Peroxisome Proliferator-Activated Receptor-Gamma Agonist 4-O-Methylhonokiol Induces Apoptosis by Triggering the Intrinsic Apoptosis Pathway and Inhibiting the PI3K/Akt Survival Pathway in SiHa Human Cervical Cancer Cells

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Introduction

The Magnolia bark derived from Magnolia officinalis has been used in traditional Chinese medicine [2]. Several bioactive compounds of the Magnolia bark, including 4-O-methylhonokiol, honokiol, and magnolol, exhibit many biological effects such as anti-inflammatory, antithrombocytic, anti-anxiety, antimicrobial, and anti-human immunodeficiency virus activities [1, 14, 17, 21, 27, 37]. In particular, 4-O-methylhonokiol (MH)*, known as a peroxisome proliferator-activated receptor-γ (PPARγ) agonist, exhibits antitumor effects, including antiproliferative activity and induction of apoptosis in prostate and colon cancer cells [20, 26].

Therefore, PPARγ agonists are regarded as a potential strategy for cancer chemoprevention and therapy [11, 30]. Peroxisome proliferator-activated receptors belonging to the nuclear hormone receptor superfamily are one of the ligand-dependent transcription factors that regulate glucose, lipid, and amino acid metabolism [28, 30]. PPARs are classified into PPARα, PPAR β/δ, and PPARγ subtypes, which are encoded by different genes, and have common as well as distinctive activities [22]. PPARγ and its ligands especially play an important role in regulating tumor suppressive activities in many cancers [10, 31, 35].

The PI3K/Akt signaling pathway is a major pathway in regulating cell survival signals [15]. Phosphatidylinositol-3
kinase (PI3K) is one of the members of the lipid kinase family. PI3K is involved in cell survival pathways, cell metabolism, and cytoskeletal rearrangements by phosphorylating the 3'-OH group of inositol to produce phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P_3) as a second messenger [4]. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), known as an antagonist of PI3K, decreases activated PI3K signals by dephosphorylating PIP_3 [15]. Recent studies have demonstrated that decreased PTEN expression is related to the progressive feature of many cancers, including cervical cancers [13]. Akt is a main downstream target of PI3K, and activated Akt phosphorylates various downstream targets to regulate diverse cellular functions such as cell cycle progression, proliferation, cell survival, angiogenesis, tumor growth, and apoptosis [7, 9, 16].

In this study, we investigated the anticancer effect of MH in SiHa human cervical cancer cells. MH has been reported to exhibit various biological effects, including antitumor effects. However, in cervical cancer cell lines, the precise antitumor mechanism of MH remains elusive. We demonstrate that MH exerts anticancer activity in SiHa cells through inhibition of the PI3K/Akt pathway and induction of the intrinsic pathway following PPARγ activation.

Materials and Methods

Reagents

4-O-Methylhonokiol (MH) was generously supplied by Professor Heon Sang Jung (Department of Food Science, Chungbuk National University, Cheongju, Korea). DAPI staining solution and propidium iodide (PI) were obtained from Sigma (St. Louis, MO, USA). The Fluorescein isothiocyanate (FITC) annexin V Apoptosis Detection Kit I was purchased from BD Biosciences (San Diego, CA, USA). Antibodies specific to caspase-3, caspase-9, caspase-8, PTEN, polyADP ribose polymerase (PARP), Bax, Bcl-2, Bcl-XL, and α-mouse IgG horseradish-peroxidase (HRP)-conjugated secondary antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies specific to GAPDH, PPARγ, Akt, p-Akt1/2/3, and anti-goat IgG HRP-conjugated secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-rabbit IgG HRP-conjugated secondary antibody was purchased from Assay Designs (Ann Arbor, MI, USA). JC-1 (5,5',6,6'-tetrachloro-1,1',3',3'-tetrathyl benzimidazolycarbocyanine iodide) was purchased from Enzo (Farmingdale, NY, USA).

Cell Culture

High-risk HPV-16 genotype SiHa cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (all of Hyclone Laboratories, UT, USA) and incubated at 37°C with 5% CO_2.

Cell Viability Assays

Cell viability was observed trypan blue reagents. SiHa cells (3 × 10^5 cells/well) were seeded into a 6-well plate, incubated overnight, and then treated with various concentrations of MH (0, 10, 20, and 40 µM). After each well was washed using phosphate buffered saline (PBS), they were harvested using trypsin-ethylenediaminetetraacetic acid (EDTA), and then centrifuged at 100 × g for 5 min at 4°C. The supernatant was removed and the pellet was resuspended with PBS. After mixing the cell suspension with 0.4% trypan blue solution (1:1 (v/v)), the mixture was incubated for 2 min at room temperature. Stained (nonviable) and unstained (viable) cells were counted using a hemocytometer.

Cell Morphology and DAPI Staining

Cell morphology was observed using an inverted phase-contrast microscope. Apoptotic nuclear morphological changes were detected by DAPI staining. Cells were cultured on coverslips and treated with MH. The coverslips were washed twice using serum-free DMEM and fixed with 100% acetone for 10 min at room temperature. After three washings with PBS, the fixed cells were stained with DAPI for 10 sec at 37°C, washed twice with PBS, and dried completely. After mounting on microscope slides, the stained cells were imaged using an Uplight fluorescence microscope (Olympus, Japan).

Annexin V-FITC/PI Staining

SiHa cells (3 × 10^4 cells/well) were seeded into a 6-well plate, incubated overnight, and then treated with various concentrations of MH. After the MH-treated cells were incubated for 72 h, they were harvested and double stained with Annexin-FITC and PI (FITC Annexin V Apoptosis Detection Kit I) according to the manufacturer’s instructions. Stained cells were counted by flow cytometry on a FACScalibur instrument (BD Biosciences), and the percentages of cells were calculated and analyzed by the CellQuest Pro software (BD Biosciences).

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

After RNA extraction using an easy-BLUE total RNA extraction kit (iNtRon Biotechnology, Seoul, Korea), the cDNA products were obtained using M-MuLV reverse transcriptase (New England Biolabs, Beverly, MA, USA). We performed RT-PCR analysis using a PCR thermal cycler Dice instrument (TaKaRa, Otsu, Shiga, Japan) with the following primer sets; E6: 5’-GCAGCCCTTGAATTA CCCAT-3’ (forward), 5’-CAGAGGTGACGAGGAAGAA-3’ (reverse); E7: 5’-ATGATGAGATACACCTACATTGC-3’ (forward), 5’-TTATGGTTTCTGAGAACAGATGGGGC-3’ (reverse); E8: 5’-TGAAGGACATGGCTAGAAGTG-3’ (forward), 5’-GGTGCAAGG GTCACTACTT-3’ (reverse); E9: 5’-ACTCCTTTCCATTCTG-3’ (forward), 5’-CACACAGTCTTCCCCCA-3’ (reverse); DR5: 5’-
GTCTGCTCTGATCACCCAAC-3' (forward), 5'-CTGCAAACTGTAGCTGCTGGC
TGTAAC-3' (forward), 5'-TGTGGCTCTCTTCCCTGTGCT-3' (reverse); and FasL:
5'-CAAGATTGACCCCGGAAGTA-3' (forward), 5'-GGCCTGTGTCTCCTTGAGAT-3'
(reverse).

Immunoblot Assay
The MH-treated cells were scraped from plates and centrifuged
(72 × g, 3 min, 4°C). The pellets were washed with ice-cold PBS
and centrifuged (1,890× g, 5 min, 4°C). This process was repeated
twice. The pellets were then lysed in a buffer (pH7.4) containing
1.5 M sodium chloride, 50 mM Tris, 1 mM EDTA, 0.1% sodium
dodecyl sulfate (SDS), 0.25% sodium deoxycholate, 1% NP-40, and
a protease inhibitor cocktail. Protein concentration was determined
by using Bradford’s assay (Bio-Rad, Hercules, CA, USA). Samples
were subjected to 10% and 12% SDS polyacrylamide gel
electrophoresis (SDS-PAGE) and transferred to polyvinylidene
difluoride membranes (Millipore, Billerica, MA, USA). Membranes
were blocked with 5% nonfat dry milk dissolved in TBST (140 mM
NaCl, 27 mM KCl, 10 mM Na₂HPO₄·12H₂O, 1.8 mM KH₂PO₄, and
0.05% Tween-20). Primary antibodies specific to caspase-3, caspase-
9, caspase-8, PARP, Bax, Bcl-2, Bcl-XL, cytochrome C, PTEN,
PPARγ, Akt, p-Akt 1/2/3, and GAPDH were adsorbed to the
membranes. The adherent proteins were visualized using HRP-
conjugated secondary antibodies with the Westzol plus Western
blot detection system (iNtRON Biotechnology).

JC-1 Staining
To detect changes in the mitochondrial membrane potential,
which is one of the most distinctive apoptotic features, we used
the JC-1 dye. After MH-treated cells were harvested, they were
stained with JC-1 at 37°C in 5% CO₂ for 10 min. Stained cells were
detected by flow cytometry in a FACScalibur instrument and
analyzed using CellQuest pro software (BD Biosciences). We also
cultured SiHa cells on coverslips and treated with MH. MH-
treated cells were stained with JC-1 at 37°C in 5% CO₂ for 15 min,
and the coverslips were washed twice using PBS. After mounting
on microscope slides, the stained cells were imaged using the
Uplight fluorescence microscope. JC-1 monomers in apoptotic
cells were detected at 485 nm excitation/535 nm emission, and JC-1
aggregates in healthy cells were detected at 540 nm excitation/
570 nm emission.

Statistical Analysis
Data were presented as the mean ± SD values. Statistical analysis
was assessed using Student’s t test with the following significance
levels: *p < 0.05, **p < 0.01.

Results
MH Induces Antiproliferation and Apoptosis in SiHa
We treated the SiHa cells with various concentrations of
MH under incubation for 24, 48, and 72 h and measured the
cytotoxicity using the trypan blue assay. The assay result
indicated that a high concentration of MH (40 µM) was
significantly cytotoxic after 72 h treatment, as shown in
Fig. 1. Therefore, we investigated the apoptotic effect of
MH after 72 h treatment. We observed that MH induced
morphological changes and a decrease of proliferation of
cells (Fig. 2A). We investigated if these results are due to
apoptosis. We could detect chromatin condensation by
nuclear staining with DAPI dye (Fig. 2B). Cell numbers at
the Sub-G1 phase were increased by MH, and this result
described that nuclear fragmentation had occurred (Fig. 2C).
Furthermore, in the AnnexinV-FITC/PI staining result, the
proportion of cells moved to the upper-right quadrant
(Fig. 2D). There was a significant increased level of Annexin
V-FITC/PI positive cells in 40 µM MH-treated cells (Fig. 2E).
Together, these results suggest that a high concentration of
MH promotes cell death through the induction of apoptosis.

MH Reduces the Survival of SiHa Through Suppression
of the PI3K/Akt Survival Signaling Pathway
PPARγ agonists exhibit antitumor activities through PPARγ
dependent or independent effects [12, 38]. We confirmed
that PPARγ expression was increased by MH (Fig. 3A).
According to recent reports, PTEN, which is one of the
PPARγ target genes, is involved in the PI3K/Akt survival
pathway by inducing Akt dephosphorylation [36]. Therefore,
we identified the expression levels of PTEN, Akt, and p-Akt
by using immunoblot assay. The PTEN expression was
increased, but its downstream factor p-Akt was reduced

Fig. 1. Cytotoxic effects of MH in SiHa.
SiHa cells were treated with various concentrations of MH (0, 10, 20,
and 40 µM) for 24, 48, and 72 h, and the cell viability was assessed by
trypan blue reagent as described in the Methods section. The viability
of untreated cells was set at 100%. All data represent the mean ± SD of
three experiments performed in triplicate.
Fig. 2. Antiproliferative and apoptotic effects of MH in SiHa.
(A) Cells were treated with different concentrations of MH for 72 h and observed under a phase-contrast microscope (400×). (B) Nuclear morphological change was detected using DAPI staining and fluorescence microscopy (200×). (C) Sub-G1 accumulation of PI-stained SiHa cells was analyzed by flow cytometry. The data represent the mean ± SD of three experiments conducted in triplicate. *p < 0.05 compared with untreated control cells. (D) Apoptosis of SiHa cells treated with MH was detected by AnnexinV-FITC/PI staining and analyzed by flow cytometry. The data represent one of three independent experiments. (E) AnnexinV-FITC/PI data presented as bar graphs with the mean ± SD of three independent experiments. *p < 0.05, compared with untreated control cells.
by MH (Fig. 3A). To confirm whether PPARγ is involved in the PI3K/Akt pathway, we pretreated cells with the PPARγ antagonist GW9662 (5 μM) for 1 h before MH treatment. Interestingly, MH-induced PTEN expression was reduced, and Akt phosphorylation was recovered by GW9662 (Fig. 3B). However, GW9662 itself did not affect MH-induced PPARγ expression (Fig. 3B). Therefore, these results demonstrate that the effects of MH are dependent on PPARγ, and MH inhibits the PI3K/Akt pathway.

MH Induces Apoptosis Through the Caspase Cascade
Cysteine-dependent aspartate-directed proteases (caspases) play critical roles in inducing apoptosis [24]. Processing PARP, a downstream factor of caspases, is a pivotal indicator of apoptosis [24]. As shown in Fig. 4, caspase-9 and caspase-3 were cleaved while caspase-8 levels were not altered, and cleaved forms were not detected. The processing of PARP occurred in the presence of a high concentration of MH. Taken together, MH induces apoptosis through a caspase-mediated pathway.

MH Induces Apoptosis Through Intrinsic Pathways
Apoptosis can be induced through two pathways, intrinsic and extrinsic [32]. First, we confirmed that the MH induced the mitochondria-dependent apoptosis pathway. In the intrinsic pathway, the balance between pro-apoptotic and anti-apoptotic Bcl-2 family proteins is broken, and mitochondrial outer membrane permeabilization (MOMP) occurs, which consequently triggers apoptosis [33]. Therefore, the Bcl-2 family plays a significant role in the intrinsic pathway. To detect the expression levels of anti-apoptotic (Bcl-2 and Bcl-XL) and pro-apoptotic (Bax) factors of the Bcl-2 family, we performed immunoblot assays. MH treatment induced a reduction of Bcl-2 and Bcl-XL expression in a dose-dependent manner (Fig. 5A). The expression of Bax was not changed by MH.

MOMP is essential to cytochrome C release [33]. Released cytochrome C triggers executor caspase activation [33]. For that reason, we examined the mitochondrial membrane potential collapse and cytochrome C release. We first performed JC-1 staining. As can be seen in Fig. 5B, the peaks were shifted to the left. This result means that mitochondrial membrane potential loss was triggered at high concentration of MH compared with control. Furthermore, as shown in Fig. 5C, cytochrome C release into the cytosol was enhanced at a high dose of MH. Therefore, these data suggest that MH induces apoptosis by passing through the mitochondria.

MH-Induced Apoptosis Is Independent of Extrinsic Pathways and HPV E6/E7 Viral Oncogenes
The death receptor-dependent pathway also plays a role in inducing the apoptotic process [39]. Accordingly, we
investigated whether the extrinsic pathway is involved in MH-mediated apoptosis. The Fas/FasL and DR5/TRAIL expression levels were not significantly altered after MH treatment (Fig. 6A). FADD and caspase-8 expression was also independent of MH treatment (Figs. 6A and 4).

Expression of E6/E7 oncogenes is a feature of HPV-positive cervical cancer cells [18]. For this reason, to investigate whether MH has an inhibitory effect on E6 and E7 expression in SiHa, we analyzed the transcription levels of E6 and E7 by RT-PCR analysis. Interestingly, MH did not downregulate both E6 and E7 transcripts (Fig. 6B), suggesting that MH-induced apoptosis is independent of the extrinsic pathway and E6/E7 oncogenes in SiHa cells.

Discussion

Recently, 4-O-methylhonokiol has been known as a new PPARγ agonist, and it inhibits growth of several cancer cells [20, 26]. MH enhances not only the PPARγ expression level but also the transcriptional activity [20]. Activated PPARγ regulates the transcription of diverse genes [19, 36]. Of these, PTEN is involved in the PI3K/Akt pathway [15]. PTEN acts as a phosphatase of PIP3, resulting in the dephosphorylation of Akt [15]. Akt regulates various cellular functions, including apoptosis, by inducing phosphorylation of downstream targets [7, 9, 16]. In the present data, MH clearly influenced the PI3K/Akt pathway by blocking it (Fig. 3) and induced apoptosis in SiHa.

Apoptosis plays essential roles in cell survival, growth, development, and tumorigenesis, and it is mediated by two mechanisms [34]. The intrinsic pathway plays a crucial role in triggering apoptosis, and an imbalance between the Bcl-2 family leads to this pathway [6]. It has been demonstrated that Bad is one of the target genes of Akt [8]. When p-Akt is reduced, Bad phosphorylation is inhibited [8]. Unphosphorylated Bad forms a heterodimer with Bcl-XL or Bcl-2 and blocks their anti-apoptotic activity [8]. It triggers an imbalance between the Bcl-2 family, which forms a pore in the mitochondrial outer membrane. Cytochrome C is released from the mitochondrial intermembrane space [6, 23]. As a result, the caspase cascade is induced, and it stimulates PARP cleavage, which is a pivotal indicator in apoptosis initiation [25]. Our results indicated that MH destroyed the balance of the Bcl-2 family and consequently triggered caspase-9/-3 activation. However, death receptors and cognate ligands were not altered, and also caspase-8 was not activated. Taken together, it seems that MH induced the intrinsic apoptosis pathway by inhibiting Akt phosphorylation.

Cervical cancer is the second leading gynecological carcinoma in women worldwide [5]. Infection with human papillomavirus (HPV) causes most cervical cancers. HPV types have been classified into low-risk or high-risk types, both of which are frequently found in most cervical cancers [3]. The viral E6 and E7 oncogenes contribute to the development of HPV-infected cervical cancer [18]. Accordingly, much of the research regarding cervical cancer has focused on HPV-positive types.

However, in the present study, MH exerted a strong anti-cancer effect on SiHa harboring HPV without influencing the E6/E7 oncogenes. Recent studies demonstrate that the PI3K/Akt pathway is considerably activated, and it is associated with regulating the tumor metabolic response in
cervical cancers in vitro and in vivo. Increased p-Akt level is related to enhanced glucose uptake in those cells [29]. Therefore, the PI3K/Akt pathway is a therapeutic target in cervical carcinoma [29]. Our data clearly indicate that MH decreased the expression of members of the PI3K/Akt pathway in SiHa (Fig. 3). These findings suggest that MH has potential as an antitumor agent in cervical cancer.

In summary, we report, for the first time, that the new PPARγ agonist MH induces apoptosis via inhibition of the PI3K/Akt survival pathway, activating the intrinsic

Fig. 6. Effects of MH on the expression of death receptors and their related factors involved in the extrinsic pathway and E6/E7 expression in SiHa.
SiHa cells were treated with various concentrations of MH for 72 h. (A) Transcriptional levels of Fas, FasL, DR5, TRAIL, and FADD were determined by RT-PCR. (B) E6 and E7 mRNA levels were measured by RT-PCR. These levels were normalized to the GAPDH mRNA level and presented as bar graphs.
pathway in the SiHa cervical carcinoma cell line (Fig. 7). Therefore, our results demonstrate that MH can be used as an anticancer agent for human cervical cancer.

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References


