JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Piceatannol Suppresses Breast Cancer Cell Invasion through the Inhibition of MMP-9: Involvement of PI3K/AKT and NF-κB Pathways

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ABSTRACT: Cancer invasion and metastasis are the main causes of treatment failure and death in cancer patients. Piceatannol (3,3′,4,5′-tetrahydroxy-*trans*-stilbene) is a natural analogue of resveratrol. This study investigated the anti-invasive mechanisms of piceatannol in MDA-MB-231 cells. Piceatannol significantly reduced serum-induced cell invasion and migration as well as adhesion without affecting the viability of cells. Furthermore, piceatannol markedly inhibited matrix metalloproteinase-9 (MMP-9) activity and expression at both protein and mRNA levels. Piceatannol attenuated phosphoinisitide-3-kinase (PI3K) and phosphorylation of AKT and mammalian target of rapamycin (mTOR), whereas phosphatase and tensin homologue (PTEN) was increased. Moreover, piceatannol inhibited nuclear factor kappa B (NF-κB) transcriptional activity and DNA binding of NF-κB on MMP-9 promoter. In addition, piceatannol diminished NF-κB nuclear translocation through blocking the inhibitor of NF-κB alpha (IκBα) phosphorylation in the cytoplasm. These results proposed piceatannol as a potential anti-invasive agent by inhibiting MMP-9 involved in PI3K/AKT and NF-κB pathways.

KEYWORDS: *piceatannol, MDA-MB-231 cells, invasion, MMP-9, PI3K/AKT, NF-κB*

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer and the first leading cause of cancer death in females worldwide, accounting for 23% of the total new cancer cases and 14% of the total cancer deaths in 2008.[1] Approximately 15−20% of breast cancer cases are in the category of triple-negative phenotype, named due to its lack of estrogen receptor-α and progesterone receptor and amplification of epidermal growth factor receptor. Patients with triple-negative breast cancers have a very poor disease-free survival because these breast cancers are aggressive and show a high rate of metastasis compared with other types of breast cancers.[2,3]

Invasion and metastasis are predominant properties of malignant cancer cells.[4] Approximately 90% of cancer patients die as a result of invasive and metastatic growth of cancer, which remains one of the greatest causes of poor clinical outcome.[4,5] Because matrix metalloproteinases (MMPs) play critical roles in the degradation of extracellular matrix (ECM), which acts as a mechanical barrier to cell movement, the proteolytic activities of MMPs are involved in the metastasis process including cell adhesion, migration, and invasion.[6−8] Among all of the MMPs, MMP-9, known as a gelatinase B, is overexpressed in breast cancer cells[6,7] and necessary or sufficient for the metastatic spread to the brain tissue during breast cancer progression.[9] Thus, MMP-9 is an attractive target for suppressing breast cancer invasion and metastasis, and inhibition of MMP-9 may have enormous advantages in cancer therapy.

The development of novel compounds with low toxicity and excellent potential for cancer chemoprevention or treatment is an important part of cancer therapies. Some natural products have been investigated as therapeutic agents for breast cancer due to their safety and several biological activities[2,3,10] Piceatannol (3,3′,4,5′-tetrahydroxy-*trans*-stilbene) is a natural analogue of resveratrol, a natural antioxidant derived from grapes, and is a naturally occurring polyphenol present in the skins of grapes, red wine, and other foods.[11−13] It has been demonstrated to exhibit anticancer, anti-inflammatory, and cardioprotective properties.[12] Piceatannol has been reported to induce apoptosis[14,15] and to inhibit cell cycle progression[16,17] in various types of human cancers and to act as an agonist for estrogen receptor α in human breast cancer cells.[18] Although recent experimental data revealed the anticancer potency of piceatannol, the effect of piceatannol on cancer invasion and the mechanisms underlying its effect are not yet fully understood in breast cancer. A limited number of studies showed that piceatannol inhibited the invasion of prostate cancer cells[12] and lung metastasis of Lewis lung cancer cells.[19] In this study, we investigated the anti-invasive mechanisms of piceatannol in the triple-negative and highly invasive human breast cancer cell line, MDA-MB-231, and demonstrated for the first time that the inhibitory effect of piceatannol on invasion of human breast cancer cells is involved in attenuating PI3K/AKT and NF-κB-mediated MMP-9.

MATERIALS AND METHODS

Cell Culture and Treatment. MDA-MB-231 (triple-negative and highly invasive human breast cancer cell line) cell line was purchased from a Korean cell line bank (Seoul, Korea) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10000 U/L penicillin, and 10 mg/L streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were treated with various concentrations (2, 5, 10 μM) of piceatannol in a serum-free culture medium.

Received: December 15, 2011
Revised: April 4, 2012
Accepted: April 5, 2012
Published: April 5, 2012

Cell Viability Assay. The effect of piceatannol on the cell viability of MDA-MB-231 cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, St. Louis, MO, USA) assay. Cells were seeded at a density of 1×10^4 cells per well in a 96-well plate, cultured for 24 h, and then treated with piceatannol. After 24 h of incubation, 30 μL of MTT solution (1 mg/mL) was added to each well and incubated for 2 h. The viable cell number was correlated with the production of formazan, which was dissolved with dimethyl sulfoxide (DMSO), and optical density (O.D.) was measured by microplate reader (Sunrise, TECAN, Austria) at 570 nm. Cell viability was calculated by the following equation: cell viability (%) = [O.D. (piceatannol) − O.D. (blank)]/[O.D. (control) − O.D. (blank)] × 100.

Invasion Assay. An invasion assay was carried out using modified 48-well microchemotaxis chambers (Neuro Probe Inc., Gaithersburg, MD, USA). A polyvinyl-pyrrolidone-free polycarbonate filter (8 mm pore size) (Neuro Probe Inc.) was coated with matrigel. The lower chamber was filled with medium containing 10% FBS and 0.1% BSA as chemoattractant agents. The coated filter and upper chamber were laid over the lower chamber. Cells (1×10^4 cells/well) were preincubated with piceatannol for 30 min at room temperature, and then cell suspension containing piceatannol and 0.1% BSA was seeded onto the upper chamber wells. After incubation for 15 h at 37 °C, the filter was fixed and stained with Diff-Quick (Sysmex, Japan), and nonmigrated cells on the upper surface of the filter were wiped off with a swab. Then randomly chosen fields were photographed under a microscope (DFC420C, Leica, Germany), and the number of cells migrated to the lower surface was calculated.

Wound-Healing Assay. The migration ability of cells was assayed by a wound-healing assay. Cells (4×10^5 cells/2 mL) were seeded in a 6-well plate and incubated at 37 °C. The confluent cells were scratched with a 200 μL pipet tip, followed by washing with PBS, and then treated with piceatannol in a complete medium. After 24 h of incubation, the cells were fixed and stained with Diff-Quick, and randomly chosen fields were photographed under a fluorescence microscope (AXIO observer A1, Zeiss, Germany). The number of cells migrated into the scratched area was calculated.

Adhesion Assay. Cells (5×10^4 cells/well) preincubated with piceatannol for 20 min at 37 °C were seeded in a 96-well plate coated with matrigel for 10 min at 37 °C. Unattached cells were removed by washing with PBS. Attached cells were fixed in 1% glutaraldehyde in PBS for 20 min, stained with 0.02% crystal violet solution for 5 min at room temperature, and randomly chosen fields were photographed under a fluorescence microscope (AXIO observer A1, Zeiss). Then, to quantify the number of attached cells, crystal violet was dissolved with 70% ethanol and O.D. was measured by microplate reader at 570 nm, reference 405 nm. The adhesion cells were calculated as a percentage of adhesion.

Gelatin Zymography. The activity of MMP-9 in the conditioned medium (CM) was assessed by gelatin zymography. Cells (4×10^5 cells/2 mL) were seeded in a 6-well plate and treated with piceatannol for 24 h. The culture medium from treated cells was collected and concentrated using a microcon (Millipore, Bedford, MA, USA). Equal amounts of concentrated CM were mixed with sample buffer without reducing agent, incubated for 15 min at room temperature, and then separated on 10% sodium dodecyl sulfate (SDS)−polyacrylamide gel polymerized with 0.1% gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 and incubated in a reaction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02% NaN_3) for 18 h at 37 °C. After incubation, the gels were stained with 0.25% Coomassie brilliant blue R-250 (Sigma Aldrich) in 7% glacial acetic acid and 40% methanol and then destained with 10% glacial acetic acid in 10% methanol. MMP-9 activity was visualized as clear bands against the blue-stained gelatin background. The bands of gelatin zymography were quantified using Image J 1.40 g software (National Institutes of Health, Bethesda, MD, USA).

MMP-9 Enzyme-Linked Immunosorbent Assay (ELISA). Cells (4×10^5 cells/2 mL) were seeded in a 6-well plate and treated with piceatannol for 24 h. The CM was collected, and secreted MMP-9 protein was then measured using a Human MMP-9 Immunoassay kit (Invitrogen, Camarillo, CA, USA) according to the manufacturer's protocol.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA was prepared by using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RT-PCR analysis was then performed as previously described.[20] The PCR conditions were as follows: 94 °C for 30 s, 65 °C for MMP-9 and TIMP-1 or 59 °C for GAPDH for 30 s, and 72 °C for 30 s. Samples were subjected to 40 cycles for MMP-9, 25 cycles for TIMP-1, and 35 cycles for GAPDH. The primer sequences used and their product sizes were as follows: MMP-9 (196 bp) forward, 5′-CGCTACCACCTCGAACTTTG-3′; MMP-9 reverse, 5′-GCCATTCACGTCGTCCTTAT-3′; TIMP-1 (345 bp) forward, 5′-CACCCACAGACGGCCTTCTGC-3′; TIMP-1 reverse, 5′-AGTGTAGTCTTGGTGAAGCC-3′; GAPDH (700 bp) forward, 5′-TCACCATCTTCCAGGAGCGA-3′; GAPDH reverse, 5′-CACAATGCCGAAGTGGTCGT-3′. The PCR products were separated on 2% agarose gels. The bands of RT-PCR were quantified using Image J 1.40 g software (National Institutes of Health).

Nuclear Extract Preparation. The cells treated with piceatannol for 24 h were fractionated into cytoplasmic and nuclear extracts using NE-PER nuclear and cytoplasmic extraction reagents (Thermo, Rockford, IL, USA) according to the manufacturer's protocol.

Western Blot Analysis. Equal amounts of proteins (25−30 μg) were separated on 10% SDS-PAGE gel and then electrotransferred onto a Hybond ECL transfer membrane (Amersham Pharmacia, Piscataway, NJ, USA) at 300 mA for 90 min. After blocking with 5% nonfat skim milk, the membrane was probed with primary antibodies for NF-κB p65, phospho-IκBα, α-tublin, PARP, PTEN (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), PI3K p85, phospho-AKT, AKT, phospho-mTOR, and mTOR (Cell Signaling, Beverly, MA, USA) and specific secondary antibodies. The protein expression was detected by enhanced chemiluminescence system (Amersham Pharmacia).

NF-κB Luciferase Reporter Assay. Cells were transfected with the NF-κB luciferase reporter construct (SABiosciences, Frederick, MD, USA) using an X-tremeGENE HP DNA transfection reagent (Roche, Mannheim, Germany). After transfection for 18 h, the cells were treated with piceatannol for 24 h. Luciferase activity was measured using the Dual-Luciferase assay system (Promega, Madison, WI, USA) with a LUMistar OPTIMA microplate luminometer (BMG Lebtech, Germany).

Chromatin Immunoprecipitation (ChIP) Assay. The cells treated with piceatannol for 24 h were processed for ChIP assay using a SimpleChIP Enzymatic Chromatin IP kit (Cell Signaling). Briefly, immunoprecipitation was performed with NF-κB p65 (Santa Cruz Biotechnologies) or rabbit IgG as a control. The NF-κB binding site of MMP-9 promoter was detected by PCR using the following primers: 5′-GACCAAGGGATGGGGGATC-3′ and 5′-CTTGACAGGCAAGTGCTGAC-3′.

Immunofluorescence Assay. Cells (4×10^5/mL) were seeded in a coverglass bottom dish (SPL, Korea) and treated with piceatannol. After washing with PBS, the cells were fixed in 4% methanol-free formaldehyde solution (pH 7.4) at 4 °C for 25 min, permeabilized with 0.2% Triton-X 100 in PBS for 5 min, and blocked in blocking buffer (5% bovine serum albumin, 0.5% Tween-20 in PBS) for 2 h. Then the cells were incubated with anti-NF-κB p65 primary antibody (Santa Cruz Biotechnologies) for 1 h at room temperature, washed with PBS, and incubated with the FITC-labeled secondary antibody (Abcam, Cambridge, U.K.) for 1 h at room temperature. The cells were counterstained with propidium iodide (PI) solution, mounted, and visualized under a FLUOVIEW FV10i confocal microscope (Olympus, Tokyo, Japan).

Statistical Analysis. All data are presented as the mean ± SD from at least three independent experiments. All statistics were calculated by Student's t test using Sigma plot software (Systat Software Inc., San Jose, CA, USA), and a p value of <0.05 was considered to be statistically significant.

RESULTS

Piceatannol Reduces the Invasion, Migration, and Adhesion of MDA-MB-231 Cells. To investigate the anti-invasive potential of piceatannol in breast cancer cells, invasion assay was performed in the triple-negative and highly invasive human breast cancer cell line, MDA-MB-231, using 48-well microchemotaxis chambers. Piceatannol showed no significant difference in cell viability up to 10 μM by MTT assay (data not shown). The results showed that exposure of cells to 10% of serum caused a 3.1-fold increase in the invasion of cells into matrigel, a solubilized basement membrane matrix. However, serum-induced cell invasion was significantly reduced by piceatannol in a concentration-dependent manner (Figure 1A). To better understand the inhibitory effect of piceatannol on cell invasion, cell migration and cell attachment were investigated by wound-healing and adhesion assays, respectively. As shown in Figure 1B, piceatannol significantly decreased serum-induced cell migration by 13, 22, and 33% of untreated control at 2, 5, and 10 μM, respectively. Piceatannol treatment also dramatically reduced cell adhesion to matrigel in a concentration-dependent manner compared with untreated control (Figure 1C). These results indicated that piceatannol has an anti-invasive activity on breast cancer without affecting the viability of cells.

Piceatannol Inhibits the Activity of MMP-9 in MDA-MB-231 Cells. MMPs play an essential role in the degradation of matrix barriers surrounding the tumor, such as ECM, for tumor growth, invasion, angiogenesis, and metastasis.[6−8] MMP-9 is abundantly expressed in breast cancer cells, and its proteolytic activity contributes to cell invasion and metastasis.[6−9] To determine whether the inhibitory effect of piceatannol on cell invasion was associated with the activity of MMP-9 in MDA-MB-231 cells, gelatin zymography was performed using the concentrated CM from the piceatannol-treated cells. As shown in Figure 2A,B, piceatannol was markedly effective in inhibiting the gelatinolytic activity of MMP-9, which was significantly inhibited by 12, 27, and 60% of untreated control in the piceatannol-treated cells at 2, 5, and 10 μM, respectively. This result revealed that piceatannol-reduced cell invasion was attributed to the inhibition of the activity of MMP-9.

Piceatannol Down-regulates the Protein and mRNA Expression of MMP-9 in MDA-MB-231 Cells. The protein level of MMP-9 in the CM was evaluated using a MMP-9 ELISA kit. Piceatannol treatment resulted in 25 and 64% inhibition of MMP-9 secretion at 5 and 10 μM compared with untreated control, respectively (Figure 2C). To define whether the inhibitory effect of piceatannol on the protein expression of MMP-9 resulted from a depressed level of MMP-9 mRNA expression, RT-PCR was carried out. Piceatannol remarkably down-regulated MMP-9 mRNA expression level by 21 and 53% of untreated control at 5 and 10 μM, respectively. However, after piceatannol treatment, no effect was observed on the mRNA expression of tissue inhibitor of metalloproteinase-1 (TIMP-1), an inhibitor of MMP-9[21] (Figure 2D,E).

Piceatannol Attenuates the PI3K/AKT Pathway in MDA-MB-231 Cells. The PI3K/AKT pathway is frequently activated in breast cancer[22] and promotes cancer cell invasion and metastasis through up-regulation of MMP-9.[23] Thus, to identify whether the PI3K/AKT pathway was related to piceatannol-inhibited MMP-9, Western blot analysis was performed. Piceatannol significantly suppressed PI3K (p85)

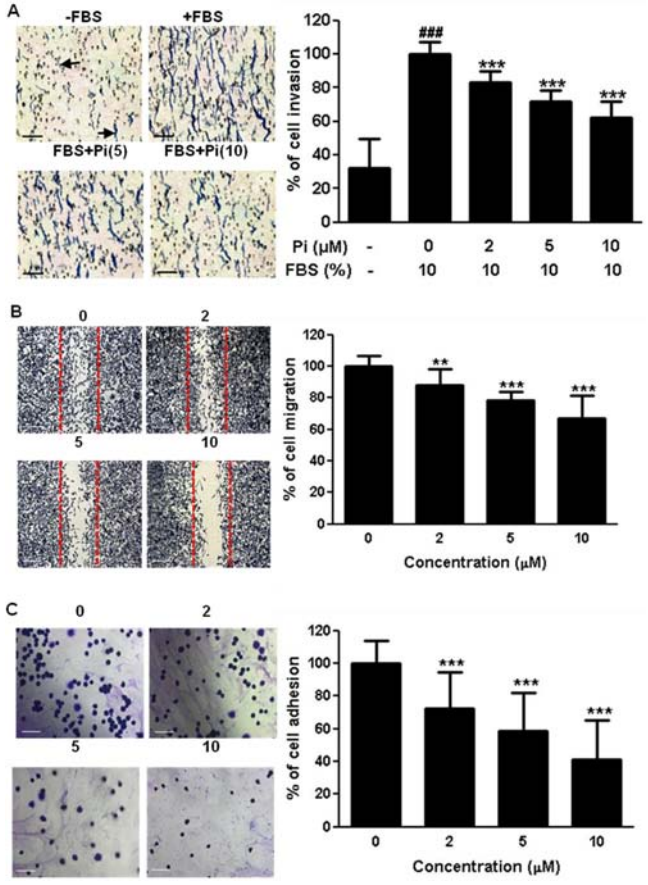

Figure 1. Piceatannol reduces the invasion, migration, and adhesion of MDA-MB-231 cells. (A) Invasion assay was carried out using modified 48-well microchemotaxis chambers. Then randomly chosen fields were photographed (×200), and the number of cells migrated to the lower surface was calculated as a percentage of invasion. Scale bar = 100 μm. Data are shown as the mean ± SD of three independent experiments by analysis of Student's t test. n = 12. ###, $p < 0.001$, vs untreated control, ***, $p < 0.001$, vs serum-treated control. Arrows indicate the invaded cells. (B) Confluent cells were scratched and then treated with piceatannol in a complete medium for 24 h. The number of cells migrated into the scratched area was photographed (×100) and calculated as a percentage of migration. Data are shown as the mean ± SD of three independent experiments by analysis of Student's t test. n = 6. **, $p < 0.01$, and ***, $p < 0.001$, vs untreated control. (C) Cells were seeded in a 96-well plate coated with matrigel and treated with piceatannol. Attached cells were photographed (×200) after crystal violet staining, and the number of attached cells was quantified by measuring O.D. Pi, piceatannol. Scale bar = 100 μm. Data are shown as the mean ± SD of three independent experiments by analysis of Student's t test. n = 12. **, $p < 0.01$, and ***, $p < 0.001$, vs untreated control.

and phospho-AKT at 5 and 10 μM and phospho-mTOR at 10 μM, whereas it had no evident effect on total ATK and mTOR. In contrast, PTEN was increased by piceatannol (Figure 3). These results suggested that piceatannol inhibited MMP-9 activity through attenuating the PI3K/AKT pathway in MDA-MB-231 cells.

Piceatannol Blocks the NF-κB Pathway in MDA-MB-231 Cells. The NF-κB pathway is a key transcription factor in the regulation of MMP-9 expression.[24,25] To further understand inhibitory mechanisms of piceatannol on MMP-9 transcriptional regulation, the NF-κB signaling pathway was investigated by reporter assay and ChIP assay. Piceatannol strikingly

dx.doi.org/10.1021/jf205171g | J. Agric. Food Chem. 2012, 60, 4083−4089

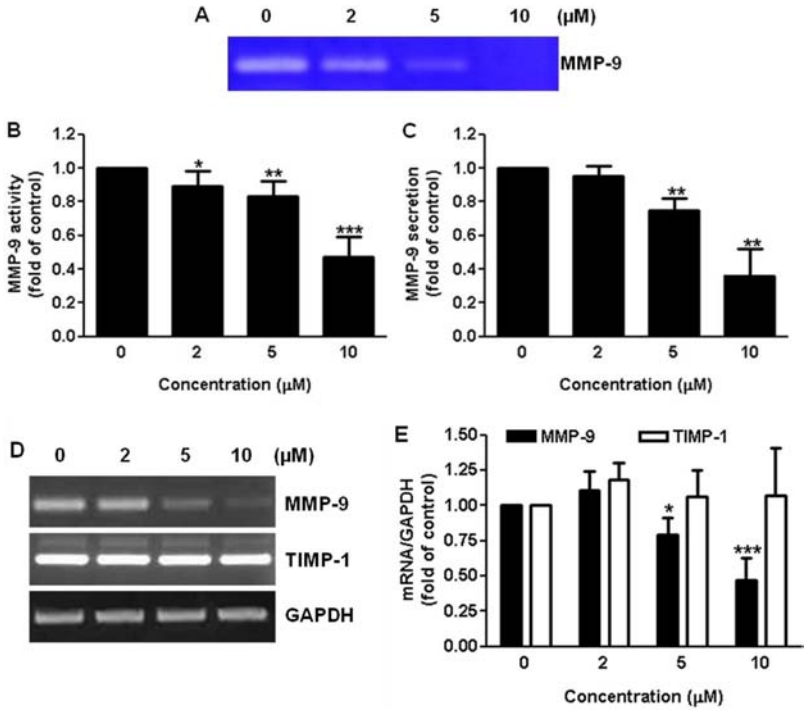

Figure 2. Piceatannol inhibits the activity and down-regulates the protein and mRNA expression of MMP-9 in MDA-MB-231 cells. Cells were treated with piceatannol for 24 h. (A) The activity of MMP-9 was assessed using the concentrated CM by gelatin zymography. (B) The bands of gelatin zymography were quantified. Data are shown as the mean ± SD of six independent experiments by analysis of Student's *t* test. $n = 6$. (C) The protein level of MMP-9 in CM was measured using an ELISA kit. Data are shown as the mean ± SD of two independent experiments by analysis of Student's *t* test. $n = 8$. (D) The mRNA expression of MMP-9 and TIMP-1 was determined by RT-PCR analysis. Then the bands of products were quantified (E), and the ratio of MMP-9/TIMP-1 mRNA expression was calculated (F). Data are shown as the mean ± SD of three independent experiments by analysis of Student's *t* test. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$, vs untreated control. $n = 3$.

inhibited NF-κB transcriptional activity at 5 and 10 μM (Figure 4A) and DNA binding of NF-κB on MMP-9 promoter at 10 μM (Figure 4B). The nuclear translocation of NF-κB was detected by immunofluorescence assay. Figure 4C showed that the NF-κB p65 subunit accumulated in the nucleus in untreated control, but not in 10 μM piceatannol-treated cells. These results were confirmed by Western blot analysis. NF-κB expression in the nucleus and the phosphorylation of IκBα in the cytoplasm were diminished by piceatannol (Figure 4D). These results demonstrated that piceatannol blocked the activation of the NF-κB signaling pathway by exerting an inhibitory effect on IκBα phosphorylation.

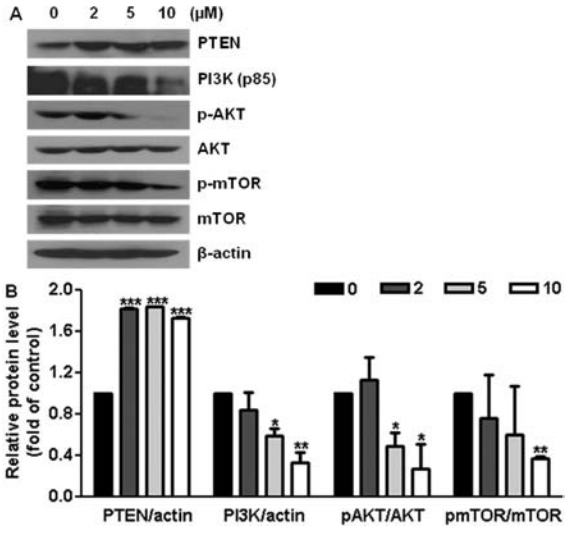

Figure 3. Piceatannol attenuates the PI3K/AKT pathway in MDA-MB-231 cells. Cells were treated with piceatannol for 24 h. (A) Equal amounts of proteins (25−30 μg) were analyzed by Western blot using indicated antibodies. (B) The bands of Western blot were quantified. Data are shown as the mean ± SD of two independent experiments by analysis of Student's *t* test. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$, vs untreated control. $n = 2$.

DISCUSSION

Cancer invasion and metastasis are the main causes of treatment failure and the primary causes of death in cancer patients.[26] Therefore, therapeutic strategies for preventing or suppressing cancer invasion and metastasis would greatly improve survival of cancer patients. A vital step in the invasive processes is the proteolytic degradation of the ECM by proteolytic enzymes, such as MMPs.[6] MMPs are involved not only in ECM degradation but also in cell migration, wound healing, and adhesion.[4,7,21]

A limited number of studies showed that piceatannol inhibited invasion of prostate cancer cells[12] and lung metastasis of Lewis lung cancer cells.[19] However, the effect of piceatannol on cancer invasion is not yet fully understood in breast cancer. We demonstrated that piceatannol strikingly inhibited the invasive (Figure 1A) and migratory (Figure 1B) abilities of MDA-MB-231 cells at noncytotoxic concentrations. The attachment of tumor cells to the ECM and their interactions were crucial steps in the invasion and metastatic processes.[27] We revealed that piceatannol treatment dramatically reduced cell adhesion to matrigel (Figure 1C). Several studies have

dx.doi.org/10.1021/jf205171g | J. Agric. Food Chem. 2012, 60, 4083−4089

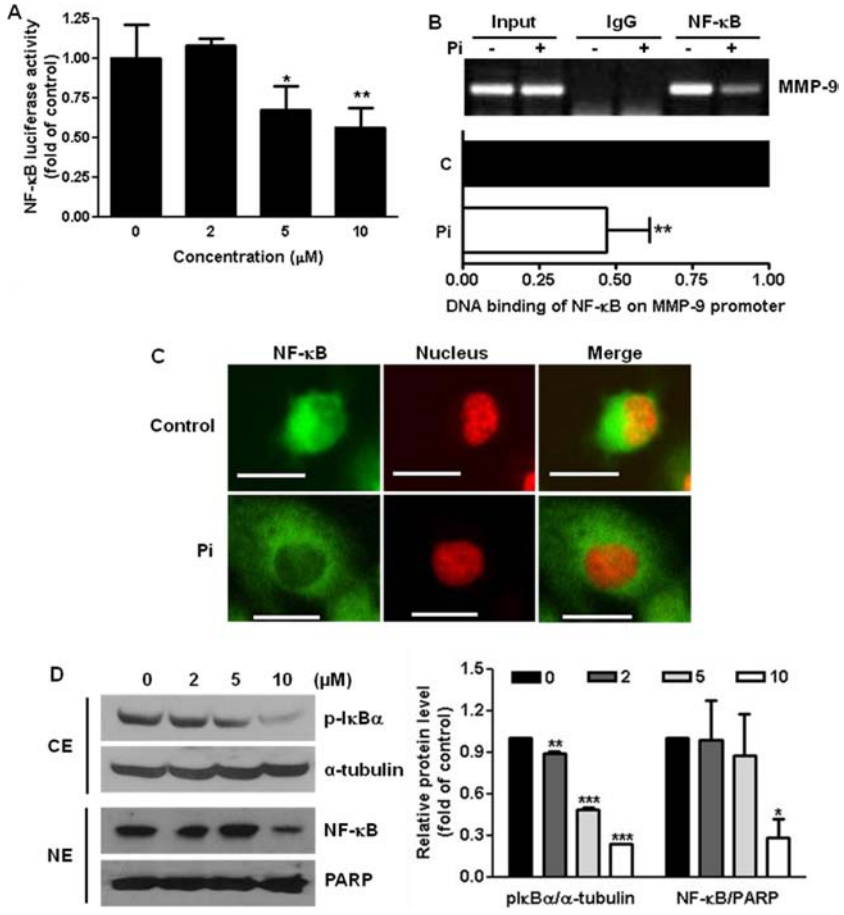

Figure 4. Piceatannol blocks the NF-κB pathway in MDA-MB-231 cells. (A) Cells were transfected with the NF-κB luciferase reporter construct for 18 h and then treated with piceatannol. Luciferase activity was measured using the Dual-Luciferase assay system. Data are shown as the mean ± SD of two independent experiments by analysis of Student's *t* test. $n = 8$. (B) The cells treated with piceatannol were processed for ChIP assay. Immunoprecipitation was performed with NF-κB p65 or rabbit IgG as a control. The NF-κB binding site of MMP-9 promoter was detected by PCR. C, control. Data are shown as the mean ± SD of three independent experiments by analysis of Student's *t* test. $n = 3$. (C) Cells were seeded in a coverglass bottom dish. After treatment with piceatannol (Pi), the cells were fixed and stained with anti-NF-κB (green). The nucleus is stained with PI solution (red). The cells were visualized under a confocal microscope (×600). Scale bar = 20 μm. (D) Cells treated with piceatannol were fractionated into cytoplasmic and nuclear extracts. Equal amounts of proteins (30 μg) were analyzed by Western blot using indicated antibodies, and the bands of Western blot were quantified. α-Tubulin and PARP were used as loading controls of cytoplasmic (CE) and nuclear (NE) extracts, respectively. Data are shown as the mean ± SD of two independent experiments by analysis of Student's *t* test. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$, vs untreated control. $n = 2$.

demonstrated that up-regulation of several MMPs, such as MMP-9, contributed to cancer invasion and metastasis.[6−9,28] We identified that piceatannol markedly inhibited the gelatinolytic activity (Figure 2A,B) and secretion of MMP-9 (Figure 2C), which resulted from regulating MMP-9 without affecting TIMP-2 at the transcriptional level (Figure 2D,E). The imbalance between MMPs and their natural inhibitors, TIMPs, plays a decisive role in the degradation of ECM to enhance the invasive and metastatic capacity of cancer.[6,21] Overall, these results suggested that piceatannol possessed the anti-invasive potential against breast cancer cells by inhibiting the activity and expression of MMP-9.

PI3K is a lipid kinase that controls multiple cellular processes through AKT activation. AKT, a well established survival factor, promotes cell growth by inducing cytoplasmic localization of $p21^{WAF1}$ and exerts an anti-apoptotic effect by inactivating pro-apoptotic factor BAD and caspase-9. Furthermore, AKT activation leads to cancer invasion and metastasis by stimulating the secretion of MMPs.[23,29−31] Previous study demonstrated that mTOR, one of the downstream targets of AKT, was one mechanism in a tightly regulated network of intracellular signal pathways and contributed to the invasiveness of human trophoblast cells by secretion of ECM-remodeling enzymes such as MMP-2 and -9.[32] PTEN is a dual-specificity lipid phosphatase and tumor suppressor, which negatively regulates the PI3K/AKT pathway by inhibiting AKT activation. On the contrary, loss of PTEN activity by mutations or deletions results in activation of AKT.[33−35] It was reported that the MMP-9 mRNA level was correlated with the PI3K/AKT/mTOR pathway and inversely correlated with PTEN.[23] Our data showed that piceatannol increased PTEN but inhibited PI3K (p85) and phosphorylation of AKT and mTOR (Figure 3).

The NF-κB pathway is a downstream target of AKT[31] and a key transcription factor in the regulation of MMP-9 expression.[24,25] NF-κB is closely linked to inflammation, tumor cell proliferation, survival, invasion, and metastasis.[36] Furthermore, NF-κB is constitutively activated in many cancers, which contributes to chemo- and radio-resistance via the expression of anti-apoptotic proteins.[37,38] Thus, the inhibition

of NF-κB signaling may achieve improved therapeutic outcome. Previous studies clarified that tumor invasion is suppressed by inhibiting NF-κB-mediated MMP-9 expression.[24,25,39] NF-κB is activated by the phosphorylation-induced degradation of IκBs, which facilitates the dissociation of IκB family proteins from NF-κB. The liberated NF-κB translocates to the nucleus and binds to the promoter region of target genes, such as MMP-9, thereby leading to gene expression.[25,36] In this study, we demonstrated that piceatannol inhibited NF-κB transcriptional activity (Figure 4A) and DNA binding of NF-κB on MMP-9 promoter (Figure 4B). According to the previous study, piceatannol inhibited TNF-induced NF-κB activation and nuclear translocation.[40] We also verified that the nuclear translocation of NF-κB was blocked by piceatannol, which was due to the inhibition of IκBα phosphorylation in the cytoplasm (Figure 4C,D). These results demonstrated that piceatannol-induced inhibition of MMP-9 was attributed to blocking of NF-κB transcriptional activity.

In conclusion, we demonstrated for the first time that piceatannol suppressed the invasive ability of MDA-MB-231 cells, including invasion, migration, and adhesion, by inhibiting MMP-9 activity and expression through increasing PTEN, attenuating the PI3K/AKT pathway, and blocking NF-κB activation. These results provided new insight into molecular mechanisms involved in the anti-invasive activity of piceatannol in breast cancer cells and strongly suggested that piceatannol is a useful anti-invasive agent for breast cancer.

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Funding

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea government [MEST] (No. 2011-0006220).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

MMP-9, matrix metalloproteinase-9; PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; PTEN, phosphatase and tensin homologue; NF-κB, nuclear factor kappa-B; IκBα, inhibitor of NF-κB alpha; ECM, extracellular matrix; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; CM, conditioned medium; TIMP-1, tissue inhibitor of metalloproteinase-1; ChIP, chromatin immunoprecipitation.

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