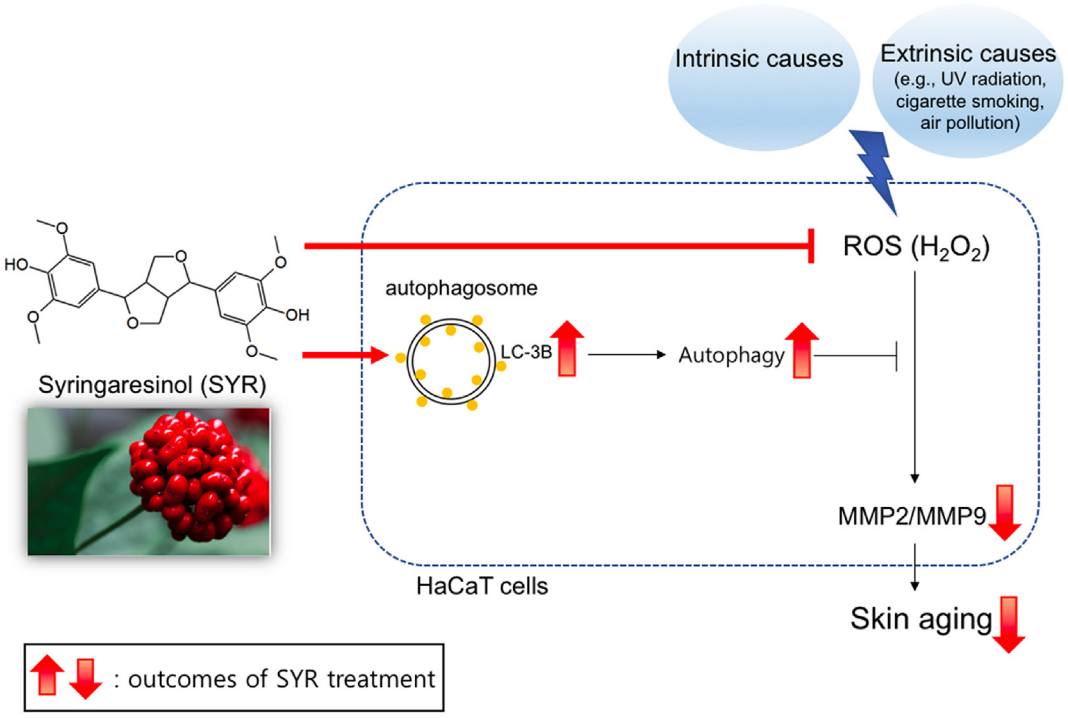

Fig. 5. Schematic summary of the anti-aging effects of SYR and its mechanisms.

suppressed by 3-MA (Fig. 4A), suggesting that autophagy activation by SYR is another important mechanism for its anti-aging activity. Autophagy generally is thought to delay aging by maintaining homeostasis through recycling mechanisms, but the role of autophagy has been expanding. For example, autophagy suppresses NF-κB signaling in the inflammatory response [46]. Given that SYR inhibits MMP-2 and -9 expression at the transcription level, and that NF-κB is an essential transcription factor for MMP expression (Fig. 1B–D) [47,48], autophagy might exert anti-aging effects by regulating transcription factors such as NF-κB. Interestingly, SYR did not activate autophagy in cells without H_2O_2, and the inhibitory effect of SYR against MMP-2 was not affected by 3-MA. These results imply that the anti-aging mechanism of SYR could vary depending on the stimulant.

5. Conclusion

Collectively, in this study, we looked over the preventive activity of SYR on skin aging. SYR showed antioxidant activity and up-regulated autophagy activity in H_2O_2-stimulated HaCaT cells, thereby decreasing the mRNA expression of MMP-9 and MMP-2 in relation to skin aging (Fig. 5). Therefore, we propose SYR as a novel active ingredient for anti-aging cosmetics.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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