



4-O-Methylhonokiol Protects HaCaT Cells from TGF- β 1-Induced Cell Cycle Arrest by Regulating Canonical and Non-Canonical Pathways of TGF- β Signaling

Sang-Cheol Kim^{1,3} Jung-Il Kang¹, Jin-Won Hyun¹, Ji-Hoon Kang¹, Young-Sang Koh¹, Young-Heui Kim², Ki-Ho Kim², Ji-Hee Ko¹, Eun-Sook Yoo¹ and Hee-Kyoung Kang^{1,*}

¹Department of Medicine, Jeju National University School of Medicine, Jeju 63243, ²R&D Center, Bioland Ltd., Cheonan 31257,

³Freshwater Bioresources Utilization Division, Nakdonggang National Institute of Biological Resources, Sangju 37242, Republic of Korea

Abstract

4-O-methylhonokiol, a neolignan compound from *Magnolia officinalis*, has been reported to have various biological activities including hair growth promoting effect. However, although transforming growth factor- β (TGF- β) signal pathway has an essential role in the regression induction of hair growth, the effect of 4-O-methylhonokiol on the TGF- β signal pathway has not yet been elucidated. We thus examined the effect of 4-O-methylhonokiol on TGF- β -induced canonical and noncanonical pathways in HaCaT human keratinocytes. When HaCaT cells were pretreated with 4-O-methylhonokiol, TGF- β 1-induced G1/G0 phase arrest and TGF- β 1-induced p21 expression were decreased. Moreover, 4-O-methylhonokiol inhibited nuclear translocation of Smad2/3, Smad4 and Sp1 in TGF- β 1-induced canonical pathway. We observed that ERK phosphorylation by TGF- β 1 was significantly attenuated by treatment with 4-O-methylhonokiol. 4-O-methylhonokiol inhibited TGF- β 1-induced reactive oxygen species (ROS) production and reduced the increase of NADPH oxidase 4 (NOX4) mRNA level in TGF- β 1-induced noncanonical pathway. These results indicate that 4-O-methylhonokiol could inhibit TGF- β 1-induced cell cycle arrest through inhibition of canonical and noncanonical pathways in human keratinocyte HaCaT cell and that 4-O-methylhonokiol might have protective action on TGF- β 1-induced cell cycle arrest.

Key Words: 4-O-methylhonokiol, Transforming growth factor- β 1, Cell cycle arrest, *Smads*, Reactive oxygen species, NADPH oxidase 4

INTRODUCTION

The growth and development of hair follicles are regulated by a variety of growth factors and cytokines. Insulin-like growth factor 1 (IGF-1), fibroblast growth factor 7 (FGF-7), and hepatocyte growth factor (HGF) are known to positively regulate hair growth, whereas IL-1, FGF-5, and transforming growth factor- β (TGF- β) are negatively regulate hair growth.

It was reported that TGF- β plays a critical role in the growth of hair follicles as well as their morphogenesis (Foitzik *et al.*, 1999; Paus *et al.*, 1999). The localization of the TGF- β isoforms in hair follicles has also been observed (Soma *et al.*, 2002). Particularly, TGF- β 1 was strongly detected in the hair cuticle, hair cortex and connective tissue sheath cells of the anagen hair follicle (Soma *et al.*, 2002). TGF- β 1 has been

considered to be a potent triggering factor of catagen induction in the murine hair cycle (Foitzik *et al.*, 2000). In addition, TGF- β 1 is involved in the regulation of hair follicle regression, and is capable of inducing premature catagen *in vivo* via the induction of apoptosis and inhibition of keratinocyte proliferation (Seiberg *et al.*, 1995; Welker *et al.*, 1997). Furthermore, androgen-inducible TGF- β 1 derived from dermal papilla cells (DPCs) has been shown to mediate the suppression of hair growth and epithelial cell growth in androgenetic alopecia (AGA) (Inui *et al.*, 2002). Collectively, TGF- β 1 operates as a catagen inducer and indirectly suppresses hair growth.

TGF- β 1 is a well-known growth inhibitory modulator in a variety of cell types including epithelial, endothelial, and myeloid cells (Silberstein and Daniel, 1987; Bascom *et al.*, 1989). Most of TGF- β effects arise from its ability to regulate transcription

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***Corresponding Author**

E-mail: pharmkhk@jejunu.ac.kr

Tel: +82-64-754-3846, Fax: +82-64-702-2687

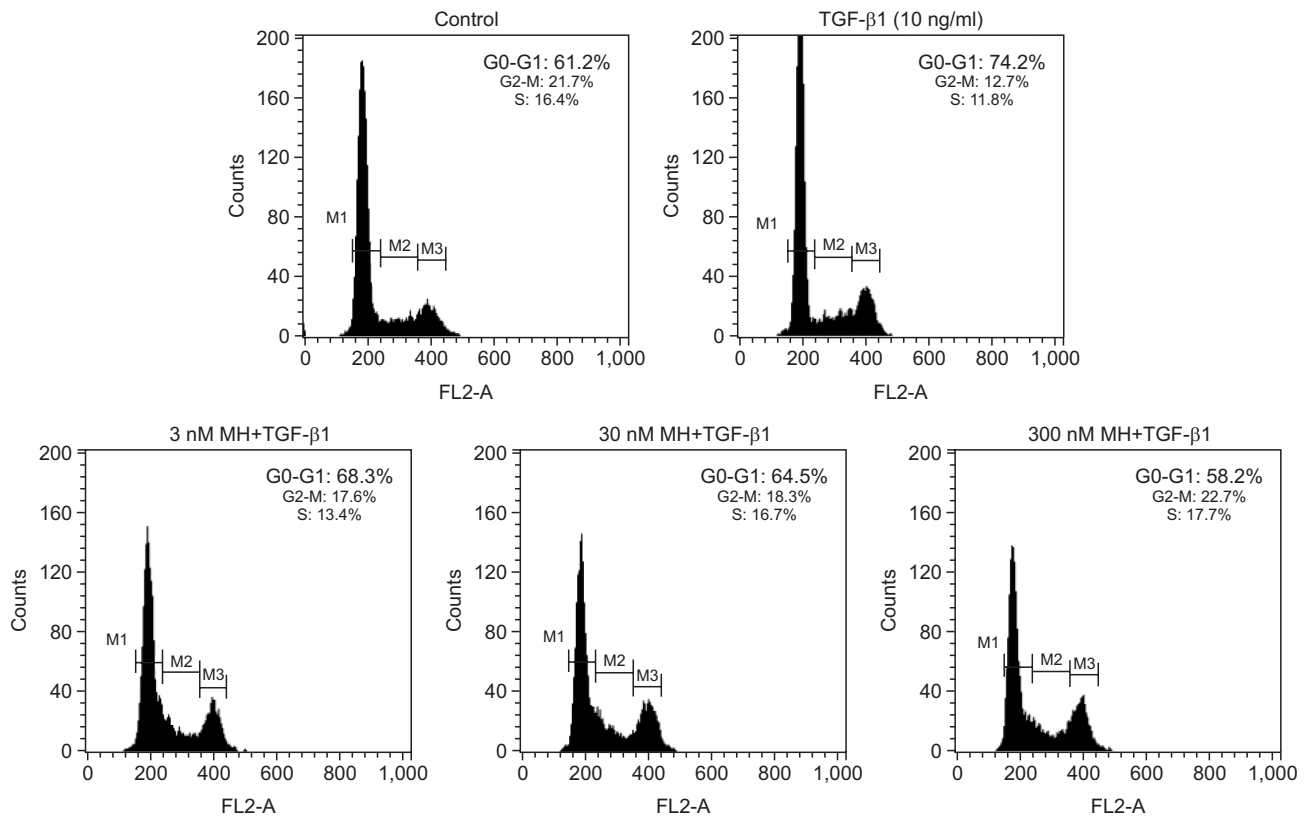


Fig. 1. The effect of 4-O-Methylhonokiol (MH) on TGF-β1-induced G1 arrest in HaCaT cell. After serum starvation for 48 h, HaCaT were pretreated with MH at final concentrations of 3, 30 and 300 nM for 1 h, and then treated with TGF-β1 (10 ng/mL) for 24 h. Cellular DNA was stained with PI staining solution and analyzed by flowcytometry.

of specific sets of genes. Among the genes, induction of p21 gives rise to the effect of TGF-β on the growth inhibition (Li *et al.*, 1995). Increase of the p21 expression in response to TGF-β results from Smad-mediated transcriptional activation. TGF-β-activated Smad2 or Smad3 forms a complex with Smad4 and the complex induces transcription by interacting with Sp1 at the p21 promoter (Pardali *et al.*, 2000). On the other hand, TGF-β has been reported to increase intracellular reactive oxygen species (ROS) in several cell types, such as vascular endothelial cells (Hong *et al.*, 1997) and human lung fibroblast cells (Thannickal *et al.*, 1998). ROS is an important regulator of cell cycle and apoptosis in many cell types (Guyton *et al.*, 1996; Reagan-Shaw *et al.*, 2006). Previous study showed that elevated ROS could induce apoptosis by activation of extracellular signal-regulated protein kinase (ERK) (Guyton *et al.*, 1996). In addition, several studies have documented significant generation of ROS in a variety of cells which is usually the consequence of mitochondrial respiration and NADPH oxidase (NOX) activity (Cheng *et al.*, 2001). NOX is expressed in many cell types and exists in at least five isoforms termed NOX1, NOX2, NOX3, NOX4 and NOX5 (Cheng *et al.*, 2001). Little is known about the role of NOX, except that it was designed for ROS generation. However, previous studies revealed that the level of NOX4 increase in response to TGF-β, which result in the apoptotic cell death (Carmona-Cuenca *et al.*, 2008; Yan *et al.*, 2014).

4-O-Methylhonokiol has been reported to have anti-inflammatory, anti-cancer, and memory improving effects (Oh *et al.*,

2009; Lee *et al.*, 2009b, 2011, 2013; Hyun *et al.*, 2015). We also found that 4-O-Methylhonokiol purified from *Magnolia officinalis* can promote the proliferation of DPCs and induce anagen phase *in vivo* via down-regulation of TGF-β1 and TGF-β2 in the outer root sheath (ORS) and epithelial strand (Kim *et al.*, 2011). In this report, we address if 4-O-Methylhonokiol can potentially protect HaCaT human keratinocytes from TGF-β1-induced cell cycle arrest and how 4-O-Methylhonokiol regulates TGF-β1 signaling pathway in HaCaT human keratinocytes.

MATERIALS AND METHODS

Compound preparation

4-O-Methylhonokiol was supplied by Bioland Ltd (Chungnam, Korea). In brief, 4-O-Methylhonokiol was isolated from the dried bark of *Magnolia officinalis* Rehd. *et* Wils. as described (Lee *et al.*, 2009b). A voucher specimen was deposited at the Herbarium of Chungbuk National University, Chungbuk, Korea (voucher specimen # CNBU2009006) (Zhang *et al.*, 2014). The dried bark of *Magnolia officinalis* Rehd. *et* Wils. was extracted twice with 95% (v/v) ethanol and concentrated under reduced pressure. The ethanol extract was suspended in H₂O and then extracted with *n*-hexane, ethyl acetate, and *n*-BuOH, respectively. The *n*-hexane fraction was subjected silica gel chromatography with *n*-hexane:ethyl acetate (9:1) gradient. The purified compound (4-O-Methylhonokiol; pu-

rity>99.5%) was then identified by $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ as described elsewhere (Lee *et al.*, 2009b). Stock solution of 4-O-Methylhonokiol was diluted in 0.05% dimethyl sulfoxide (DMSO) (final concentration, 50 mM).

Reagents

Antibodies against ERK1/2, phospho-pERK1/2, p38, phospho-p38, SAPK/JNK and phospho-SAPK/JNK were obtained from Cell signaling Technology (Beverly, MA, USA). Recombinant human TGF- β 1 was purchased from R&D systems (Minneapolis, MN, USA). Antibodies against TGF- β 1, PCNA, Smad2/3, Smad4 and monoclonal β -actin were obtained from Sigma (St. Louis, MO, USA) and p21 was obtained from BD Biosciences (San Diego, CA, USA). Antibody against Sp1 was purchased from Millipore (Billerica, MA, USA). HRP-conjugated goat anti-rabbit and horse anti-mouse IgGs were obtained from Vector (Vector Laboratories, Burlingame, CA, USA). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE) were obtained from Sigma.

Cell culture

HaCaT cells, immortalized human keratinocytes were obtained from Amore Pacific Company (Yongin, Korea). HaCaT cells were cultured in DMEM (Hyclone/Inc, UT, USA) supplemented with 10% fetal bovine serum (Gibco Inc., Grand Island, NY, USA) and penicillin/streptomycin (100 unit/mL and 100 $\mu\text{g/mL}$, respectively) at 37°C in a humidified atmosphere under 5% CO_2 .

RNA preparation and RT-PCR

To extract total RNA, we used the Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) protocol following the manufacturer's instructions. RNA isolation was carried out in an RNase-free environment. The 1 μg aliquots of RNA were reverse transcribed using MuLV reverse transcriptase (Promega, Madison, WI, USA), oligo (dT)₁₅ primer, deoxyribonucleotide triphosphate (dNTP) (0.5 μM) and 1 URNase inhibitor. The primer sequences of NOX1, NOX2, and NOX4 were as follows; NOX1-sense: gatcaattvagtgtgavgaccac; antisense: cagactgcaatatcggtgacagca (420 bp); NOX2-sense: ggagttcaagatcggtgaaacta; antisense: cagactgcaatatcggtgacagca (550 bp); NOX4-sense: ctgagcggaaatcaatcagctgtg; antisense: agaggaaacacgacaatcagccttag (251 bp). The polymerase chain reaction (PCR) was performed with a C1000™ Thermal cycler (Bio-Rad, HC, USA), and the amplification was followed by 35 cycles of 94°C for 30 sec (denaturing), 60°C for 30 sec for (annealing) and 72°C for 30 sec (extension). The PCR products were electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide staining.

Cell cycle analysis

HaCaT cells (1.0×10^5 cells/mL) were pre-incubated for 24 h, and then washed 3 times with PBS, cultured 48 h in serum-free DMEM. Serum starvation-arrested cells were stimulated by 10% FBS without or with TGF- β 1 (10 ng/mL) and in the absence or presence of 4-O-Methylhonokiol. For the flow cytometric analysis to determine cell cycle phase distribution, the treated cell were harvested, washed twice with PBS, and fixed in 70% ethanol for 30 min at 4°C. The fixed cells were washed twice with cold PBS, incubated with 50 $\mu\text{g/mL}$ RNase A at 37°C for 30 min, and stained with 50 $\mu\text{g/mL}$ propidium iodide (PI) in the dark for 30 min at 37°C. The stained cells were ana-

lyzed using fluorescence activated cell sorter (FACS) caliber flow cytometry (Becton Dickinson, San Jose, CA, USA). Histograms were analyzed with the software program Cell Quest (Becton Dickinson) (Krishan, 1975).

Western blot analysis

To obtain whole cell protein, the HaCaT cells were lysed with a lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM NaVO_3 , 10 mM NaF, 1 mM dithiothreitol, 1 mM Phenylmethylsulfonyl fluoride, 25 $\mu\text{g/mL}$ aprotinin, 25 $\mu\text{g/mL}$ leupeptin, 1% Nonidet P-40) for 30 min at 4°C. The cell lysates were centrifuged at 15,000 rpm at 4°C for 15 min. The supernatant was stored at -20°C until analysis. In some experiments, nuclear fraction was prepared using NE-PER nuclear extraction reagents (Pierce Biotechnology, Rockford, IL, USA) by following the manufacturer's instructions. Protein concentration was determined by Bradford method (Bradford, 1976). Equal amount of protein was loaded onto a SDS-PAGE gel. After electrophoretic separation, proteins were transferred onto a polyvinylidene fluoride membrane (Bio-Rad) with a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)) at 120 V for 1.5 h. After blocking with 1% bovine serum albumin in TBS-T (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20), the membrane was incubated with specific primary antibodies at 4°C overnight. Primary antibody (1:500-1:1000) incubation was followed by washing with TBS-T and then incubating with a secondary HRP antibody (1:5000; Vector Laboratories) at room temperature for 1 h. A chemiluminescence reaction (ECL, Intron Biotechnology, Sungnam, Korea) was used to visualize protein bands on X-ray films (AGFA, Mortsel, Belgium). All blots were probed with β -actin to confirm that equal amounts of protein were loaded.

Measurement of intracellular ROS

Intracellular ROS was analyzed by flow cytometry using DCFH-DA and DHE. HaCaT cells (1.0×10^5 cells/mL) were seeded on 35-mm dishes and incubated for 24 h, then washed 3 times with PBS, cultured for 24 h in serum-free DMEM. After 24 h, HaCaT cells were preincubated with 4-O-Methylhonokiol (3, 30 and 300 nM) for 1 h and treated with TGF β 1 (10 ng/mL) for different times. The cells were washed with PBS and incubated in the medium containing DCFH-DA (10 μM) or DHE (10 μM) for 30 min at 37°C with 5% CO_2 in the dark. The HaCaT cells were washed twice with PBS and intracellular accumulation of fluorescence DCF-DA or DHE were measured (10,000 cells each) using a FACScan Flow Cytometer (Becton Dickinson). For DCF-DA assay, excitation was at 488 nm with an emission at 540 nm. For DHE assay, excitation was at 535 nm with an emission at 610 nm. Histograms were analyzed with the software program Cell Quest (Becton Dickinson).

Superoxide dismutase (SOD) and catalase (CAT) activity

The HaCaT cells were seeded at 1×10^5 cells/mL. After 16 h incubation, the cells were treated with various concentrations of 4-O-Methylhonokiol for 1 h and harvested. The cells were suspended in 10 mM potassium phosphate buffer (pH 7.5) and then lysed on ice by sonication twice for 15 sec. Triton X-100 (0.1%) was then added to the lysates and the lysates were incubated for 10 min on ice. The lysates were centrifuged at 5000 \times g for 30 min at 4°C to remove the cellular debris. The protein amount of the supernatant was determined by Bradford method (Bradford, 1976).

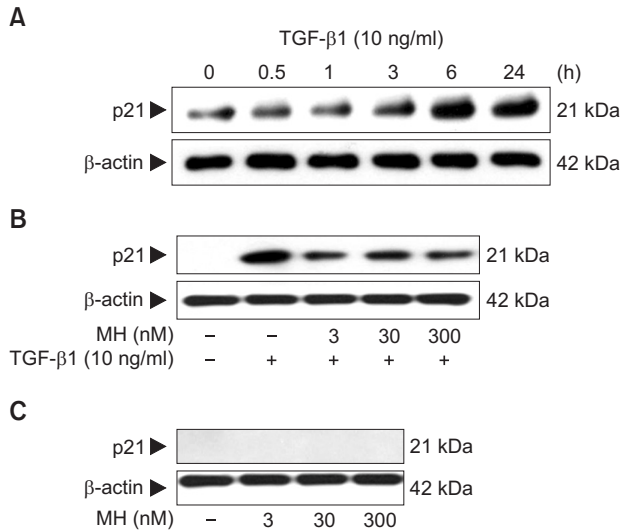


Fig. 2. The effect of 4-O-Methylhonokiol (MH) on TGF-β1-induced p21 expression in HaCaT cell. (A) HaCaT cells were treated with TGF-β1 (10 ng/mL) for the indicated times. Whole lysates (30 μg proteins) were examined by 10% SDS-PAGE and analyzed with Western blotting using for p21. (B) After pretreatment with MH (3, 30 and 300 nM) for 1 h, HaCaT cells were treated with TGF-β1 (10 ng/mL) for another 24 h. The expression level of p21 was analyzed by Western blotting. (C) HaCaT cells were incubated with MH (3, 30 and 300 nM) for 24 h, and the expression level of p21 was analyzed. Blots were then probed with the corresponding antibodies after which they were stripped and reprobed with an antibody to β-actin to ensure equivalent loading and transfer.

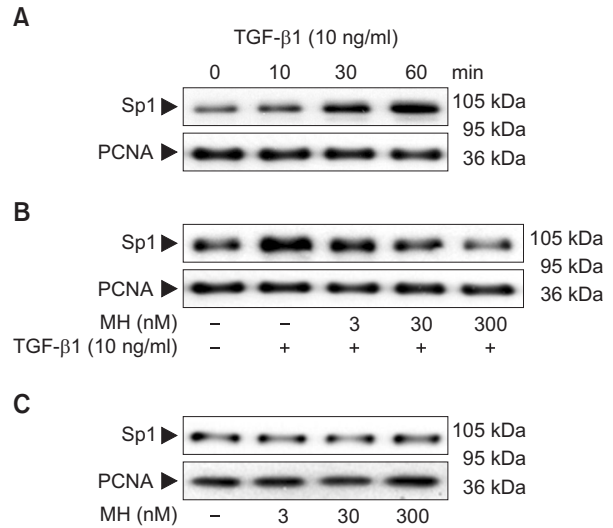


Fig. 4. The effect of 4-O-Methylhonokiol (MH) on TGF-β1-induced nuclear translocation of Sp1 in HaCaT cell. (A) HaCaT cells were treated with TGF-β1 (10 ng/mL) for the indicated times. Nuclear fractions were examined by 8% SDS-PAGE and analyzed with Western blotting using antibody against Sp1. (B) After pretreatment with MH (3, 30 and 300 nM) for 1 h, HaCaT cells were treated with TGF-β1 (10 ng/mL) for another 1 h. The nuclear translocation of Sp1 was analyzed by Western blotting. (C) HaCaT cells were incubated with MH (3, 30 and 300 nM) for 1 h, and the expression of Sp1 was analyzed. Blots were then probed with the corresponding antibodies after which they were stripped and reprobed with an antibody to PCNA to ensure equivalent loading and transfer.

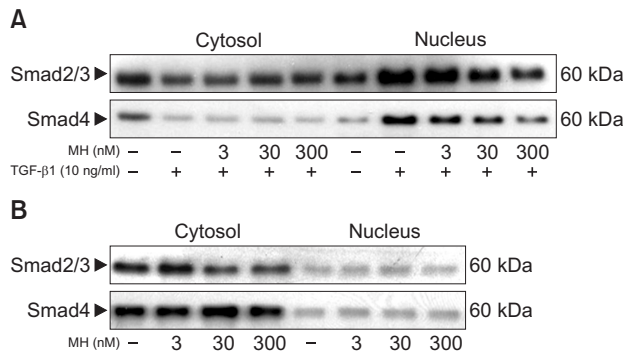


Fig. 3. The effect of 4-O-Methylhonokiol (MH) on TGF-β1-induced Smads translocation in HaCaT cell. Nuclear translocation of Smad proteins was analyzed by separating cytoplasmic and nuclear fractions after treating HaCaT cells with TGF-β1 and/or MH for 1 h. Smad proteins were detected by Western blotting using antibodies against Smad2/3 and Smad4, respectively.

The SOD activity has been used to detect the inhibition level of epinephrine auto-oxidation (Misra and Fridovich, 1972). 5 μg of the protein was added to 50 mM potassium phosphate buffer (pH 10.2) containing 0.1 mM EDTA and 0.4 mM epinephrine. Epinephrine rapidly undergoes auto-oxidation at pH 10 to produce adrenochrome, a pink colored product, which can be measured at 480 nm using a UV/VIS spectrophotometer in kinetic mode. SOD inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored at 480 nm. The SOD activity was expressed as units/mg protein and one unit

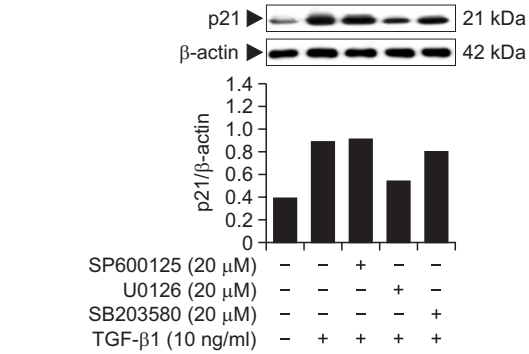


Fig. 5. Effect of MAPK inhibitors on TGF-β1-induced p21 expression in HaCaT cells. HaCaT cells were pretreated with U0126 (20 μM), SB203580 (20 μM), and SP600125 (20 μM) for 1 h, and further incubated in the absence or presence of TGF-β1 (10 ng/mL) for 24 h. Whole lysates (30 μg proteins) of the HaCaT cells were examined by 10% SDS-PAGE and analyzed with Western blotting using antibodies for p21.

of enzyme activity was defined as the amount of enzyme required for 50% inhibition of auto-oxidation of epinephrine.

For measurement of the CAT activity, 5 μg of protein was added to 50 mM potassium phosphate buffer (pH 7) containing 100 mM (v/v) H₂O₂. The reaction mixture was incubated for 2 min at 37°C and the absorbance was monitored at 240 nm for 5 min. The change in absorbance with time was proportional to the breakdown of H₂O₂ (Carrillo *et al.*, 1991). The CAT activity was expressed as units/mg protein and one unit

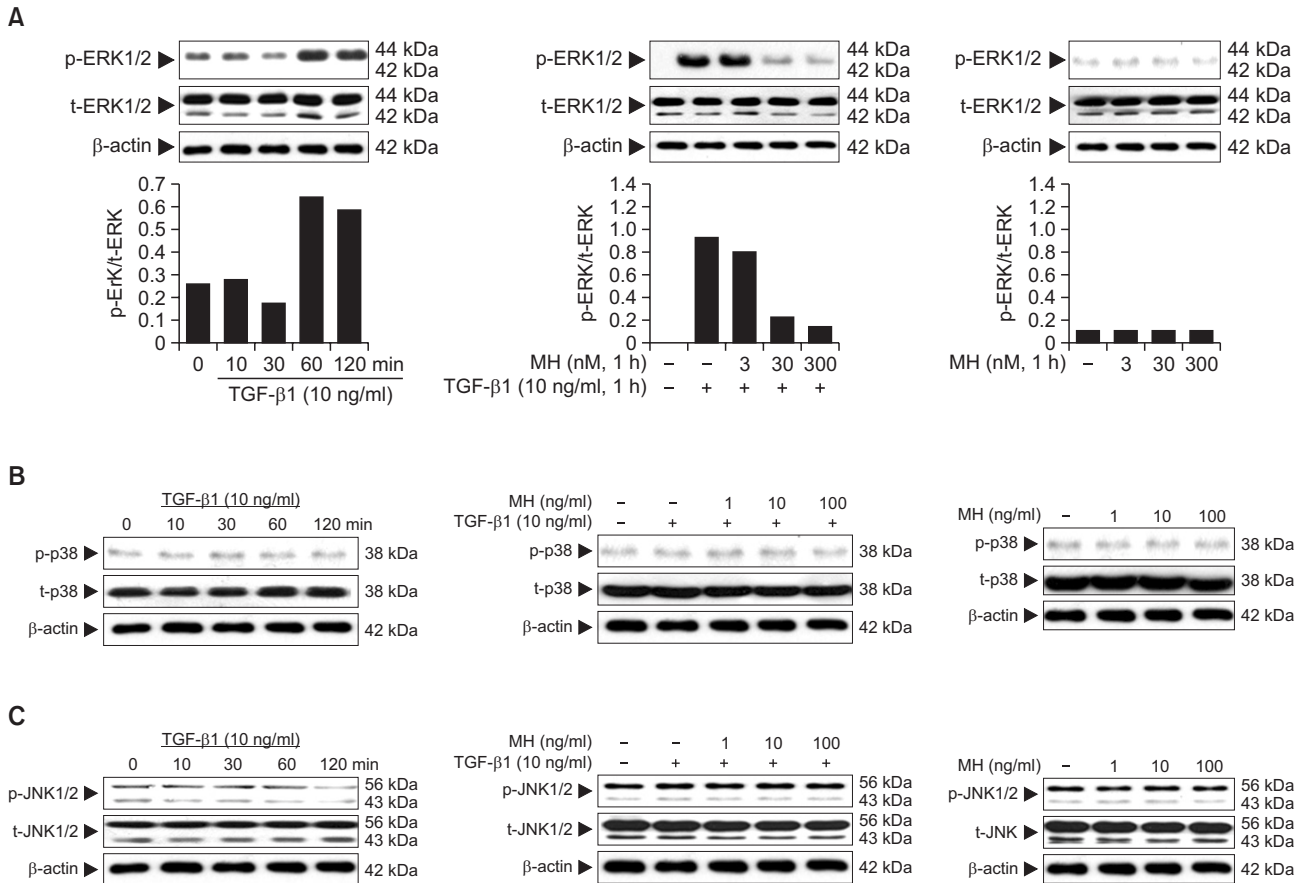


Fig. 6. Effect of 4-O-Methylhonokiol (MH) on TGF- β 1-induced MAPKs activation in HaCaT cells. After serum starvation for 24 h, HaCaT cells were stimulated with TGF- β 1 (10 ng/mL) for the indicated times. Whole lysates (30 μ g proteins) were examined by 10% SDS-PAGE and analyzed with Western blotting using antibodies for phospho-ERK1/2 or ERK1/2 (A), phospho-p38 or p38 (B), phospho-JNK or JNK (C). After pretreatment with MH (3, 30 and 300 nM) for 1 h, HaCaT cells were stimulated with TGF- β 1 (10 ng/mL) for another 1 h. Phosphorylations of ERK1/2, p38, and JNK were analyzed by Western blotting.

of enzyme activity was defined as the amount of enzyme required to breakdown of 1 mM H₂O₂.

RESULTS

4-O-Methylhonokiol inhibited TGF- β 1-induced G1 arrest in HaCaT cell

To assess whether 4-O-Methylhonokiol can inhibit TGF- β 1-induced G1 arrest in HaCaT cells, we performed propidium iodide staining followed by flowcytometric analysis. As shown in Fig. 1, TGF- β 1 (10 ng/mL) arrested cells in the G0-G1 phase (74.2%) of the cell cycle. Pretreatment with 4-O-Methylhonokiol at 3, 30 and 300 nM before TGF- β 1 (10 ng/mL) treatment decreased the percentage of G1 phase cells to 68.3%, 64.5% and 58.2%, respectively, in a dose-dependent manner.

4-O-Methylhonokiol inhibited the increase of TGF- β 1-induced p21 expression in HaCaT cell

Previous studies have shown that p21 is an important cell cycle regulator and is induced by TGF- β 1. The induction of p21 by TGF- β 1 plays a causative role in TGF- β 1-mediated inhibition of cell growth (Moustakas *et al.*, 2002). Firstly, we

confirmed that TGF- β 1 treatment led to an increase in the expression level of p21 in human keratinocytes HaCaT cells in a time-dependent manner (Fig. 2A). We examined 4-O-Methylhonokiol effect on TGF- β 1-induced p21 expression. HaCaT cells were pretreated with 4-O-Methylhonokiol at final concentrations of 3, 30 and 300 nM for 60 min, and then treated with 10 ng/mL of TGF- β 1 for 24 h. As shown in Fig. 2B, 4-O-Methylhonokiol treatment decreased TGF- β 1-induced p21 expression. When treated with 4-O-Methylhonokiol alone (3, 30 and 300 nM) for 24 h, the expression of p21 was not observed (Fig. 2C).

4-O-Methylhonokiol inhibited TGF- β 1-mediated nuclear translocation of Smads

It has been reported that Smads are translocated into the nucleus compartment in response to TGF- β 1 stimulation, which was essential for induction of TGF- β 1-target gene (Massague and Wotton, 2000). To determine the effect of 4-O-Methylhonokiol on TGF- β 1-mediated nuclear translocation of Smad proteins, we analyzed levels of Smads in the cytoplasmic and nuclear fractions after treating HaCaT cells with TGF- β 1 and/or 4-O-Methylhonokiol. As shown in Fig. 3, TGF- β 1 induced nuclear translocation of Smad2/3, and

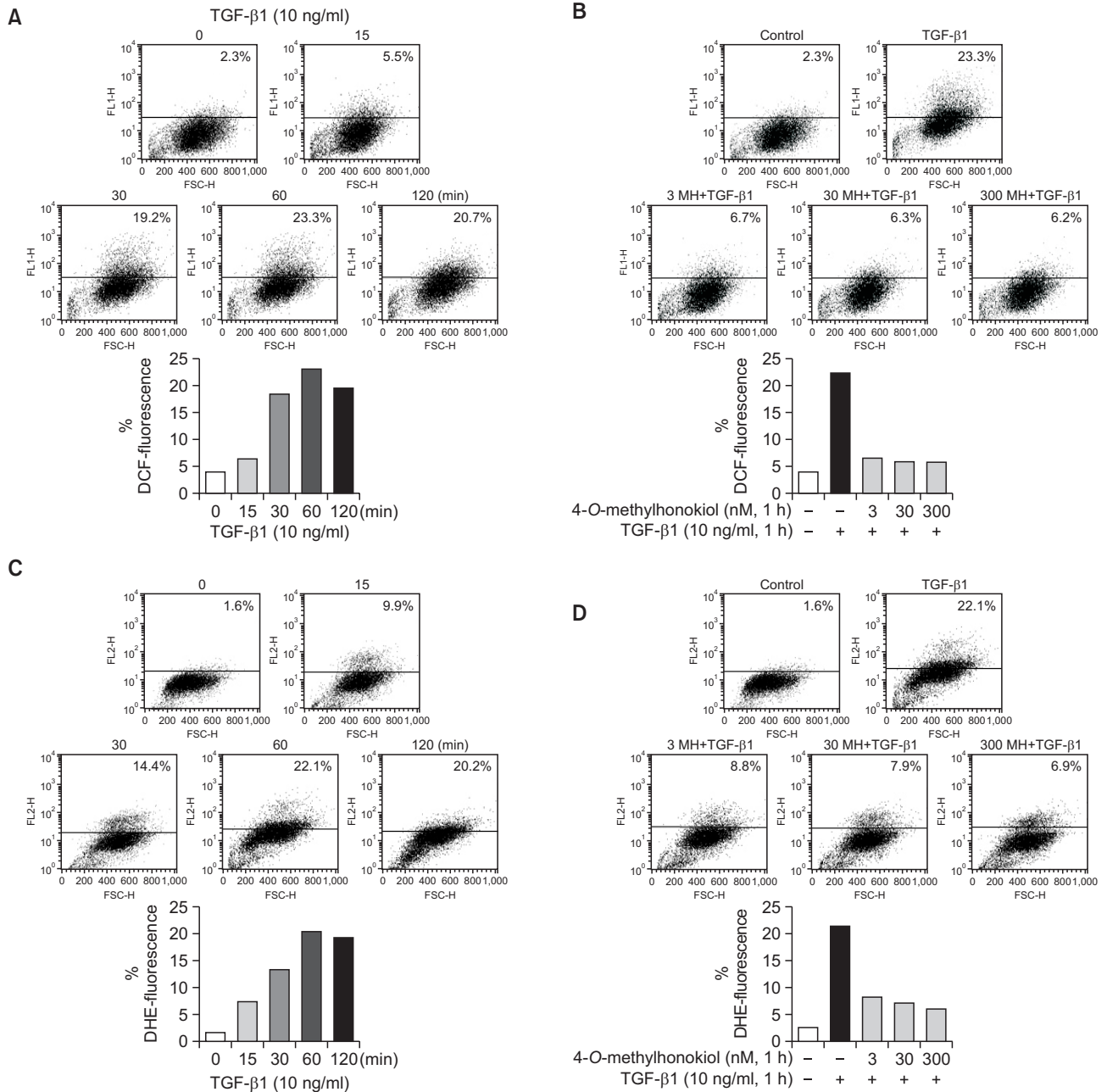


Fig. 7. Effect of 4-O-Methylhonokiol (MH) on the TGF- β 1-induced ROS production in HaCaT cells. (A) After incubation of serum-starved HaCaT cells in the presence of TGF- β 1 (10 ng/mL) for the indicated times, DCF fluorescence was measured with flow cytometry. (B) HaCaT cells were pretreated with MH (3, 30 and 300 nM) for 1 h, and further incubated following treatment with TGF- β 1 (10 ng/mL) for 1 h. DCF fluorescence was measured with flow cytometry. (C) After incubation of serum-starved HaCaT cells in the presence of TGF- β 1 (10 ng/mL) for the indicated times, DHE fluorescence was measured with flow cytometry. (D) HaCaT cells were pretreated with MH (3, 30 and 300 nM) for 1 h, and further incubated following treatment with TGF- β 1 (10 ng/mL) for 1 h. DHE fluorescence was measured with flow cytometry.

Smad4 within 60 min, which was blocked by 4-O-Methylhonokiol. 4-O-Methylhonokiol alone did not show any effect on the localization of Smad proteins. These results demonstrated that 4-O-Methylhonokiol could block TGF- β 1-mediated nuclear translocation of Smad2/3, and Smad4.

4-O-Methylhonokiol inhibited TGF- β 1-induced nuclear translocation of Sp1 in HaCaT cell

It has been demonstrated that p21 induction by TGF- β 1 is mediated through binding of Sp1 transcription factor to Sp1 binding site in the promoter region (Pardali *et al.*, 2000), and interaction between Sp1 transcription factor and Smad proteins is critical for the induction of TGF- β 1 target genes (Pardali *et al.*, 2000). To determine whether 4-O-Methylhonokiol

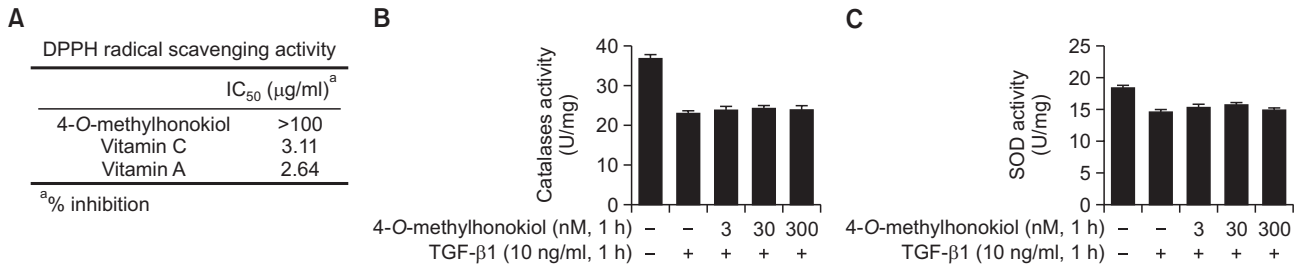


Fig. 8. Effect of 4-O-Methylhonokiol (MH) on enzymatic and non-enzymatic antioxidant systems. (A) DPPH radical scavenging activity. Data are expressed as the concentration necessary to scavenge 50% of DPPH radical. (B) Catalase and (C) SOD activity in HaCaT cells. The enzyme activities were expressed as average enzyme unit per milligram protein.

could affect the TGF-β1-induced nuclear translocation of Sp1, HaCaT cells were pretreated with 4-O-Methylhonokiol at final concentrations of 3, 30 and 300 nM for 1 h, and then treated with TGF-β1 (10 ng/mL) for 1 h. Treatment with TGF-β1 (10 ng/mL) increased the level of Sp1 in the nucleus of HaCaT cells (Fig. 4A), while pretreatment with 4-O-Methylhonokiol inhibited the TGF-β1-mediated increase of Sp1 nuclear level in a dose-dependent manner (Fig. 4B). However, 4-O-Methylhonokiol alone did not affect level of Sp1 (Fig. 4C).

Effect of 4-O-Methylhonokiol on TGF-β1-induced MAPKs activation in HaCaT cells

It has been known that the mitogen-activated protein kinase (MEK) pathway is required for the TGF-β1-induced stimulation of p21 (Hu *et al.*, 1999). We examined the effect of U0126, a pharmacological inhibitor of MEK, on the TGF-β1-induced increase of p21. As shown in Fig. 5, pretreatment of HaCaT cells with U0126 dramatically inhibited the TGF-β1-induced increase of p21. To assess whether the other ERK family members such as p38 and JNK, are involved in induction of p21 by TGF-β1, we examined the effect of SB203580 and SP600125, specific inhibitors of p38 and JNK, respectively, on the TGF-β1-induced increase of p21. Pretreatment of HaCaT cells with these inhibitors did not affect the level of TGF-β1-induced p21 (Fig. 5). These data indicate that induction of p21 by TGF-β1 requires the activation of ERK signaling pathway, but not of p38 or JNK pathway.

We addressed if 4-O-Methylhonokiol could affect the TGF-β1-induced activation of ERK pathway. The increase of ERK phosphorylation following TGF-β1 treatment was apparent with maximum activity at 60 min, which was sustained longer than 120 min thereafter (Fig. 6A). 4-O-Methylhonokiol decreased the TGF-β1-induced activation of ERK in a dose-dependent manner (Fig. 6A), whereas TGF-β1 did not affect the activation of p38 and JNK (Fig. 6B, 6C).

Effect of 4-O-Methylhonokiol on the TGF-β1-induced ROS production in HaCaT cells

Recent accumulating evidences suggest that ROS is an important intracellular messenger to mediate the activation of various signaling molecules in various cells (Yoon *et al.*, 2005). We examined the effect of 4-O-Methylhonokiol on the TGF-β1-induced ROS production. When HaCaT cells were incubated with TGF-β1 (10 ng/mL) for 15, 30, 60 and 120 min, the increase of DCF fluorescence appeared from 30 min, with maximum production at 60 min, which was slightly decreased at 120 min (Fig. 7A). However, when HaCaT cells were pre-

treated with 4-O-Methylhonokiol followed by the treatment with TGF-β1 (10 ng/mL) for 60 min, pretreatment of 4-O-Methylhonokiol significantly reduced the TGF-β1-induced ROS generation (Fig. 7B). In addition, we also evaluated whether 4-O-Methylhonokiol could block production of superoxide by TGF-β1. Fig. 7C shows that the DHE fluorescence increased in TGF-β1-treated HaCaT cells, whereas the pretreatment of 4-O-Methylhonokiol reduced the TGF-β1-induced ROS generation (included superoxide form) (Fig. 7D).

To assess how 4-O-Methylhonokiol could inhibit TGF-β1-induced ROS production, we examined the effect of 4-O-Methylhonokiol on the DPPH free radical scavenging activity, the SOD and CAT activities. As shown in Fig. 8, 4-O-Methylhonokiol did not affect the DPPH free radical scavenging activity, the SOD and CAT activities.

Effect of 4-O-Methylhonokiol on the TGF-β1-induced NADPH oxidase NOX4 in HaCaT cells

It has been previously reported that NOX4 mediates TGF-β1-induced ROS production (Carmona-Cuenca *et al.*, 2008). We examined the effect of 4-O-Methylhonokiol on TGF-β1-induced NOXs expressions. As shown in Fig. 9A, NOX4 mRNA expression increased at 30 min after TGF-β1 treatment. TGF-β1 did not affect the expression of NOX1 and NOX2. However, pretreatment of 4-O-Methylhonokiol reduced increase of NOX4 mRNA level upon TGF-β1 (Fig. 9B).

DISCUSSION

In this study, we addressed the effect and mechanism of 4-O-Methylhonokiol, a neolignan compound from *M. officinalis*, on TGF-β1-induced cell cycle arrest in human keratinocyte HaCaT cells. To the best of our knowledge, this is the first study to demonstrate that 4-O-Methylhonokiol could inhibit TGF-β1-induced cell cycle arrest through down regulation of canonical and noncanonical pathways.

TGF-β1 is a multifunctional cytokine that has the ability to regulate cell differentiation, survival, and death. In many cell types, TGF-β1 inhibits cellular proliferation by causing growth arrest in the G1 phase of the cell cycle (Senturket *et al.*, 2010). The TGF-β1 induced G1 arrest has been attributed to the regulatory effects of TGF-β1 on the levels and activities of cyclins-cyclin-dependent kinase (CDK) complexes and cyclin dependent kinase inhibitors (p21^{WAF1/Cip1}, p27^{kip1}, p18, p16, and p15) (Senturket *et al.*, 2010). We found that 4-O-Methylhonokiol could prevent TGF-β1 induced keratinocyte growth arrest in

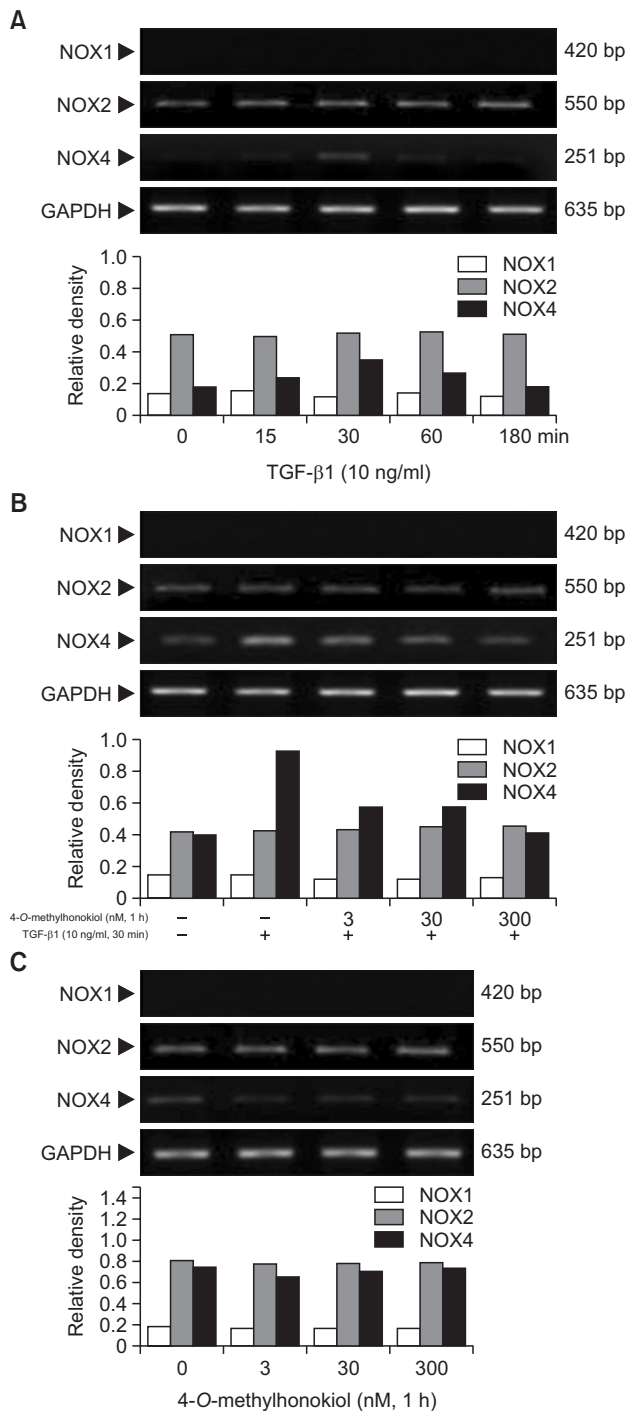


Fig. 9. Effect of 4-O-Methylhonokiol (MH) on the TGF-β1-induced NADPH oxidase NOX4 in HaCaT cells. (A) After serum starvation for 24 h, HaCaT cells were treated with TGF-β1 (10 ng/mL) for the indicated times. RNA extraction was carried out in RNase-free environment and the mRNA expression of NOX1, NOX2 and NOX4 were determined by RT-PCR. (B) After pretreated with MH (3, 30, 300 nM) for 1 h, HaCaT cells were incubated with TGF-β1 (10 ng/mL) for 30 min. The mRNA expression of NOX1, NOX2 and NOX4 were determined by RT-PCR as described above. (C) HaCaT cells were treated with MH (3, 30, 300 nM) for 1 h, and the mRNA expression of NOX1, NOX2 and NOX4 were determined by RT-PCR as described above.

G1 phase via the decrease of p21, one of downstream effectors of TGF-β1 pathway (Fig. 1, 2). On the other hand, 4-O-methylhonokiol increased p21 and apoptotic proteins, which are followed by the inhibition of prostate tumor growth (Lee *et al.*, 2013). Smad proteins may play a role in regulation of CDK inhibitor p21 gene expression by TGF-β1, because TGF-β1-induced p21 gene expression is further enhanced by overexpression of Smad3 and Smad4. In contrast, dominant negative mutants of Smad3 and Smad4 inhibit TGF-β1-induced p21 gene expression (Pardali *et al.*, 2000). In the present study, Smad2/3, Smad4 and Sp1 were significantly translocated into the nucleus following treatment with TGF-β1, whereas this conventional TGF-β1 cascade was blocked by 4-O-Methylhonokiol (Fig. 3, 4). It has been demonstrated that the ability of Smads to induce specific transcription programs in response to TGF-β1 results from a functional cooperation with other transcription factors in multiprotein complexes in nucleus (Datta *et al.*, 2000; Massague and Wotton, 2000). In addition, the requirement of functional and physical interaction between Smad proteins and Sp1 transcription factors for the induction of p21 in response to TGF-β1 has been reported (Ellenrieder, 2008). The interaction of SMAD3/4 with Sp1 and FoxO1 transcription factors by TGFβ signaling could modulate oligodendrocyte progenitor cell cycle withdrawal and differentiation through the transcriptional modulation of c-myc and p21 gene expression (Palazuelos *et al.*, 2014). Collectively, 4-O-Methylhonokiol might inhibit TGF-β1-induced cell cycle arrest through inhibition of canonical pathway in human keratinocyte HaCaT cell.

In addition to the Smad pathway, the canonical pathway of TGF-β signaling, there are several examples of interactions between TGF-β signaling and noncanonical pathways such as mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), β-catenin and phosphatidylinositol 3-kinase (PI3K) (Li *et al.*, 2001). Importantly, several studies have demonstrated that MAPKs are involved in TGF-β-induced p21 expression on a variety of cell types (Hu *et al.*, 1999; Senturk *et al.*, 2010). In addition, both Smad- and Ras/MAPK mediated pathways target a proximal sequence of the p21 promoter that can recruit transcription factors of the Sp1 family together with Smads (Moustakas *et al.*, 2002). The pretreatment of HaCaT keratinocytes with U0126, MEK inhibitor, dramatically inhibited induction of p21 by TGF-β1, whereas pretreatment of cells with SB203580 and SP600125, specific inhibitors of p38 and JNK, did not affect the induction of p21 by TGF-β1 (Fig. 5). These data indicate that induction of p21 by TGF-β1 may require the activation of ERK signaling pathway, but neither of p38 nor JNK signaling pathway. TGF-β1-induced ERK activation was completely inhibited by pretreatment with 4-O-Methylhonokiol (Fig. 6A). 4-O-Methylhonokiol induces neurotrophic factors through ERK activation in rat embryonic neuronal cells (Lee *et al.*, 2009a), whereas memory improving effect of 4-O-Methylhonokiol may be associated with the suppression of ERK activation in presenilin 2 mutant mice (Lee *et al.*, 2011). On the other hand, the growth arrest in response to TGF-β1 was accompanied by ROS production (Yoon *et al.*, 2005). Various mechanisms are involved in ROS production by TGF-β1. ROS generation in response to TGF-β1 stimulation is rapid and engages non-SMAD (e.g., EGFR, Src kinase, MAPKs, and p53) and SMAD2/3 pathways (Samarakoon *et al.*, 2013). In addition, it has been previously reported that NOX4 mediates TGF-β1-induced ROS production (Carmona-Cuenca *et al.*,

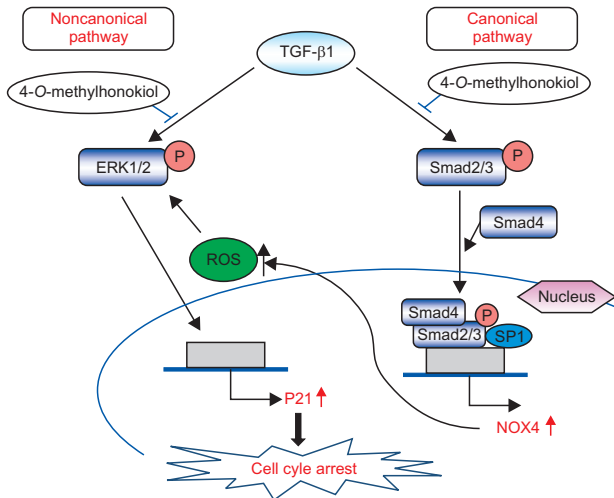


Fig. 10. The scheme for target of 4-O-Methylhonokiol on the TGF- β pathway.

2008; Peshavariya *et al.*, 2014). Pretreatment of HaCaT cells with 4-O-Methylhonokiol reduced the TGF- β 1-induced ROS generation (Fig. 7). NOX4 mRNA expression increased at 30 min after TGF- β 1 treatment, whereas the expression of NOX4 was reduced by pretreatment of 4-O-Methylhonokiol (Fig. 9). The results suggest that 4-O-Methylhonokiol could protect keratinocytes against TGF- β 1-induced cell cycle arrest by blocking ROS production through NADPH oxidase system.

Taken together, the results of this study demonstrate that 4-O-Methylhonokiol could inhibit TGF- β 1-induced cell growth arrest through inhibition of canonical and noncanonical pathways in human keratinocyte HaCaT cell and that the hair-growing activity of 4-O-Methylhonokiol might be at least related to its protective action on TGF- β -induced catagen induction in hair cycle (Fig. 10).

CONFLICT OF INTEREST

No competing financial interests exist for any of the authors of this study.

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