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Sargahydroquinoic acid inhibits TNFα-induced AP-1 and NF-κB signaling in HaCaT cells through PPARα activation

Youngsic Jeon [a], Yujung Jung [a], Min Cheol Kim [a], Hak Cheol Kwon [a], Ki Sung Kang [b], Yong Kee Kim [c,*], Su-Nam Kim [a,*]

[a] Natural Medicine Center, KIST Gangneung Institute, Gangneung 210-340, Republic of Korea
[b] College of Korean Medicine, Gachon University, Seongnam 461-701, Republic of Korea
[c] College of Pharmacy, Sookmyung Women's University, Seoul 140-742, Republic of Korea

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ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors and expressed in various cell types in the skin, including keratinocytes, fibroblasts and infiltrating immune cells. Thus, their ligands are targets for the treatment of various skin disorders, such as photo-aging and chronological aging of skin. Intensive studies have revealed that PPARα/γ functions in photo-aging and age-related inflammation by regulating matrix metalloproteinases (MMPs) via activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB). However, the detailed mechanism of PPARα/γ's role in skin aging has not yet been elucidated. In this study, we confirmed that sargahydroquinoic acid (SHQA) as a PPARα/γ ligand significantly decreased Tumor Necrosis Factor-alpha (TNFα)-induced MMP-2/-9 expression by downregulating TNFα-induced transcription factors, subsequently reducing IκBα degradation and blocking NF-κB p65 nuclear translocation in HaCaT human epidermal keratinocyte cells. Treatment of cells with SHQA and GW6471 (PPARα antagonist) not bisphenol A diglycidyl ether (PPARγ antagonists), reversed the effect on TNFα-induced inflammatory signaling pathway activation. Taken together, our data suggest that SHQA inhibit TNFα-induced MMP-2/-9 expression and age-related inflammation by suppressing AP-1 and NF-κB pathway via PPARα.

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that play an important role in the remodeling of the extracellular matrix during developmental morphogenesis, angiogenesis and tissue repair and in tissue destruction during pathological processes, such as arthritic inflammation, skin aging, tumor invasion and metastasis [1,2]. MMP expression is very low in unstimulated skin cells and healthy tissues, but some MMPs are induced by extracellular stimuli, such as UV, growth factors, cytokines and tumor promoters [3–5]. The expression and activity of gelatinase A (MMP-2) and B (MMP-9) were reported in human cultured keratinocyte and hairless mice chronically exposed to UVB involved in epidermal hyperplasia and skin wrinkles [6,7]. The inhibition of gelatinases suppresses UVB-induced epidermal thickness enhancement and wrinkle formation and may provide and effective therapeutic method for counteracting photo-aging. MMPs expressed in the human skin are regulated by inflammation-related transduction pathways, such as activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) [8,9]. AP-1 forms heterodimer complexes with c-Jun and c-Fos, induced by various inputs, including growth factors, cytokines and UV exposure [10]. NF-κB is an important MMP mediator that regulates the immune response, cell survival, cell proliferation and inflammation [11,12].

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily and consist of three isotypes: PPARα, β/δ and γ. They are ligand-receptor-dependent transcription factors that heterodimerize with RXR to allow binding to peroxisome proliferator-activated receptor response element (PPRE) and activate PPAR-responsive genes. These genes were previously thought to only regulate lipid metabolism and glucose homeostasis; however, recent studies have revealed roles for PPARs in age-related inflammation and photo-aging as regulators of NF-κB and MMPs [13–16]. Furthermore, PPARα and PPARγ negatively interact with the transcription factors AP-1 and NF-κB,

* Corresponding authors. Addresses: Natural Medicine Center, KIST Gangneung Institute, 290 Daejeon-dong, Gangneung-si, Gangwon-do 210-340, Republic of Korea. Fax: +82 33 650 3529 (S.-N. Kim). College of Pharmacy, Sookmyung Women's University, 100 Cheongpa-ro 47-gil, Yongsan-gu, Seoul 140-742, Republic of Korea. Fax: +82 2 710 9871. (Y.K. Kim).
E-mail addresses: yksnbk@sm.ac.kr (Y.K. Kim), snkim@kist.re.kr (S.-N. Kim).

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which control MMP expression [17–19]. The combined activation of PPARα and PPARγ by a dual agonist could lead to complementary or synergistic effects to improve lipid homeostasis and insulin sensitivity and to control inflammation [14]. In addition, the role of PPARα/γ dual activation in inhibiting UV-induced inflammatory cytokine and MMP expression makes them potential therapeutic targets for age-related inflammation [20].

Previously, we screened active marine natural products able to increase PPARα/γ transcriptional activity, then found that *Sargassum yezoense* (SY) extract and its active ingredients, sargaquinoic acid (SQA) and sargahydroquinoic acid (SHQA) have strong therapeutic potential for metabolic disorders, but their anti-skin aging potential was not investigated [21,22].

In this study, we found that SHQA inhibits MMP-2/-9 overexpression in HaCaT human epidermal keratinocyte cells. In addition, we explored the molecular mechanisms by which SHQA inhibits Tumor Necrosis Factor-alpha (TNFα)-induced inflammatory responses through PPARα or PPARγ.

2. Materials and methods

2.1. Cell culture and chemicals

HaCaT cells (N.E. Fusenig, Deutsches Krebsforschungszentrum, Heidelberg, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories Inc., Logan, UT, USA) and 1% penicillin/streptomycin (Invitrogen) at 37 °C and 5% CO_2 [23]. SQA and SHQA was isolated from methanol extract of SY extract as described previously [21,22]. Troglitazone and WY14643 were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA), and GW6471 and bisphenol A diglycidyl ether (BADGE) were purchased from Tocris (Ellisville, MO, USA).

2.2. Cell-based transactivation assay

HaCaT cells were seeded into 24-well plates and cultured for 24 h before transfection. Prior to transfection, the medium was replaced with 10% charcoal dextran-treated FBS–DMEM. After 4 h, a DNA mixture containing a 3X multimerized PPRE-luciferase reporter plasmid (0.3 μg) and the internal control plasmid pRL-SV-40 (5 ng) were transfected using the TransFast™ transfection reagent (Promega, Madison, WI, USA). Twenty-four hours after transfection, the cells were treated with 10 μM WY14643, 10 μM troglitazone or the indicated concentrations of SQA, SHQA or SY and were incubated for an additional 24 h. The luciferase activities of the cell lysates were measured using the Dual-Luciferase® Reporter Assay System according to the manufacturer's instructions (Promega). The relative luciferase activity was normalized to the corresponding Renilla luciferase activity to determine the transfection efficiency.

Transactivation of AP-1 or NF-κB was evaluated using pAP-1-luc or pNF-κB-Luc (Stratagene, La Jolla, CA, USA) with pRL-SV40 in HaCaT cells. Twenty-four hours after transfection, the cells were treated with a PPAR antagonist (PPARα; GW6471 or PPARγ; BADGE) in 10% charcoal dextran-treated FBS–DMEM for 1 h and with the indicated concentration of SHQA for an additional hour. The cells were then treated with the inflammatory cytokine TNFα (20 ng/ml) for 12 or 4 h. Transactivation of AP-1 or NF-κB in HaCaT cells was determined as described above.

2.3. Reverse transcriptase-PCR

Total RNA was isolated from HaCaT cells using the easy-Blue™ total RNA extraction Kit (iNTRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. The concentration of each sample was measured by spectrophotometry at 260 nm; the integrity of each RNA sample was evaluated using the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). cDNA was synthesized from 1 μg of total RNA in 20 μl with random primers using the ImProm-II Reverse Transcription System (Promega). PCR was performed using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The reactions were performed in 50 μl containing 5 μl of 10× Taq buffer, 1 μl of 10 mM dNTPs, 1.25 U of Taq DNA polymerase (Solgent, Seoul, Korea), 1 μl of cDNA (corresponding to 25 ng reverse-transcribed total RNA) and 5 pmol of each primer. The RT-PCR amplification protocol was as follows: activation of Taq polymerase at 95 °C for 2 min, followed by 31 cycles of cDNA denaturation at 95 °C for 20 s, primer annealing at 58 °C for 40 s, and elongation at 68 °C for 30 s, with a final extension step of 10 min. The primer sets were as follows: PPARα, forward 5′-CTT CGC AAA CTT GGA CCT GA-3′ and reverse 5′-AGC ATC CGA CTC CGT CTT CT-3′; PPARγ, forward 5′-AGA GCC TTC CAA CTC CCT CA-3′ and reverse 5′-CAA GGC ATT TCT GAA ACC GA-3′; GAPDH, forward 5′-ACC ACA GTC CAT GCC ATC AC-3′ and reverse 5′-TCC ACC ACC CTG TTG CTG TA-3′. The PCR product was analyzed by agarose gel electrophoresis and ethidium bromide staining.

2.4. Preparation of nuclear and cytoplasmic fractions

Nuclear and cytoplasmic protein fractions were prepared as described previously [24].

2.5. Western blotting

Supernatants from cells were precipitated with trichloroacetic acid (final concentration: 10% v/v), and the protein concentration of each sample was determined using the QuantiPro™ BCA assay kit (Sigma–Aldrich). Whole cell lysates were prepared in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate, 1 mM Na_3VO_4) containing a protease inhibitor (Roche, Penzberg, Germany) and a phosphatase inhibitor cocktail (Sigma–Aldrich). Loading dye was added to the precipitated supernatants and lysates (each containing 10 or 25 μg protein), and the samples were heated for 10 min at 95 °C and subjected to electrophoresis on a 10% SDS–polyacrylamide gel. The proteins were transferred to Immobilon® SQ PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked in 5% skim milk at room temperature for 1 h and were probed with primary antibodies against p-c-Jun, c-Jun, p-c-Fos, c-Fos, p65, p-IκBα, IκBα, LDH, GAPDH (Cell Signaling Technology, Beverly, MA, USA), β-actin or Lamin A/C (Santa Cruz, CA, USA) for 24 h at 4 °C. After washing, bound antibodies were detected with corresponding horse radish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Signals were detected with SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and were visualized with the LAS-4000 Luminescent Image Analyzer (Fuji Film, Tokyo, Japan).

2.6. Statistical analyses

The data are expressed as the means ± SD. Differences between the mean values in the two groups were analyzed using one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. SHQA increases PPARα/γ transactivation and inhibits MMP-2/-9 expression

In our previous study, we have demonstrated transcriptional activation of PPARα/γ by SQA, SHQA or SY in CV-1 cell systems

[21]. SQA, SHQA or SY treatment led to an increase in PPARα and PPARγ reporter gene activities in a dose-dependent manner. However, SQA, SHQA or SY had no detectable effect on PPARδ and RXRα transcriptional activation (data not shown). To determine whether SQA, SHQA or SY acted as a PPARα/γ agonist in skin, we transfected human epidermal keratinocyte HaCaT cells with PPRE reporters. SQA, SHQA or SY increased PPRE-driven transcriptional activation in a dose-dependent manner in HaCaT cells (Fig. 1A). To explore whether SQA, SHQA or SY inhibited TNFα-induced MMP-2/-9 expression, we examined MMP-2/-9 protein levels in supernatants from SQA, SHQA or SY-treated cells by western blot. Treatment with SQA, SHQA and SY significantly attenuated MMP-2 levels in

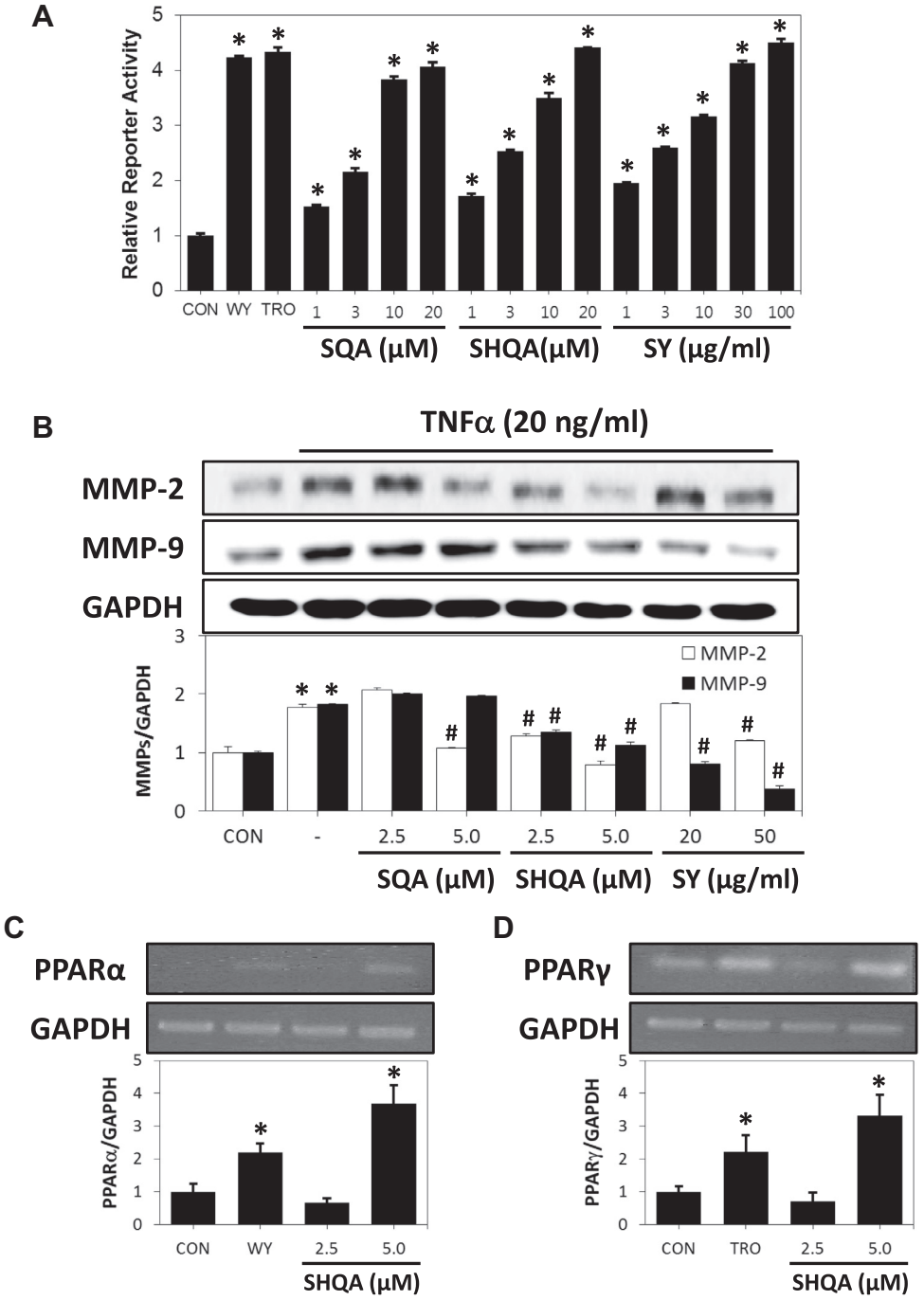

Fig. 1. Sargaquinoic acid increases PPARα/γ transactivation and inhibits MMP-2/-9 expression. (A) HaCaT cells were transiently cotransfected with the PPRE-luciferase reporter construct and the pRL-SV40 vector. The cells were treated with 10 μM WY14643 (WY), 10 μM troglitazone (TRO), or various concentrations of *S. yezoense* extract (SY; 1, 3, 10, 30 or 100 μg/ml), sargaquinoic acid (SQA; 1, 3, 10 or 20 μM) or sargahydroquinoic acid (SHQA; 1, 3, 10 or 20 μM) for 24 h. The harvested cells were analyzed by reporter assay as described in Section 2. Each bar represents the mean ± SD of duplicates. $^{*}P < 0.05$ vs. control. (B) HaCaT cells were treated with SQA (2.5 or 5.0 μM), SHQA (2.5 or 5.0 μM) or SY (20 or 50 μg/ml) in combination with 20 ng/ml TNFα for 48 h to measure MMP-2/-9 expression. Relative MMP-2/-9 protein expression was determined by Western blot analysis and normalized to GAPDH expression. Each bar represents the mean ± SD of duplicates. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. TNFα-treated group. The relative gene expression levels of PPARα (C) and PPARγ (D) from WY-, Tro- or SHQA (2.5 and 5.0 μM)-treated HaCaT cells were determined by RT–PCR and confirmed by agarose gel electrophoresis with ethidium bromide staining. Each bar represents the mean ± SD of duplicates. $^{*}P < 0.05$ vs. control.

a dose-dependent manner, but SHQA and SY significantly attenuated MMP-9 levels (Fig. 1B). These findings suggest that SHQA from SY can blocks MMP-2/-9 protein production more effectively than SQA.

Then, to identify whether SHQA enhanced PPARα and PPARγ expression, we evaluated PPARα and PPARγ mRNA levels by RT–PCR. Treatment of SHQA significantly increased PPARα and PPARγ mRNA levels in a dose-dependent manner (Fig. 1C and D). These findings support that SHQA block MMP-2/-9 protein production via PPARα or PPARγ.

3.2. SHQA inhibits TNFα-induced AP-1 and MAPK signaling

To explore whether SHQA affects TNFα-induced MAPK signaling, we performed Western blot analysis of c-Jun and c-Fos phosphorylation and expression. As shown in Fig. 2A, SHQA inhibited TNFα-induced c-Jun phosphorylation, but had no detectable effect on c-Fos phosphorylation and expression. These results indicate that AP-1 is important targets of SHQA. To investigate SHQA transactivate AP-1, we stimulated cells with TNFα and performed AP-1 reporter gene assays in the absence or presence of SHQA. As shown in Fig. 2B, SHQA treatment markedly decreased TNFα-induced AP-1 transcriptional activity in a dose-dependent manner.

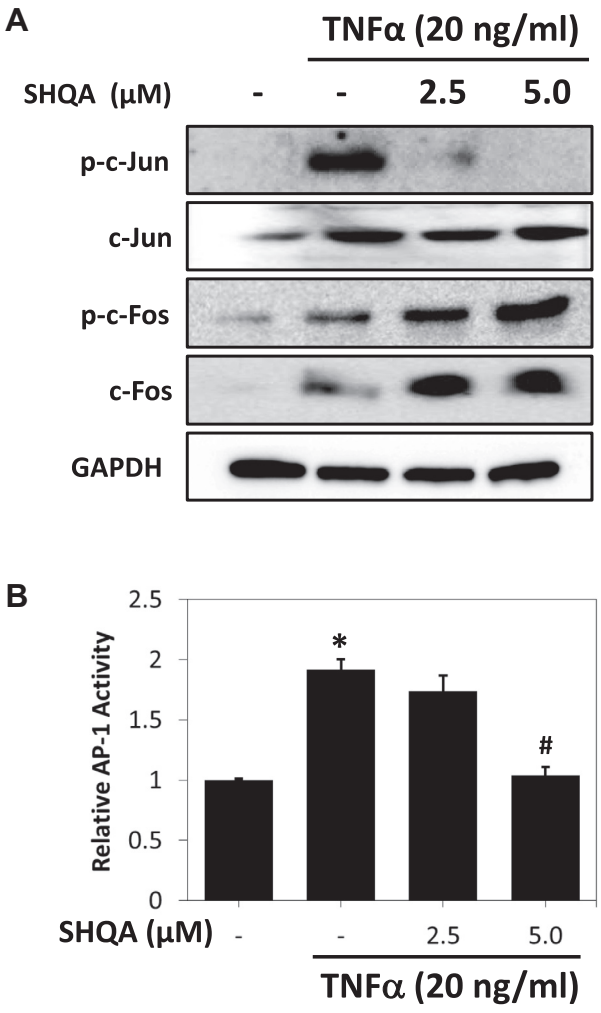

Fig. 2. SHQA inhibits TNFα-induced AP-1 and MAPK signaling. (A) HaCaT cells were treated with SHQA for 1 h, stimulated by TNFα (20 ng/ml) and incubated with the indicated concentrations of SHQA (2.5 and 5.0 μM) for 12 h. Protein expression levels of p-c-Jun, c-Jun, p-c-Fos and c-Fos were assessed by Western blot analysis. (B) Transcriptional activation of AP-1 was analyzed as described in Section 2. Each bar represents the mean ± SD of triplicates. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. TNFα-treated group.

Taken together, our results suggest that SHQA inhibits TNFα-induced AP-1 activation, an important step in MMP-2/-9 expression.

3.3. SHQA inhibits TNFα-induced p65 translocation and IκBα phosphorylation

We next determined whether SHQA could affect TNFα-induced NF-κB activation. Thus we examined the nuclear level of the p65 subunit of NF-κB and IκBα expression/phosphorylation by Western blot after TNFα treatment. As shown in Fig. 3A and B, SHQA strongly inhibited p65 nuclear translocation and decreased IκBα phosphorylation in a dose-dependent manner. To examine NF-κB transcriptional activation, we stimulated cells with TNFα and performed NF-κB reporter gene assays in the absence or presence of SHQA. As shown in Fig. 3C, SHQA treatment markedly inhibited TNFα-induced NF-κB transcriptional activity in a dose-dependent manner. Taken together, these results suggest that SHQA inhibits TNFα-induced NF-κB transactivation by affecting IκBα phosphorylation, leading to the inhibition of NF-κB nuclear translocation, another important step in the regulation of MMP-2/-9 expression.

3.4. SHQA inhibits TNFα-induced AP-1 and NF-κB signaling via PPARα

To investigate SHQA's effect on AP-1 transactivation via PPARα or PPARγ, we stimulated cells with TNFα and performed AP-1 reporter gene assays in the absence or presence of SHQA or co-treatment with a PPAR antagonist (GW6471 or BADGE). As shown in Fig. 4A, SHQA treatment markedly decreased AP-1 transcriptional activity, but co-treatment with GW6471 enhanced AP-1 transcriptional activity. In particular, BADGE had no effect on AP-1 transactivation. These results suggest that SHQA inhibits TNFα-induced AP-1 activation and GW6471 reversed this effect, suggesting that SHQA acts through PPARα to regulate AP-1 activation.

To examine NF-κB transcriptional activation, we stimulated HaCaT cells with TNFα and performed NF-κB reporter gene assays in the absence or presence of SHQA or co-treatment with PPAR antagonists. As shown in Fig. 4B, SHQA treatment markedly inhibited NF-κB transcriptional activity, but co-treatment with PPARα antagonist enhanced NF-κB transcriptional activity. To explore whether SHQA acted through PPARα or PPARγ to affect TNFα-induced NF-κB signaling, we incubated HaCaT cells with SHQA alone or in combination with a PPAR antagonist (GW6471 or BADGE). We found that only GW6471 significantly restored TNFα-induced p65 translocation (Fig. 4C). Taken together, these results suggest that PPARα activation suppresses TNFα-induced NF-κB transactivation by inhibition of NF-κB nuclear translocation.

4. Discussion

Marine natural products provide a rich source of chemical diversity that can be used to design and develop new potentially useful therapeutic agents [25–27]. SHQA is a natural compound that acts through PPARα/γ dual activation to control glucose and lipid metabolism in 3T3-L1 cells and *db/db* mice [21,22]. PPAR activity was previously thought to only regulate lipid/glucose homeostasis and metabolic diseases. However, accumulating evidence suggests that PPARs play important roles in age related-inflammatory diseases and skin aging. We demonstrate here benficial efetcs of SHQA on age-related inflammatory disease and skin aging.

Skin aging is the hallmark of prolonged UV exposure and intrinsic aging, causing collagen breakdown by increasing the expression

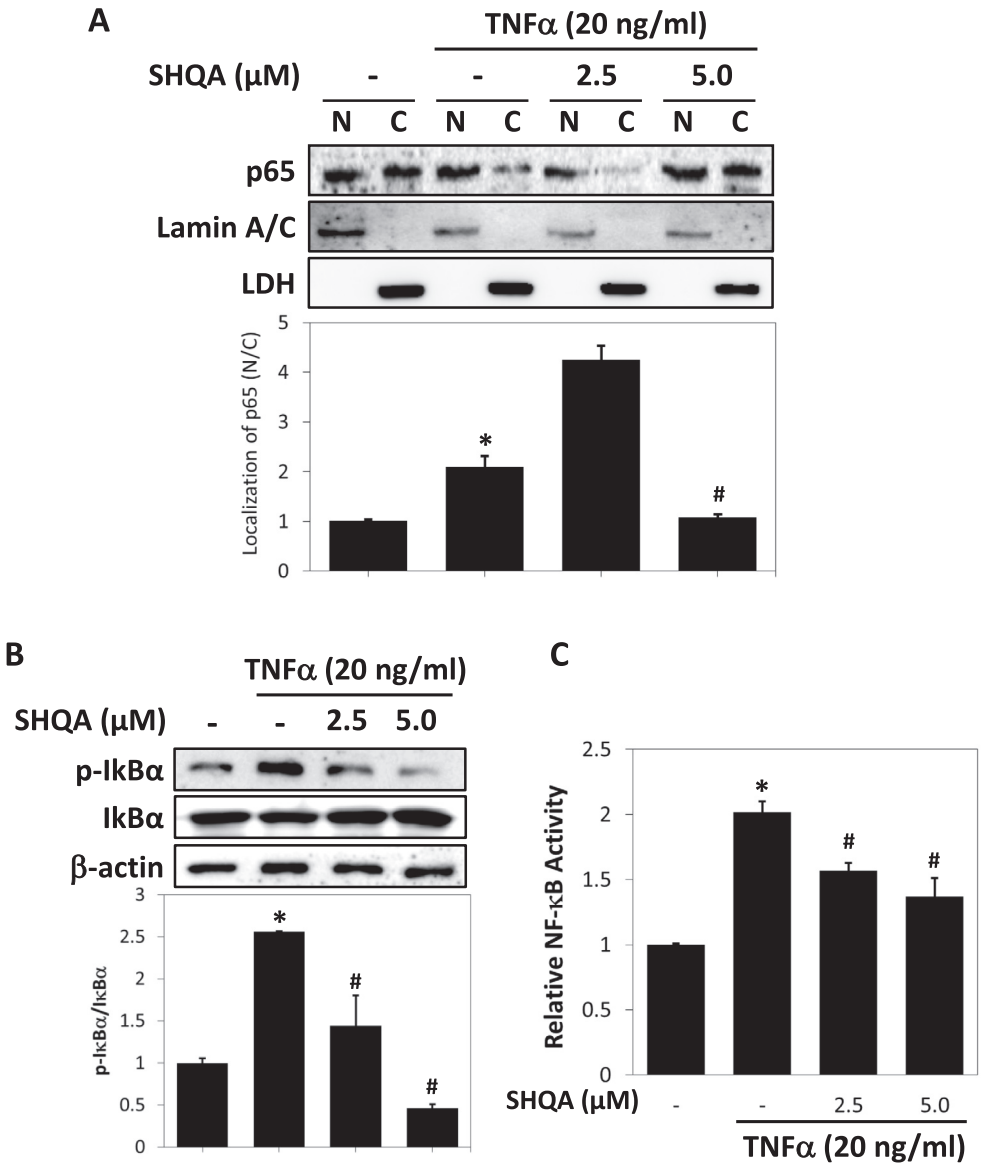

Fig. 3. SHQA inhibits TNFα-induced p65 translocation and IκBα phosphorylation. (A) HaCaT cells were treated with SHQA for 1 h, stimulated by TNFα (20 ng/ml) and incubated with the indicated concentrations of SHQA (2.5 and 5.0 μM) for 1 h. The amount of the NF-κB p65 subunit that translocated from the cytosol (C) into the nucleus (N) was assessed by Western blot analysis after nuclear and cytoplasmic fractionation. Lamin A/C and lactate dehydrogenase (LDH) were used as nuclear and cytoplasmic markers, respectively. Each bar represents the mean ± SD of duplicates. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. TNFα-treated group. (B) HaCaT cells were treated with SHQA for 1 h, stimulated by TNFα (20 ng/ml) and incubated with the indicated concentrations of SHQA (2.5 and 5.0 μM) for 1 h. p-IκBα and IκBα expression levels were assessed by Western blot analysis and normalized to GAPDH expression. Each bar represents the mean ± SD of duplicates. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. TNFα-treated group. (C) Transcriptional activation of NF-κB was analyzed as described in Section 2. Each bar represents the mean ± SD of triplicates. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. TNFα-treated group.

levels of MMP enzymes [28]. AP-1 and NF-κB activation regulate MMP expression [3,9,20]. Furthermore, a recent study reported that PPARα and PPARγ activation inhibit MMPs, IL-6 and IL-8 expression via the AP-1 and NF-κB pathway to induce anti-inflammatory and anti-aging effects [20]. In this study, we found that SHQA increased the transactivation and expression of PPARα and PPARγ (Fig. 1A, C and D) and inhibits TNFα-induced enhancement of MMP-2/-9 expression (Fig. 1B) and that the PPARα/γ dual activator SHQA inhibits this expression by suppressing c-Jun phosphorylation and NF-κB activity via PPARs.

AP-1 is mainly composed of heterodimer complexes with c-Jun and c-Fos and is induced by a variety of stimulations, such as growth factors, cytokines and UV [5]. In recent studies, PPARs have been shown to play a crucial role in inflammation by regulating AP-1. PPARα can repress the AP-1 signaling pathway by interacting with and sequestering c-Jun [29]. Indeed, PPARα activation has anti-inflammatory effects, as it ameliorated the inflammatory response activated by whole-brain irradiation by negatively regulating the AP-1 and NF-κB pathway in microglial cells [18]. Furthermore, PPARα has been shown to modulate NF-κB activation by inducing IκBα in IL-1β-induced smooth muscle cells [30]. Additionally, several studies have indicated that PPARγ elicits its anti-inflammatory effects by inducing AP-1 and NF-κB interactions. Moreover, PPARγ activation can inhibit AP-1 and p65 translocation by enhancing IκBα, the main inhibitor of NF-κB [29,30]. In our study, SHQA inhibited TNFα-induced phosphorylation of c-Jun (Fig. 2A), which potentiates AP-1 transcriptional activity (Fig. 2B). But, SHQA has no effect on TNFα-induced c-Fos expression

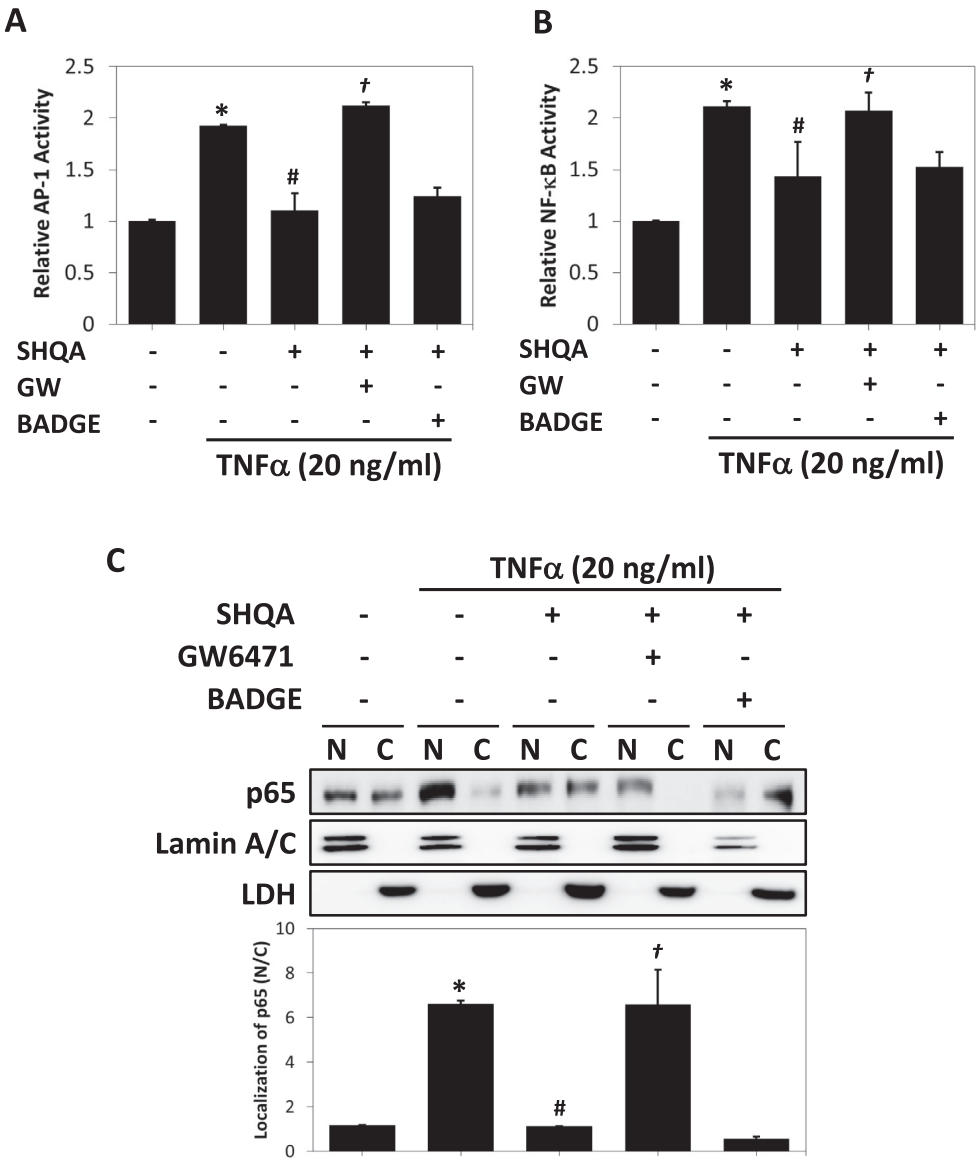

Fig. 4. SHQA inhibits TNFα-induced AP-1 and NF-κB signaling via PPARα. Transcriptional activation of AP-1 (A) and NF-κB (B) was analyzed as described in Section 2. Each bar represents the mean ± SD of triplicates. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. TNFα-treated group; $^{\dagger}P < 0.05$ vs. SHQA-treated group. (C) HaCaT cells were treated with 20 μM GW or 20 μM BADGE for 1 h, stimulated by TNFα (20 ng/ml) and treated with 5.0 μM SHQA alone or in combination with 20 μM GW or 20 μM BADGE for 4 h. The amount of the NF-κB p65 subunit that translocated from the cytosol (C) into the nucleus (N) was assessed by Western blot analysis after nuclear and cytoplasmic fractionation. Lamin A/C and lactate dehydrogenase (LDH) were used as nuclear and cytoplasmic markers, respectively. Each bar represents the mean ± SD of duplicates. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. TNFα-treated group; $^{\dagger}P < 0.05$ vs. SHQA-treated group.

(Fig. 2A). Additionally, GW6471 not BADGE strongly enhanced AP-1 transcriptional activity compared to the TNFα-treated control (Fig. 4A).

NF-κB plays crucial roles in skin homeostasis and in the transcriptional response to UV irradiation and oxidative stress. Some studies have shown that UV irradiation gradually degrades the NF-κB inhibitor IκBα, induces p65 translocation, and induces NF-κB DNA-binding activity [30]. Furthermore, PPARα activation suppresses NF-κB activation through two mechanisms; first, PPARα interacts with the Rel domain of the p65 subunit of NF-κB and then inhibits its transcriptional activity. Second, PPARα induces the expression of IκBα, a major NF-κB inhibitor [29,31]. Here, we found that SHQA inhibits p65 translocation (Fig. 3A) by inducing IκBα phosphorylation (Fig. 3B) and NF-κB transcriptional activation (Fig. 3C) in TNFα-treated HaCaT cells. Furthermore, SHQA acts through PPARα, as these effects of SHQA are reversed by GW6471 not BADGE (Fig. 4B and C). These results indicate that the effect of SHQA is PPARα-dependent and suppresses TNFα-induced NF-κB transactivation and affects IκBα phosphorylation, leading to the inhibition of NF-κB nuclear translocation.

In summary, we demonstrate that SHQA inhibits NF-κB and AP-1 signaling by selectively activating PPARα and PPARγ, and ultimately inhibits TNFα-induced MMP-2/-9 expression in a dose-dependent manner. Mechanistically, SHQA suppresses TNFα-induced NF-κB transactivation by inducing IκBα phosphorylation and inhibiting p65 nuclear translocation and AP-1 activation, which is mediated by c-Jun phosphorylation. Importantly, our study shows that SHQA's inhibitory effects on the NF-κB and AP-1 signaling pathway relate to PPARα activation. Thus, these findings suggest that SHQA is a potential agent for treating and preventing age-related inflammatory disease and skin aging.

Conflict of interest

The authors have declared no conflicting interests.

Acknowledgments

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