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ARTICLE

Omega-7 inhibits inflammation and promotes collagen synthesis through SIRT1 activation

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Abstract Excessive accumulation of reactive oxygen species (ROS) during oxidative stress accelerates the skin aging process. ROS stimulate inflammatory processes in the skin, leading to activation of matrix metalloprotease-1 (MMP-1). Silent information regulator 1 (SIRT1) controls a broad range of cellular functions including the expression of MMP-1. Omega-7 fatty acids such as palmitoleic acid have many beneficial effects on health, including improvement in cardiovascular risk factors and increased insulin sensitivity. However, the effectiveness of omega-7 fatty acids (herein referred to as omega-7) related to skin aging, characterized by the degradation of collagen and loss of elasticity, remains unclear. We here investigated the effects of palmitoleic acid, a representative omega-7, on collagen regeneration through its ability to activate SIRT1 and inhibit MMP-1 in the presence of hydrogen peroxide (H_2O_2)-induced oxidative stress in human HaCaT cells. SIRT1 activation by omega-7 decreased signaling levels of nuclear transcription factor kappa B (NF-κB) and inflammatory cytokines. However, inhibition of SIRT1 by sirtinol counteracted the advantage effects of omega-7 in H_2O_2-treated HaCaT cells. In addition, omega-7 significantly counteracted the decrease in collagen abundance and loss of elasticity induced by H_2O_2. Consistent with this observation, omega-7 significantly decreased H_2O_2-induced upregulation of MMP-1 in HaCaT cells. Together, these studies suggest the potential efficacy of SIRT1 in collagen regeneration and indicate that omega-7 is a possible functional food to improve skin health for the prevention of aging.

Keywords Anti-inflammation · Collagen regeneration · Omega-7 · Oxidative stress · Silent information regulator 1

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Introduction

Oxidative stress is considered as a main factor that cause toxic effects through the generation of free radicals and can cause disruptions in normal cell function [1]. In the skin, oxidative stress is generated by various external stimuli such as excessive exposure to UV, environmental toxins, and heat [2] and leads to the production of reactive oxygen species (ROS) [3]. ROS products can accelerate skin aging by decreasing collagen regeneration and elasticity while simultaneously increasing the abundance of inflammatory factors [4]. Collagen regulation factors such as metalloproteinase-1 (MMP-1) and pro-collagen type 1 (PCOL1) have important roles in oxidative stress generated in the skin [4]. Likewise, collagen degradation by oxidative stress is a major feature of damaged skin, and collagen regulation factors are used as a distinctive marker of oxidative stress and inflammation in aging skin.

Silent information regulator 1 (SIRT1) is the most extensively studied members of the class III histone deacetylase family in mammals. SIRT1 plays a key role in various physiological processes such as aging, inflammation, senescence, and apoptosis [5]. Moreover, SIRT1 regulates inflammatory responses through deacetylation of nuclear transcription factor kappa B (NF-κB), a major

factor associated with the regulation of pro-inflammatory cytokines [6]. Along these lines, external stimuli such as UV irradiation, environmental toxins, and heat reduce SIRT1 expression as demonstrated in human keratinocytes [7].

Palmitoleic acid (16:1, *Cis*-9-hexadecenoic acid) is an omega-7 fatty acid (herein referred to as omega-7) found in cold-water fish and plants such as macadamia and sea buckthorn berry [8]. Various studies have reported that omega-7 is associated with a number of beneficial effects such as improved cardiovascular risk [9] and increased insulin sensitivity [10]. Similar to omega-7, omega-3 unsaturated fatty acids inhibit inflammation by activating the SIRT1 pathway in mouse microglia cells [11]. In addition, omega-3 has demonstrated to increase SIRT1 gene expression in coronary artery disease as well as to generate antioxidant effects [12]. Likewise, omega-9 unsaturated fatty acids have been shown to ascent rates of complete fatty acid oxidation by SIRT1-PGC1α complex activation in skeletal muscle cells [13]. However, the effects of omega-7 with respect to SIRT1 and skin aging have not yet been reported. Thus, we here investigated the physiological activity and effects of omega-7 in hydrogen peroxide (H_2O_2)-treated human keratinocyte cells. Our results suggested that natural resource of omega-7 is helpful for collagen regeneration and elasticity through SIRT1 activation.

Materials and methods

Cell culture

Human keratinocyte cell lines (HaCaT) (ATCC, Rockville, MD, USA) were kept in Dulbecco's modified Eagle medium (DMEM) (HyClone, Logan, UT, USA) containing 10% FBS (Gibco, CA, USA) and 1% antibiotics (penicillin [100 U/mL], streptomycin [100 μg/mL]) (Sigma-Aldrich, St. Louis, MO, USA) in a 37 °C humidified atmosphere with 5% CO_2. Cell culture media was changed every 2–3 days. Cells were subcultured in 100-mm dishes (Nunc, Rochester, NY, USA) 24 h before treatment. Omega-7 (Sigma-Aldrich) was dissolved in 99.5% ethanol and stocked at − 80 °C. Every aliquot was used not more than two times.

Cell viability assay

Cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit from sigma [14]. HaCaT cells were plated at a density of 2×10^4 cells/well in 48-well plates (Nunc, NY, USA). When cell confluency was 80% after incubation for 24 h, cells were treated with omega-7 (1–20 μg/mL) or H_2O_2 (0.1–1 mM) for 24 h. We also investigated the effects of a 1-h pretreatment with omega-7 (5–20 μg/mL) on HaCaT cell viability treated with 1 mM H_2O_2 for 24 h. After incubation, 10 μL of the MTT solution (500 μg/mL) was added to each well, and cells were incubated for 2 h in 37 °C humidified incubator without light. Absorbance was determined at 540 nm using a microplate reader (BioTek, Winooski, VT, USA).

Intracellular reaction oxygen species (ROS) production assay

Intracellular ROS production was determined by fluorescence assay using dichlorofluorescin diacetate (DCF-DA; Invitrogen, Carlsbad, CA, USA). The ROS production was determined using the method described by Gerber and Dubery [15] with slight modification. HaCaT cells were plated in 48-well plates at a density of 4×10^5 cell/well. When cell confluency was 80% after incubation for 24 h, we investigated the effect of a 1-h pretreatment with omega-7 (20 μg/mL) on ROS production in HaCaT cells treated with 1 mM H_2O_2 and/or sirtinol (10 μM) for 24 h. After incubation, cells were washed with PBS and stained with 10 μM DCF-DA in PBS for 20 min in the dark at 37 °C. Fluorescence was determined with an excitation wavelength 530 nm.

Preparation of nuclear extracts

To prepare nuclear extracts, HaCaT cells were plated in 6-well plates at a density of 5×10^6 cell/well. We investigated the effect of a 1-h pretreatment with omega-7 (20 μg/mL) on ROS production in HaCaT cells treated with 1 mM H_2O_2 and/or sirtinol (10 μM) for 24 h. After washing the treated cells three times with PBS, the cells were collected, pelleted by centrifugation, and lysed in buffer containing 10 mM HEPES (pH 7.8), 2 mM $MgCl_2$, 1 M DTT, 0.1 mM EDTA, 0.1 mM PMSF, and protease inhibitor for 20 min at 4 °C. Next, 10% NP-40 was added for 15 s, and the cells were centrifuged at 12,000 rpm for 20 min at 4 °C. After centrifugation, the pellet was washed with PBS and resuspended in nuclear extraction buffer containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail and incubated on ice for 30 min. After incubation, the nuclear extract was prepared by centrifugation at 12,000 rpm for 15 min at 4 °C. The protein concentrations of extracts were determined using a commercial kit (Bio-Rad Laboratories, Hercules, CA, USA).

Western blotting

For Western blotting, cells were treated in the same manner as for measuring viability and ROS and preparing nuclear extracts. Total proteins from HaCaT cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 12% gels, transferred to PVDF membranes (BioRad, Hercules, CA, USA), and blocked with 5% skim milk in PBS, after which the membranes were incubated with a 1:1000 v/v dilution of primary antibodies against COX-2 and PCOL1 (Santa cruz Biotechnology, Santa Cruz, CA, USA); β-actin and NF-κB (Cell signaling, Danvers, MA, USA); and PGE2, histone H3, MMP-1, and elastin (Abcam, Cambridge, MA, USA) with 1% skim milk overnight at 4 °C. After the Blots were washed, the membranes were next incubated with peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (1:5000 v/v, Millipore, CA, USA) for 1 h at room temperature. The immunoreactions were visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, San Jose, CA, USA) and analyzed using a chemiImager analyzer (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis

All experiments were carried out at least in triplicate, and results are presented as mean ± SEM. The data were analyzed using Student's *t* test (for two groups) and one-way ANOVA, and Tukey's test (for more than two groups). Differences with P values < 0.05 were considered statistically significant.

Results

SIRT1 is up-regulated by omega-7 in HaCaT cells

We first used MTT assays to assess the cytotoxicity of omega-7 in HaCaT cells. HaCaT cells were treated with omega-7 at various concentrations (1, 5, 10, and 20 µg/mL) for 24 h. Compared to control cells, omega-7 did not show any cytotoxic effects until a concentration of 20 µg/mL (Fig. 1A). We next evaluated the ability of omega-7 to regulate expression of SIRT1 in HaCaT cells and found that omega-7 increased SIRT1 expression in dose-dependent manner (Fig. 1B). Thus, these data confirmed that omega-7 is not cytotoxic up to a concentration of 20 µg/mL in HaCaT cells and is able to up-regulate expression of SIRT1.

Omega-7 decreases oxidative stress, reduces MMP-1 expression, and up-regulates the expression of PCOL1 and elastin in H_2O_2-treated HaCaT cells

We first established the effects of oxidative stress in HaCaT cells. Cell viability was decreased by 40% after a 24-h treatment with 1 mM H_2O_2 (Fig. 2A). Based on these data, we used 1 mM H_2O_2 in all subsequent experiments. HaCaT cells pretreated with omega-7 exhibited significantly increased viability compared with cells treated with H_2O_2 alone (Fig. 2B). Next, to evaluate the effects of omega-7 on collagen regeneration, we measured levels of MMP-1, PCOL1, and elastin using three different concentrations of omega-7 in HaCaT cells treated with 1 mM H_2O_2. The expression of MMP-1 in cells treated with H_2O_2 alone was significantly increased, whereas co-treatment with omega-7 significantly decreased expression of MMP-1 induced by H_2O_2 (Fig. 2C). In addition, the expression of PCOL1 and elastin in cells treated with H_2O_2 alone was significantly decreased, whereas omega-7 significantly increased the expression of both in a dose-dependent manner (Fig. 2D, E). Thus, these results suggest that omega-7 rescues collagen regulation and improves elasticity in H_2O_2-treated HaCaT cells.

Omega-7 down-regulates ROS production and inflammatory factors induced by H_2O_2 in HaCaT cells

We next investigated the association between omega-7 (20 µg/mL) and SIRT1 on H_2O_2-induced NF-κB, ROS, COX-2, and PGE_2 expression in the presence of sirtinol, an inhibitor of SIRT1 (10 µM). Omega-7 significantly increased the protein expression of SIRT1, which was decreased by H_2O_2 exposure in HaCaT cells. Sirtinol significantly counteracted the inhibitory effect of omega-7 on H_2O_2-induced SIRT1 expression (Fig. 3A). On the other hand, omega-7 significantly decreased the expression of H_2O_2-induced expression of NF-κB, ROS, COX-2, and PGE_2. Sirtinol significantly counteracted the suppressive effects of omega-7 on H_2O_2-induced NF-κB, ROS, COX-2, and PGE_2 (Fig. 3B–E). These results indicate that omega-7 has a protective effect on H_2O_2-induced cell damage as well as anti-inflammatory effects in HaCaT cells, mediated by activation of SIRT1.

Omega-7 increases collagen regeneration through SIRT1 activation

We confirmed the association between omega-7 (20 µg/mL) and SIRT1 with MMP-1, PCOL1, and elastin expression in H_2O_2-treated HaCaT cells using sirtinol (10 µM). Omega-7 significantly diminished H_2O_2-induced

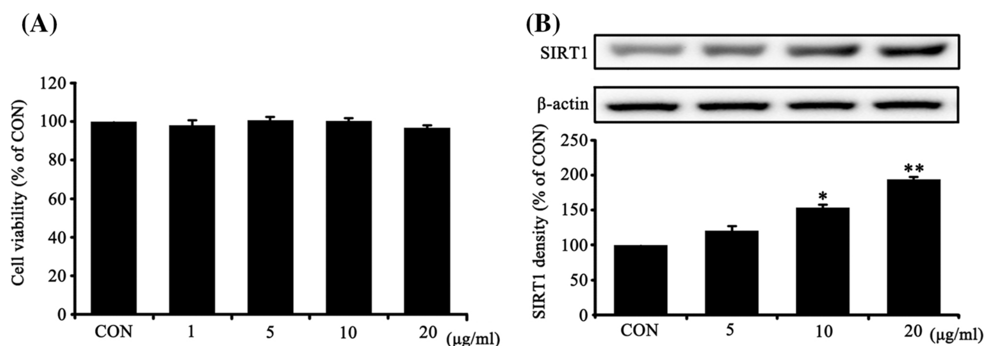

Fig. 1 Cytotoxicity and SIRT1 expression of omega-7 in HaCaT cells. (**A**) Viability of HaCaT cells after treatment with increasing concentrations of omega-7 (1–20 μg/mL) for 24 h. (**B**) SIRT1 protein expression was determined in cells treated with increasing concentrations of omega-7 (1–20 μg/mL) for 24 h. Data are expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus CON

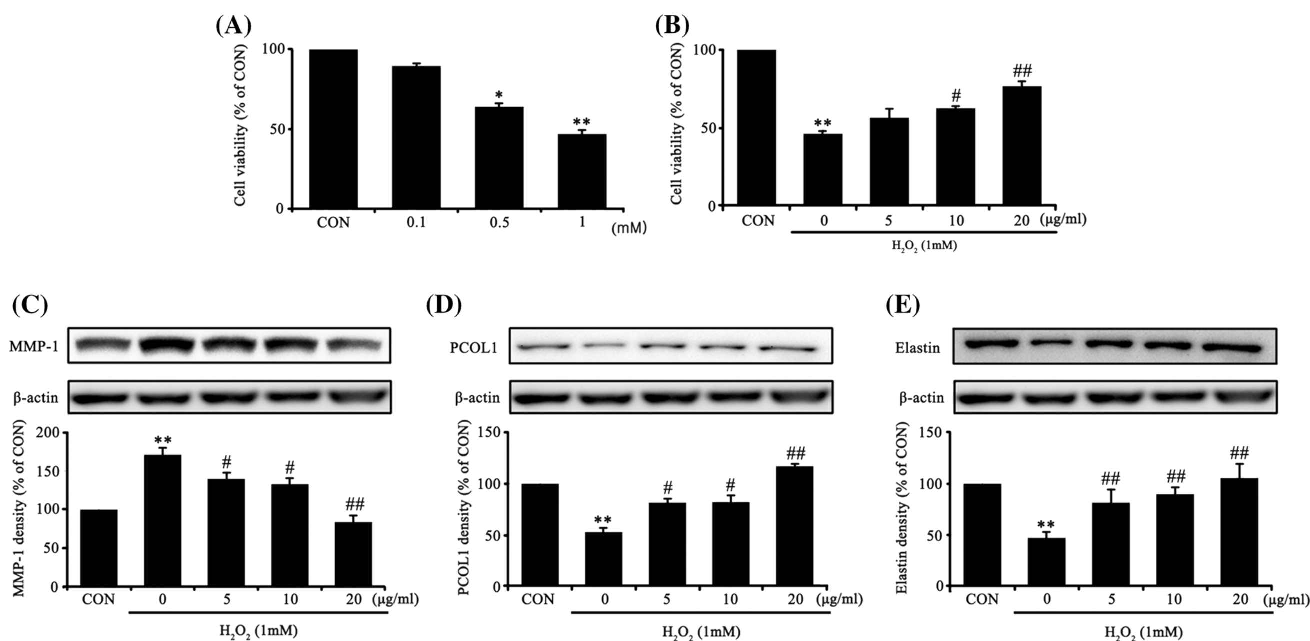

Fig. 2 Effects of omega-7 on cell viability, collagen regeneration, and elasticity factor expression against oxidative stress in H_2O_2-treated HaCaT cells. (**A**) Viability of HaCaT cells after treatment with increasing concentrations of H_2O_2 (0.1–1 mM) for 24 h. (**B**) HaCaT cells after treatment with 1 mM H_2O_2 for 24 h after pretreatment with omega-7 (1–20 μg/mL) for 1 h. (**D–E**) Whole cell lysates were subjected to Western blot analysis to evaluate MMP-1, PCOL1, and elastin expression. $^{*}P < 0.05$, $^{**}P < 0.01$ versus CON, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus H_2O_2

MMP-1 expression in HaCaT cells. In addition, sirtinol significantly reversed the inhibitory effects of omega-7 on H_2O_2-induced MMP-1 expression (Fig. 4A). On the other hand, omega-7 significantly increased the expression of PCOL1 and elastin, which was decreased by H_2O_2 exposure in HaCaT cells. Sirtinol also significantly counteracted the ability of omega-7 to rescue the H_2O_2-mediated decrease in PCOL1 and elastin expression (Fig. 4B, C). Together, these results showed that activation of SIRT1 by omega-7 significantly decreases H_2O_2-induced MMP-1. In addition, the activation of SIRT1 by omega-7 significantly increased PCOL1 and elastin in H_2O_2-treated HaCaT cells.

Discussion

Palmitoleic acid (16:1, *Cis*-9-hexadecenoic acid) is an omega-7 fatty acid present in animals such as cold-water fish and plants such as macadamia and sea buckthorn [8]. Omega-7 fatty acids have been shown to have beneficial effects as health supplements to improve cardiovascular disease risk and increase insulin sensitivity [9]. In the present study, we showed that omega-7 inhibits inflammatory factors and rescues collagen production through activation of SIRT1 and subsequent downregulation of NF-κB and MMP-1 in H_2O_2-treated HaCaT cells. SIRT1 is the

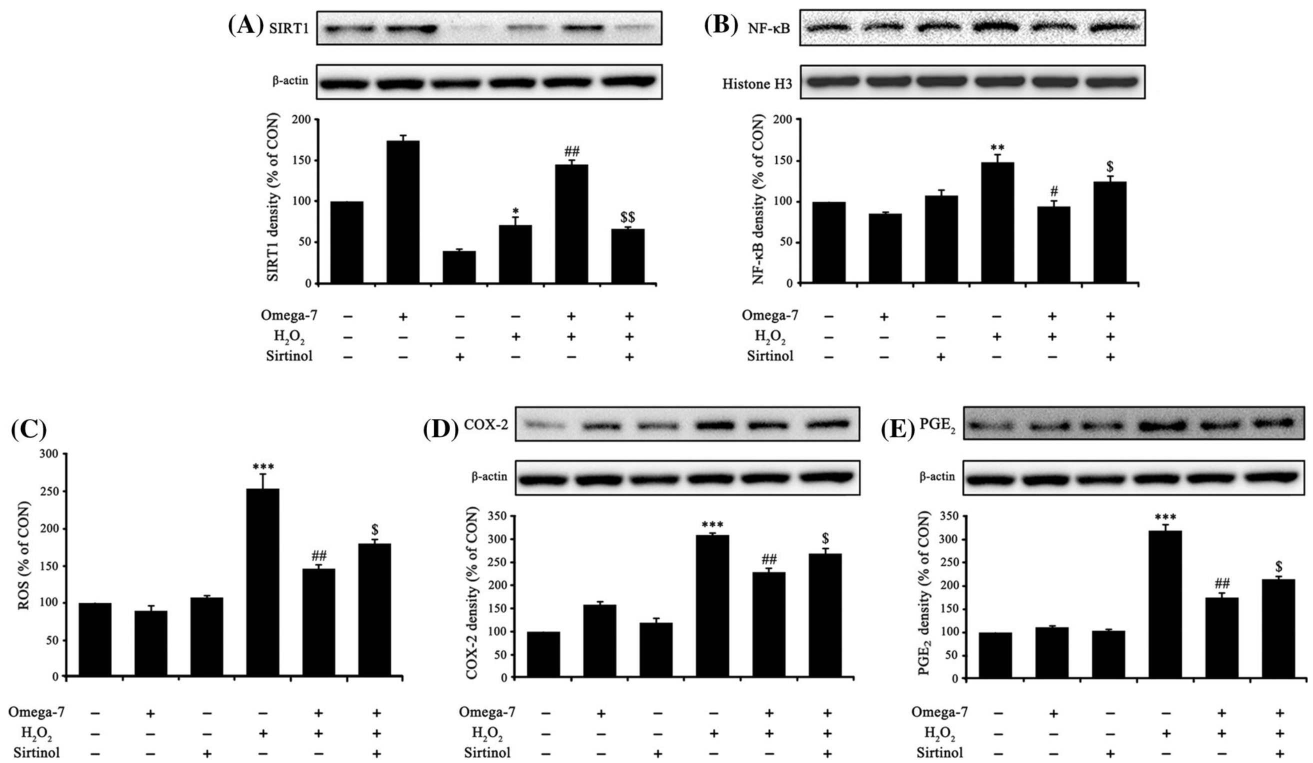

Fig. 3 Effect of omega-7 on the activation of SIRT1 and inflammatory factors in HaCaT cells co-treated with H_2O_2 and a SIRT1 inhibitor. HaCaT cells were pretreated for 1 h with omega-7 (20 µg/mL), followed by incubation with 1 mM H_2O_2 and/or sirtinol (10 µM) for 24 h. (**A, D, E**) Whole cell lysates were subjected to Western blot analysis to evaluate expression of SIRT1, COX-2, and PGE_2. (**B**) Nuclear extracts were prepared for Western blot analysis of NF-κB. (**C**) HaCaT cells were pretreated with omega-7 (1–20 µg/mL) for 1 h, followed by H_2O_2 (1 mM) and/or sirtinol (10 µM) for 24 h, after which ROS levels were evaluated by DCF-DA. Data are expressed as the mean ± SEM of three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{**}P < 0.001$ versus CON, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus H_2O_2 alone, $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ versus omega-7 co-treated with H_2O_2

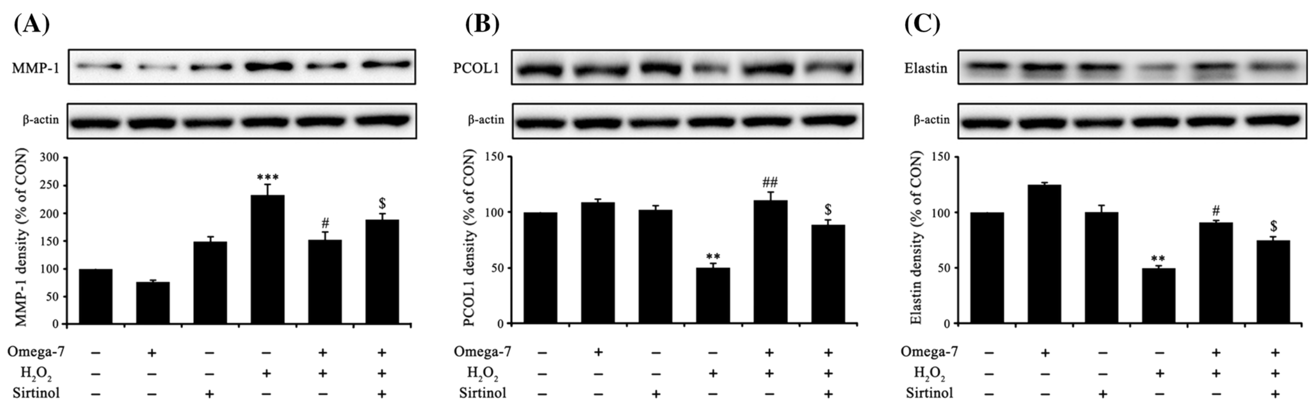

Fig. 4 Effect of omega-7 on activation of MMP-1, PCOL1, and elastin in HaCaT cells co-treated with H_2O_2 and a SIRT1 inhibitor. HaCaT cells were pretreated for 1 h with omega-7 (20 µg/mL), followed by treatment with 1 mM H_2O_2 and/or sirtinol (10 µM) for 24 h. (**A–C**) Whole cell lysates were subjected to Western blot analysis to evaluate the expression of MMP-1, PCOL1, and elastin. Data are expressed as the mean ± SEM of three independent experiments, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus CON, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus H_2O_2 alone, $^{\$}P < 0.05$ versus omega-7 co-treated with H_2O_2

mainly studied member of the sirtuin protein family, which are conserved NAD^+-dependent deacetylases [16]. SIRT1 has been showed to play a key role in a variety of physiological activity associated with aging [17]. Consistently, we showed that omega-7 increases SIRT1 activation in a dose-dependent manner, and that this activation could be decreased by the SIRT1 inhibitor sirtinol (Figs. 1A, 3A).

External stimuli such as toxins, heat, and UVB are major skin irritants that are all capable of generating oxidative stress in skin [18]. Excessive ROS production during

oxidative stress interrupts the maintenance of normal skin functions by increasing inflammation and inhibiting collagen synthesis, ultimately promoting skin aging [19]. In particular, ROS up-regulate MMP-1 and down-regulate factors involved in collagen synthesis in damaged skin cells [20]. Among a number of different collagenases, MMP-1 is the major player, serving primarily to cleave type I collagen. In this way, MMP-1 can cause disruption on the balance between synthesis and degradation of collagen and plays a key role in the formation of wrinkles and decreased elasticity [21]. In this study, we showed that omega-7 significantly decreased the expression of MMP-1 while increasing the expression of PCOL1 and elastin (Fig. 2C–E). These results indicate that omega-7 rescues collagen synthesis by inhibiting MMP-1 activation.

NF-κB is a major transcription factor involved in the inflammatory response [22]. NF-κB is located in the cytosol in an inactivated state and, upon stimulation by ROS, translocates to the nucleus to regulate the expression of inflammatory proteins such as COX-2 and chemical regulators such as PGE_2, together leading to accelerated skin aging [23]. A recent study demonstrated that SIRT1 also plays a critical role as a suppressor of the NF-κB signaling pathway by deacetylating NF-κB [24]. In this way, inhibition of NF-κB signaling pathway by SIRT1 activation decreases pro-inflammatory cytokines and gene products involved in inflammation and apoptosis [25]. In the present study, we confirmed whether omega-7-mediated activation of SIRT1 could regulate oxidative stress and inflammation in H_2O_2-treated HaCaT cells. Omega-7 up-regulated SIRT1 expression in H_2O_2-treated HaCaT cell, whereas the increased level of SIRT1 was reversed by sirtinol, a specific SIRT1 inhibitor. These results show that omega-7 exerts its effects primarily by increasing SIRT1 activation (Fig. 3A). Likewise, omega-7 inhibits ROS production and inflammatory factors such as NF-κB, COX-2, and PGE_2 in H_2O_2-treated HaCaT cells. As expected, inhibition of ROS production and inflammation factors was reversed by co-treatment with sirtinol (Fig. 3B–E). Importantly, these results suggest that the increase in SIRT1 expression by omega-7 was account for the anti-inflammatory effects of omega-7 in H_2O_2-treated HaCaT cells. Lastly, SIRT1 can accelerate the decrease in MMP-1 expression though deacetylation of NF-κB [26]. Taken together, our results showed that omega-7 decreases MMP-1 expression and increases PCOL1 and elastin expression in H_2O_2-treated HaCaT cells by activating SIRT1, and that these effects could be reversed by a SIRT1 inhibitor (Fig. 4A–C).

In conclusion, our results suggest that omega-7 has anti-inflammatory effects in HaCaT cells, where it promotes collagen regeneration in the presence of H_2O_2-induced cytotoxicity. The anti-inflammatory effects of omega-7 appear to be mediated through SIRT1 activation. Our results provide strong evidence for omega-7 as a functional food for promoting skin health to prevent aging.

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