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N-(4-Hydroxyphenyl)Retinamide Inhibits Breast Cancer Cell Invasion Through Suppressing NF-KB Activation and Inhibiting Matrix Metalloproteinase-9 Expression

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ABSTRACT

Synthetic retinoid *N*-(4-hydroxyphenyl)retinamide (4-HPR) has been reported to exhibit anti-invasive and anti-metastatic activities by suppressing the enzymatic activity of matrix metalloproteinase (MMP)-9, but the underlying mechanism remains unclear. Here, we show that 4-HPR blocks the activity of MMP-9 in two ways: by reducing phorbol 12-myristate 13-acetate (PMA)-induced MMP-9 secretion and by suppressing cell invasion through the downregulation of MMP-9 gene transcription in MCF-7 breast cancer cells. 4-HPR inhibits the transcriptional activity of MMP-9 by reducing the DNA-binding activity of NF-κB on the MMP-9 promoter as well as by inhibiting the degradation of IκBα, leading to cytoplasmic accumulation of NF-κB. We also found that 4-HPR inhibits invasion and MMP-9 expression in the highly metastatic breast cancer cell line MDA-MB-231. Thus, 4-HPR might be a potent anti-invasive agent that works by suppressing MMP-9 expression via the NF-κB signaling pathway. J. Cell. Biochem. 113: 2845–2855, 2012.

KEY WORDS: 4-HPR; MMP-9; CELL INVASION; NF-κB; BREAST CANCER

For malignant tumors to progress and metastasize, the tumor cells must produce matrix-degrading enzymes that allow them to disseminate from the primary tumor and invade the surrounding tissue. Several families of matrix proteases have been implicated in cancer invasion and metastasis, but most of the available data focus on the matrix metalloproteinases (MMPs) [Fingleton, 2006; Tai et al., 2008]. Expression of MMP-2 and MMP-9 has been shown to be involved in the regulation of metastasis in various cancers, including breast cancer. MMP-9 degrades type IV collagen, which is the major structural component of the basement membrane and extracellular matrix (ECM), and its activity increases with the degree of malignancy [Tai et al., 2008].

MMP-9 activity in various tumor cells is tightly controlled, mainly at the transcriptional level [Stamenkovic, 2000; Liang et al., 2009]. The MMP-9 promoter is highly conserved and contains multiple functional elements, including nuclear factor-κB (NF-κB)- and activator protein 1 (AP-1)-binding sites [Sato and Seiki, 1993; Kim et al., 2011], and previous studies have suggested that NF-κB is one of the most important transcription factors regulating MMP-9 expression [Park et al., 2009; Chou et al., 2010; Kim et al., 2011]. NF-κB is retained in the cytoplasm by the inhibitory protein IκBα. Phosphorylated IκBα is ubiquitinated, and subsequently degraded by the proteasome, resulting in the liberation of NF-κB [Karin and Ben-Neriah, 2000; Chen and Greene, 2004]. The NF-κB released translocates into the nucleus and binds to the promoter region of MMP-9, leading to gene expression.

The retinoids are vitamin A derivatives that play important roles in growth, vision, reproduction, immune function, differentiation, and apoptosis [De Luca, 1991; Nagy et al., 1998; Tang and Gudas, 2011]. *N*-(4-Hydroxyphenyl)retinamide (4-HPR), a synthetic derivative of retinoic acid, has been reported to inhibit cell growth in a variety of human cancer, including neuroblastomas and colorectal, prostate, breast, ovarian, and small cell lung cancers, as well as both lymphoid and myeloid leukemia cell lines [Moon et al., 1979; Kelloff et al., 1994; Reynolds and Lemons, 2001]. In addition, 4-HPR has been evaluated in several clinical trials, and it appears to have

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effective chemo-preventive properties against various malignant tumors [Pienta et al., 1997; Simeone et al., 2006]. Studies to define the therapeutic mechanism of 4-HPR-9 have shown that it suppresses tumor cell invasion and inhibits MMP-9 expression [Simeone et al., 2006], but the molecular mechanism underlying the anti-invasive and anti-migratory effects of 4-HPR is not yet fully understood in breast cancer. In the present study, we found that 4-HPR reduced the invasion and migration of human breast cancer cells by inhibiting MMP-9 gene expression through the suppression of NF-κB activation.

MATERIALS AND METHODS

CELLS AND REAGENTS

The human breast cancer cell line MCF-7 and MDA-MB-231 cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and penicillin–streptomycin (100 μg/ml) at 37°C with 5% CO_2 atmosphere in a humidified incubator. Gelatin was obtained from DIFCO (Lexington, KY). Lipofectamine 2000 reagent was purchased from Invitrogen (Carlsbad, CA). PMA was purchased from Calbiochem (La Jolla, CA). Anti-p65, anti-tubulin, and anti-IκBα antibodies were from Santa Cruz (Santa Cruz, CA). Anti-p-IκBα antibody was from Cell signaling (Beverly, MA). All the chemicals not included above were from Sigma.

CELL PROLIFERATION AND VIABILITY ASSAY

All proliferation and viability assays were based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. The cells were treated with various concentration of 4-HPR presence or absence 100 nM PMA, and allowed to grow for 24 and 48 h. At the end of the experiment, the media were removed and DMSO was added with MTT solubilization solution. Absorbance was measured at 550 nm.

COLONY FORMING ASSAY

MCF-7 cells were seeded into 6-well plate and allowed to attach for 24 h at 37°C in culture medium. Cells were then treated with various concentration of 4-HPR. After 4 days, colonies were fixed with fixing solution (methanol:acetic acid = 3:1) for 10 min at room temperature and stained with 0.01% crystal violet solution. Plates were washed with PBS and were photographed.

IN VITRO INVASION ASSAY

Matrigel invasion assays were used to assess the effect of 4-HPR in MCF-7 and MDA-MB-231 cells. The 8-μm pore size polycarbonate nucleopore filter inserts in a 24-well transwell chamber (BD Biosciences) were coated with 30 μg/well matrigel (Sigma). 4-HPR treated-MCF-7 cells were seeded into the upper part of the matrigel-coated filter, and serum-free RPMI with or without 100 nM PMA was added to the lower part. After 36 h, the cells that had migrated through the matrigel and the 8-μm pore-size membrane were fixed, stained, and counted under a light microscope.

RNA EXTRACTION AND SEMI-QUANTITATIVE RT–PCR

Total RNA was extracted from cells with Trizol (Invitrogen) according to the manufacturer's protocol. Approximately 2 μg of total RNA was used to prepare cDNA using the Superscript First Strand cDNA Synthesis Kit (Bioneer, Daejeon, South Korea). The following primers were used in this study: 5′-TCCCTGGAGACCT-GAGAACC-3′ and 5′-CGGCAAGTCTTCCGAGTAGTT-3′ for MMP-9; 5′-CCATCACCATCTTCCAGGAG-3′, and 5′-CCTGCTTCACCACG-TTCTTG-3′ for GAPDH. PCR was performed with Platinum Tap polymerase (Invitrogen) under the following conditions: 30 cycles of 96°C for 40 s, 55°C (MMP-9) or 60°C (GAPDH) for 40 s, 72°C for 1 min followed by 10 min at 72°C. The PCR products were electrophoresed on a 2% (w/v) agarose gel in 1× Tris-acetate-EDTA (TAE) buffer, and stained with ethidium bromide solution. All the PCR reactions were repeated at least three times. GAPDH was amplified as an internal control. The intensity of each band amplified by RT–PCR was analyzed using MultiImageTM Light Cabinet (version 5.5, Alpha Innotech Corp., San Leandro, CA), and normalized to that of GAPDH mRNA in corresponding samples.

GELATIN ZYMOGRAPHY

The presence of MMP-9 in the supernatants of DMSO or 4-HPR treated MCF-7 cells was analyzed with gelatin zymograms. Briefly, cells were incubated in serum-free RPMI and the supernatants were collected after incubation for 24 h, clarified by centrifugation, normalized to the total protein concentration of the cell lysate, mixed with non-reducing Laemmli sample buffer, and separated by electrophoresis in 10% SDS–PAGE containing 1 mg/ml gelatin (DIFCO). After electrophoresis, gels were re-natured by washing in 2.5% Triton X-100 solution twice for 30 min to remove all SDS. The gels were then incubated in 50 mmol/L Tris–HCl (pH 7.4), 5 mmol/L $CaCl_2$, and 1 μM $ZnCl_2$ at 37°C overnight. After incubation, the gels were stained with 0.05% Coomassie brilliant blue R-250 for 30 min at room temperature and then destained in distilled water. MMP-9 activities were visible as clear bands on a blue background where the gelatin substrate had been hydrolyzed by enzyme activity.

ELISA

The supernatants were collected for measuring secreted-MMP-9 protein. The total and active MMP-9 protein was assayed according to SensoLyte PlusTM 520 MMP-9 assay system (AnaSpec, San Jose, CA). MMP-9 activity unit was expressed as a change in fluorescence intensity at excitation of 490 nm/emission of 520 nm.

WOUND HEALING ASSAY

The MDA-MB-231 cells were incubated until 90–100% confluent. After cells were scratched by P-10 pipette tip, cells were incubated for various time periods. The pictures were taken at 0, 12, 24, and 36 h. Phase contrast images were taken by Nikon microscopy system (Nikon Instrument). Measurement of the wound-healing gap distance was performed using the computer program Image J. Results were expressed as the mean ± SE. $P \leq 0.05$ was considered significant (Student's *t*-test).

TRANSIENT TRANSFECTION AND LUCIFERASE REPORTER ASSAY

The transcriptional activities of MMP-9, NF-κB, and AP-1 were measured by luciferase reporter assay using the pMMP-9-Luc, and pNF-κB-Luc reporter plasmids. MCF-7 cells were seeded into 6-well plates. Cells at 70–80% confluence were co-transfected with 0.2 μg of MMP-9, or NF-κB reporter constructs and 0.2 μg pSV-β-galatosidase for 24 h. The transfected cells were incubated with 4-HPR and then stimulated with 100 nM PMA for 9 h. Luciferase and β-galactosidase activities were assayed according to the manufacture's protocol (Promega), using a Luminometer 20/20n (Turner BioSystems, Sunnyvale, CA). Luciferase activity was normalized by β-galactosidase activity in cell lysate and expressed as an average of three independent experiments.

CELL FRACTIONATION AND IMMUNOBLOTTING

Nuclear and cytosolic fractions were prepared using Nuclear and Cytoplasmic Extraction Reagents kit (Fermentas). PCNA and α-tubulin were used as markers for nuclear and cytosol proteins, respectively. Lysate proteins were resolved by SDS–PAGE and transferred onto nitrocellulose membranes. The membranes were incubated with TBS containing 0.1% Tween 20 and 5% skimed milk, and then exposed to the desired primary antibodies. After treatment with anti-rabbit or -mouse antibodies conjugated with horseradish peroxidase, the immunoreactive bands were visualized by standard ECL method.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Double-stranded oligonucleotides containing the NF-κB (5′-AGTT-GAGGGGACTTTCCCAGGC-3′) or consensus sequences were 5′-end-labeled with γ-^{32}P ATP using T4 polynucleotide kinase. Unincorporated nucleotide was removed by passage over a Bio-Gel P-6 spin column (Bio-Rad, Inc., Hercules, CA). Nuclear extract was incubated with radiolabeled probe for 20 min, and protein–DNA complexes were separated from free probes by electrophoresis on a 4% native polyacrylamide gel in 0.5× Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM EDTA, 4% glycerol, 0.5 mM DTT, and 50 mg/ml of poly (dI–dC). Dried gels were visualized by autoradiography.

IMMUNOFLUORESCENT STAINING

MCF-7 cells were treated with or without 5 μM 4-HPR for 30 min and then incubated with 100 nM PMA for 30 min. Cells were fixed with 4% paraformaldehyde in PBS at room temperature for 10 min. Nonspecific sites were blocked by incubating with 200 μl of 1% BSA in PBS at 37°C for 15 min. A rabbit polyclonal antibody against p65 was diluted 1:200 in PBS containing 1% BSA and incubated with the coverslips at 37°C for 1 h. Cells were then washed with 1% BSA/PBS for 10 min at room temperature before incubating with a 1:200 dilution of FITC-labeled goat anti-rabbit IgG antibody at room temperature for 45 min, and then the coverslips were rinsed with a 1% BSA/PBS solution for 10 min. Then the cells were stained with 4,6-diamidino-2-phenylindole (DAPI) for another 1 min at room temperature. The coverslips containing the cells were then mounted with AquaMount (Lerner Laboratories, New Haven, CT) containing 0.01% 1,4-diazobicyclo(2,2,2)octane. Fluorescent images were taken by OLYMPUS IX71 fluorescence microscope.

STATISTICS ANALYSIS

Statistical analysis was performed by computer program Prism (GraphPad Software, La Jolla, CA). The results are presented as mean ± SE. The statistical significance of differences between groups was analyzed via repeated measures of one-way analysis of variance followed by Student's *t*-test. A P value ≤ 0.05 was considered to be significant.

RESULTS

4-HPR SUPPRESSES PMA-INDUCED PROLIFERATION AND INVASION OF MCF-7 CELLS

We first examined the effect of 4-HPR on PMA-induced proliferation and invasion of MCF-7 cells. Treatment with PMA for 24 and 48 h significantly increased the viability of MCF-7 cells, but treatment with >3 μM 4-HPR decreased cell viability in the presence or absence of PMA (Fig. 1B). To further investigate the inhibitory effects of 4-HPR on cell proliferation, we performed a colony formation assay. PMA used as a positive stimulator markedly enhanced the colony-forming ability of MCF-7 cells, compared with the control cells (Fig. 1C). PMA-induced colony formation was slightly suppressed in the cells treated with 4-HPR at 1 μM, whereas 5 μM 4-HPR inhibited the colony-forming ability of MCF-7 cells by approximately 2.5 folds (Fig. 1C). We next examined whether 4-HPR inhibits PMA-induced invasion in MCF-7 cells. PMA induced a 20-fold increase in the invasion of MCF-7 cells; however, 4-HPR inhibited this invasion in a dose-dependent manner (Fig. 1D). These results indicate that 4-HPR is an effective inhibitor of cell migration, invasion, and metastasis in PMA-induced MCF-7 breast cancer cells.

4-HPR INHIBITS PMA-INDUCED INVASION OF MCF-7 CELLS BY REDUCING EXPRESSION AND SECRETION OF MMP-9

MMP-9 is an important enzyme involved in the degradation of the ECM [Woessner, 1991], and has also been reported to be involved in cancer cell invasion and metastasis [Sato and Seiki, 1993]. To confirm whether MMP-9 activity is involved in the PMA-induced invasion of MCF-7 cells, we treated cells with PMA, primary antibody against MMP-9, or 1 μM 4-HPR, alone or in combination. Treatment with MMP-9 primary antibody and 4-HPR (1 μM) significantly blocked PMA-induced cell invasion (Fig. 2A). We next studied the effect of 4-HPR on the expression of MMP-9 and its activity in conditioned media. Western blotting (Fig. 2B top and second panels), RT–PCR (Fig. 2B third and bottom panels), gelatin zymography, and ELISA indicated that the basal level of MMP-9 in MCF-7 cells was low. However, MMP-9 protein levels, RNA expression, and secretion were all markedly induced by PMA treatment. Interestingly, 4-HPR inhibited both the increase in MMP-9 expression (Fig. 2B) as well as the secretion (Fig. 2C,D), but it had no effect on the mRNA expression of TIMP-1 (data not shown). These results indicate that 4-HPR inhibits cell invasion by downregulating MMP-9 expression and secretion in PMA-treated MCF-7 cells.

4-HPR INHIBITS PMA-INDUCED TRANSCRIPTION OF MMP-9

To determine whether MMP-9 expression was regulated at the transcriptional level by 4-HPR, we performed a promoter assay

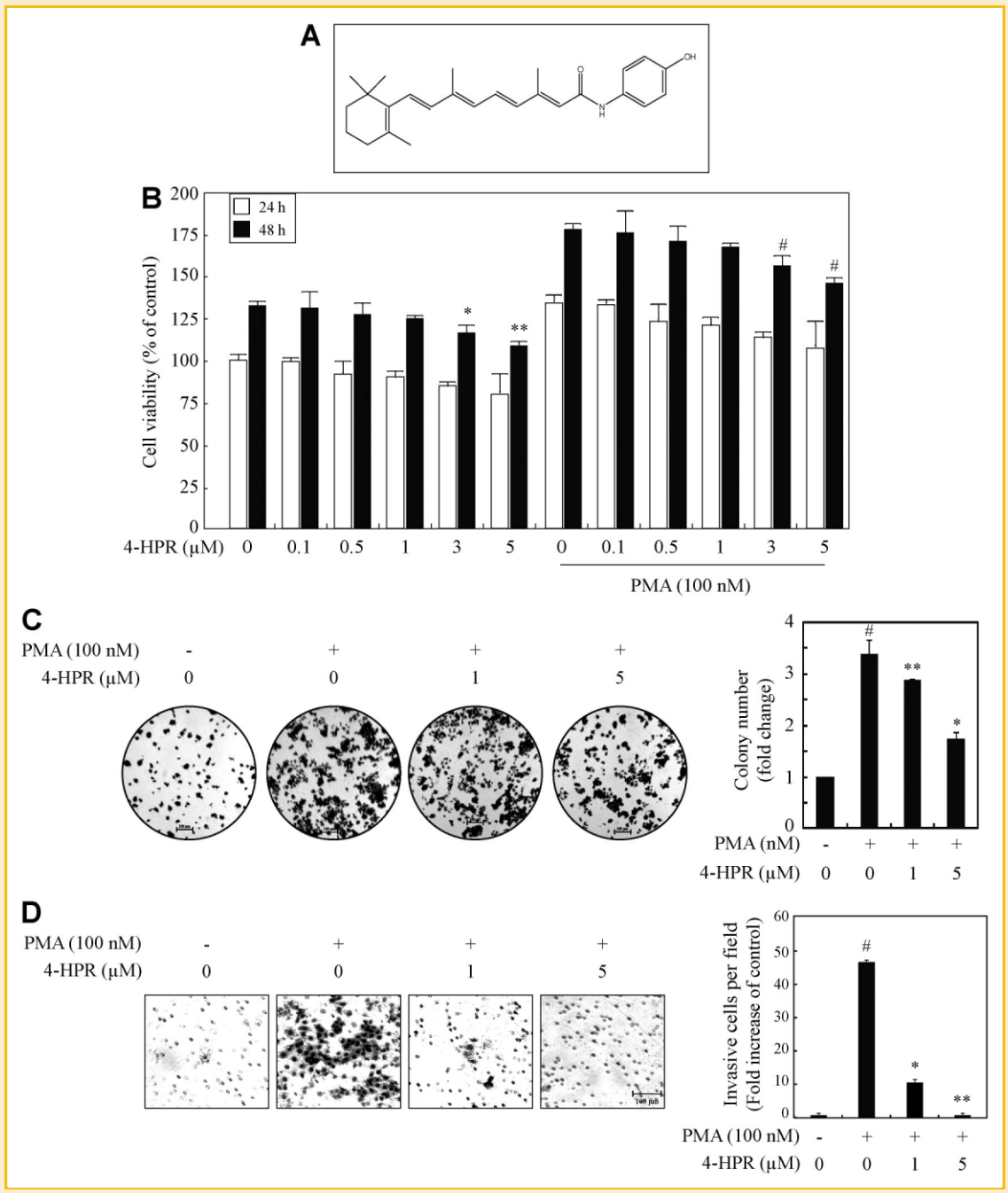

Fig. 1. 4-HPR suppresses PMA-induced proliferation and invasion of MCF-7 cells. A: Chemical structure of 4-HPR. B: MCF-7 cells were treated with 0–5 μM 4-HPR in the absence or presence of 100 nM PMA for 24 h (white bars) and 48 h (black bars), and cell viability was measured using a MMT assay; $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle alone-treated cells, $^{\#}P < 0.01$ versus PMA alone-treated cells. C: MCF-7 cells were treated with the indicated concentration of 4-HPR for 30 min and then incubated with 100 nM PMA for 4 days. After 4 days, colonies were fixed with fixing solution for 10 min at room temperature and stained with 0.01% crystal violet solution. Representative photographs demonstrating colony formation are shown. Original magnification was ×100. Scale bars, 100 μm. The number of colonies from triplicate plates was quantified using Image J; $^{\#}P < 0.01$ versus vehicle alone-treated cells, $^{*}P < 0.01$, $^{**}P < 0.001$ versus PMA alone-treated cells. D: MCF-7 cells were incubated with 100 nM PMA and the indicated concentration of 4-HPR for 36 h. The invasion ability of MCF-7 cells was determined by a Matrigel invasion assay. Original magnification was 200×. Scale bars, 100 μm. The cell invasion ability was quantified; $^{\#}P < 0.001$ versus vehicle alone-treated cells, $^{*}P < 0.01$, $^{**}P < 0.001$ versus PMA alone-treated cells. Data represent the mean ± SE of three independent experiments.

using MCF-7 cells transiently transfected with a MMP-9 promoter (−925/+13). As shown in Figure 3A, PMA treatment upregulated the promoter activity of MMP-9 by 6.5 folds, compared with the control cells. 4-HPR inhibited this PMA-induced promoter activity in a dose-dependent manner (Fig. 3A), indicating that 4-HPR inhibits MMP-9 expression at the transcriptional level. Since NF-κB and AP-1 play important roles in controlling MMP-9 expression in various cancer cell lines [Liu et al., 2002], we next looked at the

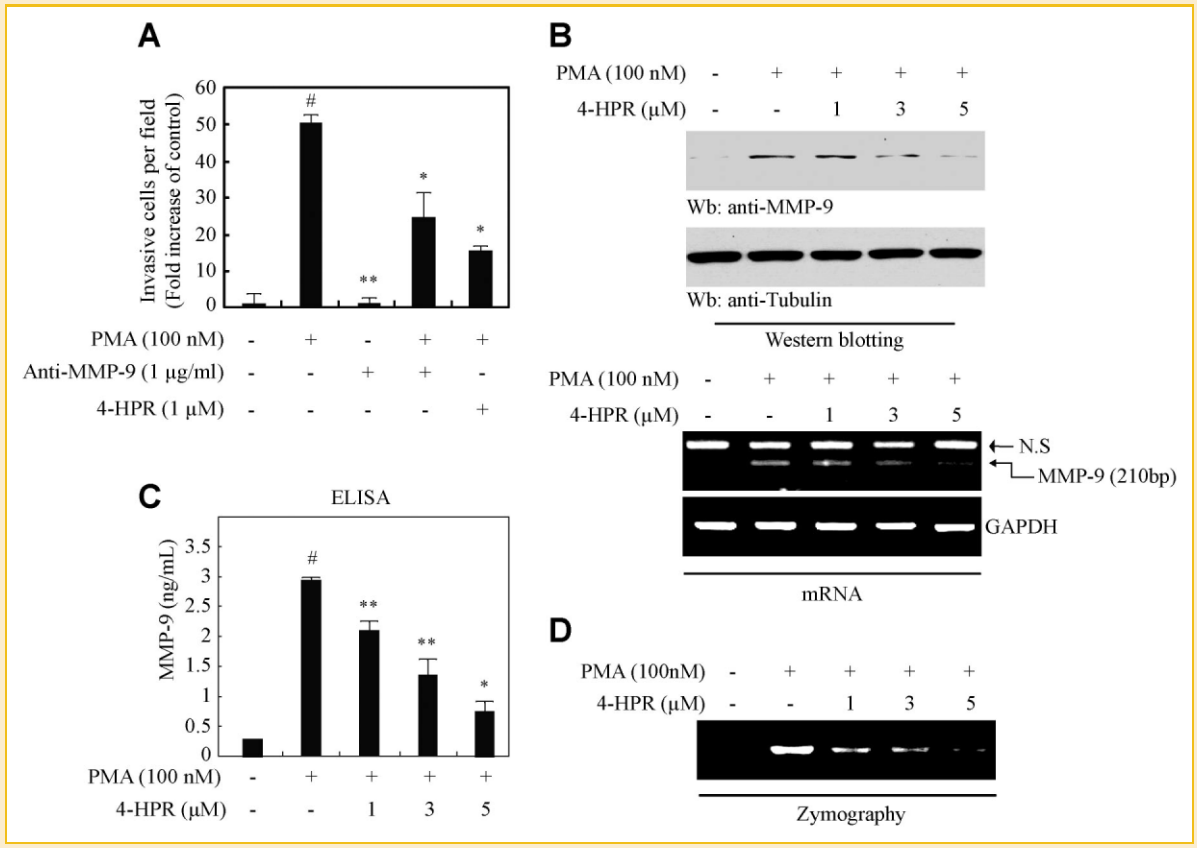

Fig. 2. 4-HPR reduces PMA-induced expression of MMP-9 in MCF-7 cells. A: MCF-7 cells were incubated in Matrigel-coated transwell with or without PMA and anti-MMP-9 antibody or 1 μM 4-HPR for 36 h. The cell invasion ability was quantified; $^{\#}P < 0.001$ versus vehicle alone-treated cells, $^{*}P < 0.01$, $^{**}P < 0.001$ versus PMA alone-treated cells. Data represent the mean ± SE of three independent experiments. B: MCF-7 cells were incubated with the indicated concentration of 4-HPR for 30 min followed by 100 nM PMA stimulation for 24 h. After 24 h, the protein level and the mRNA level of endogenous MMP-9 were measured by Western blotting (top and 2nd panels) and RT–PCR (3rd and bottom panel). Tubulin and GAPDH was used as an internal control. C,D: MCF-7 cells were pretreated with 4-HPR for 30 min and stimulated with 100 nM PMA for 24 h. After 24 h, the conditioned medium was collected and assayed for the secreted MMP-9 using ELISA (C) and gelatin zymography (D); $^{\#}P < 0.001$ versus vehicle alone-treated cells, $^{*}P < 0.01$, $^{**}P < 0.001$ versus PMA alone-treated cells. Data represent the mean ± SE of three independent experiments.

effect of 4-HPR on the binding of these transcription factors to the MMP-9 promoter. Sequence analysis of the MMP-9 promoter revealed binding sites for NF-κB (−600 bp), and AP-1 (−533 and −79 bp). To verify the transcription factors that participate in the regulation of MMP-9 transcription, we constructed mutants in which different transcription factor-binding sites in the MMP-9 promoter were deleted. We found that 4-HPR had no effect on the promoter activities of the NF-κB-binding site deletion mutants (−580/+13 and −450/+13; Fig. 3B), suggesting that the NF-κB (−600 bp)-binding site in the MMP-9 promoter is involved in the inhibitory effect of 4-HPR on PMA-induced transcriptional activation of MMP-9. To confirm these findings, we generated a promoter containing a mutation in the NF-κB (−600 bp)-binding site. The results obtained showed that 4-HPR did not affect the PMA-induced activity of this mutated promoter (−925/+13; Fig. 3C). To further investigate this observation, we used luciferase reporter vectors containing tandem repeats of the NF-κB- or AP-1-binding sites. As shown in Figure 3D,E, the luciferase activity in the cells transfected with the NF-κB reporter plasmid was reduced in a dose-dependent manner by the 4-HPR treatment, whereas no significant changes were observed in the cells transfected with the AP-1 reporter plasmid. These results indicate that the binding of NF-κB to the MMP-9 promoter contributes to the inhibitory effect of 4-HPR on PMA-induced MMP-9 transcription.

4-HPR INHIBITS THE DNA BINDING ACTIVITY OF NF-κB IN THE MMP-9 PROMOTER

To examine the effect of 4-HPR on the DNA-binding activities of NF-κB, we performed electrophoretic mobility shift assay. As shown in Figure 4A, PMA induced the DNA-binding activities of NF-κB, while 4-HPR decreased the DNA-binding activity of NF-κB in a dose-dependent manner. NF-κB is sequestered in the cytoplasm by binding to the IκB family, and is activated by IκBα phosphorylation and subsequent degradation in the proteasome, thus allowing the NF-κB subunits p65 and p50 to enter the nucleus and activate target genes [Magnani et al., 2000]. We used immunofluorescence to determine whether 4-HPR affects the phosphorylation of IκBα and the nuclear translocation of the NF-κB p65 subunit. Our results show that p65 translocated into the nucleus of MCF-7 cells after treatment with 100 nM PMA, but it remained in the cytoplasm when the cells were treated with 5 μM 4-HPR treatment (Fig. 4B). To further

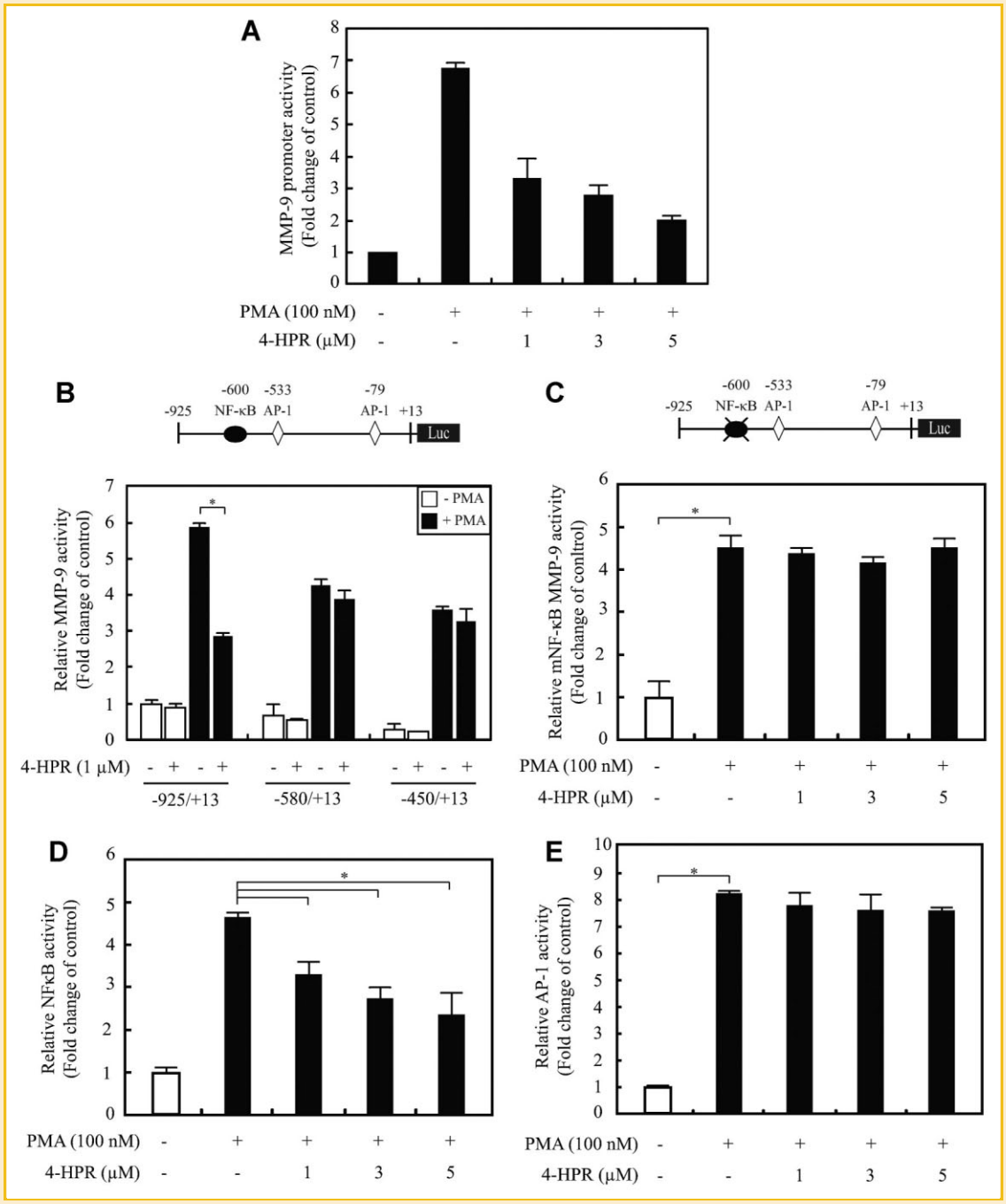

Fig. 3. 4-HPR inhibits the transcription of MMP9 promoter constructs via suppression of PMA-stimulated NF-κB activity. A: MCF-7 cells were transfected with the pMMP-9-luciferase (−925/+13) and the pSV40-β-galactosidase vectors. The transfected cells were treated with the indicated concentrations of 4-HPR for 30 min and incubated with 100 nM PMA for 9 h. The luciferase activity was normalized by β-galactosidase activity. Each value represents the mean ± SD of three-independent experiments and is expressed relative to a control. B,C: MCF-7 cells were transfected with the deletion mutants pMMP-9-Luc (B) and NF-κB binding site mutant (mNF-κB) pMMP-9-Luc (C). The transfected cells were treated with 4-HPR for 30 min and incubated with 100 nM PMA for 9 h. The luciferase activity was normalized by β-galactosidase activity. Each value represents the mean ± SD of three-independent experiments and is expressed relative to a control. One-way ANOVA was performed to determine statistical significance ($^{*}P < 0.05$). D,E: MCF-7 cells were transfected with the luciferase reporter plasmids containing tandem NF-κB (D) or AP-1 (E) binding sites. The transfected cells were treated with 4-HPR for 30 min and incubated with 100 nM PMA for 9 h and the luciferase activities were determined. Each value represents the mean ± SD of three-independent experiments and is expressed relative to a control. One-way ANOVA was performed to determine statistical significance ($^{*}P < 0.05$).

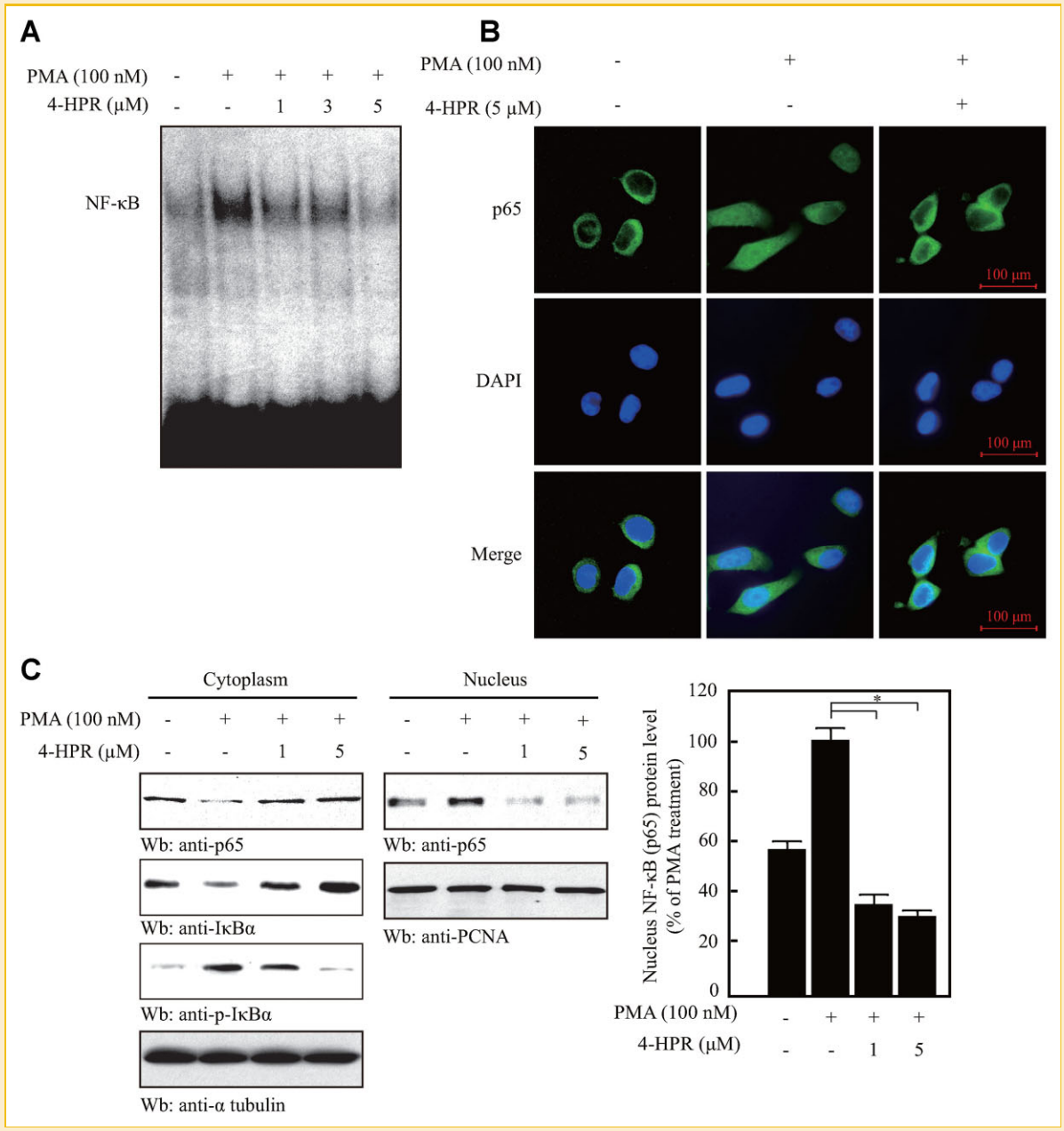

Fig. 4. 4-HPR inhibits the NF-κB activity in PMA-stimulated MCF-7 cells. A: MCF-7 cells were pretreated with the indicated concentration of 4-HPR for 30 min and incubated with 100 nM PMA for 30 min. Nuclear extracts were prepared and incubated with radiolabeled oligonucleotides containing the NF-κB motif in the MMP-9 promoter. B: MCF-7 cells were pretreated with 5 μM 4-HPR for 30 min and incubated with 100 nM PMA for 30 min. The cells were fixed with paraformaldehyde, incubated with polyclonal anti-p65 antibody for 1 h, and incubated with FITC-conjugated anti-rabbit antibody for 45 min. After mounting, fluorescent images were taken by OLYMPUS IX71 fluorescence microscope. C: MCF-7 cells were pretreated with the indicated concentration of 4-HPR for 30 min and incubated with 100 nM PMA for 30 min. Cells were harvested and fractionated into the cytoplasm and the nucleus. Lysates were then separated on a 10% SDS–polyacrylamide gel and subjected to Western blotting with anti-p65, anti-p-IκBα, and anti-IκBα antibodies. The analysis was repeated in three times, and α-tubulin and PCNA were used as markers for the cytoplasmic and nuclear fractions. The NF-κB protein level that was translocated to the nucleus was quantified by densitometric analyses. The bars represent the mean ± SD. One-way ANOVA was performed to determine statistical significance ($^{*}P < 0.05$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

confirm this observation, we analyzed the levels of p65 in the cytosolic and nuclear fractions. The results obtained showed that PMA increased the phosphorylation of IκBα in the cytoplasm, leading to increased nuclear translocation of the NF-κB p65 subunit (Fig. 4C). However, 4-HPR inhibited this PMA-induced phosphorylation of IκBα in a dose-dependent manner (Fig. 4C), and decreased the amount of NF-κB p65 subunit in the nucleus (Fig. 4C, right panel). These results indicate that 4-HPR inhibits NF-κB activation by suppressing IκBα phosphorylation and subsequent nuclear translocation of NF-κB in PMA-treated MCF-7 cells.

4-HPR INHIBITS THE EXPRESSION AND SECRETION OF MMP-9 IN MDA-MB-231 CELLS

The results obtained with MCF-7 cells suggest that 4-HPR inhibits PMA-induced MMP-9 expression by suppressing the nuclear translocation of NF-κB. To further demonstrate the functional consequence of 4-HPR in metastatic breast cancer, we used MDA-MB-231 cells, a highly metastatic breast cancer cell line that highly expresses MMP-9 and constitutively activated NF-κB [Kousidou et al., 2004; Matsumoto et al., 2005]. We treated these cells with 4-HPR (0–5 μM) and monitored changes in MMP-9 expression. As shown in Figure 5A, treatment of MDA-MB-231 cells with 4-HPR for 24 h decreased MMP-9 expression in a dose-dependent manner. We further assessed the MMP-9 secretion in 4-HPR treated MDA-MB-231 cells by zymography and ELISA. The results of gelatin zymography and ELISA revealed that 4-HPR inhibited the secretion and the proteolytic activity of MMP-9 (Fig. 5B,C). These findings support our results obtained with PMA-induced MCF-7 cells (Fig. 2).

INHIBITION OF CONSTITUTIVELY ACTIVATED NF-κB BY 4-HPR IN DMA-MB-231 CELLS

NF-κB is often constitutively activated in breast and prostate carcinomas [Palayoor et al., 1999], and a previous study showed that constitutive activation of NF-κB contributed to the progression of breast cancer [Nakshatri et al., 1997; Romieu-Mourez et al., 2001]. As shown in Figure 6A, NF-κB was constitutively activated in MDA-MB-231 in the EMSA assay (Fig. 6A, lane 1). 4-HPR inhibited the activation of NF-κB in a dose-dependent manner (Fig. 6A). These results suggest that 4-HPR inhibits the nuclear translocation and transcriptional activity of NF-κB. To further assess the effect of 4-HPR on NF-κB translocation, we used immunostaining to show that p65 in the vehicle-treated cells was localized in both the cytoplasm and the nucleus, whereas p65 in the 4-HPR-treated cells was exclusively found in the cytoplasm. Subcellular fractionation followed by immunoblotting revealed that the nuclear translocation of p65 was substantially augmented after treatment with 4-HPR (Fig. 6C, top panel), further supporting our immunostaining results. These data indicate that 4-HPR inhibits the constitutive activation of NF-κB by suppressing the nuclear translocation of NF-κB in metastatic breast cancer MDA-MB-231 cells.

4-HPR INFLUENCES MIGRATION OF METASTATIC BREAST CANCER CELLS

MMP-9 expression has been implicated in tumor invasion and metastasis [Sato and Seiki, 1993]. Our results shown that 4-HPR decreases MMP-9 expression in MDA-MB-231 cells (Fig. 5). To confirm the anti-invasive activity of 4-HPR, we investigated its inhibitory effects on invasion and migration in MDA-MB-231 cells. As shown in Figure 7A, treatment with 4-HPR inhibited the invasion of MDA-MB-231 cells in dose-dependent manner, corresponding to the results obtained with the PMA-induced MCF-7 cells (Fig. 1). 4-HPR also inhibited the migration of MDA-MB-231 cells in a dose-dependent manner (Fig. 7B). Collectively, these data support the idea that 4-HPR inhibits the migration and invasion of breast cancer cells through inhibition of NF-κB-mediated MMP-9 transcription.

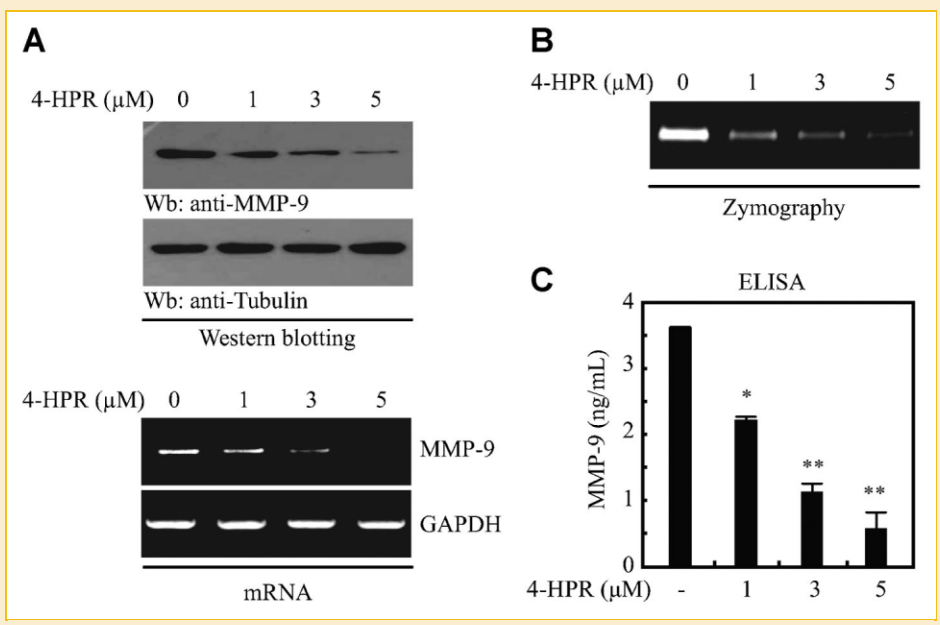

Fig. 5. 4-HPR reduces the expression of MMP-9 in MDA-MB-231 (A) MDA-MB-231 cells were treated with the indicated concentration of 4-HPR for 24 h. After 24 h, the protein level and the mRNA level of endogenous MMP-9 were determined by Western blotting (top and 2nd panels) and RT-PCR (3rd and bottom panel). Tubulin and GAPDH was used as an internal control. B,C: MDA-MB-231 cells were treated with the indicated concentration of 4-HPR for 24. After 24 h, the conditioned medium was collected and assayed for the secreted MMP-9 using ELISA (C) and gelatin zymography (D); $^{*}P < 0.01$, $^{**}P < 0.001$ versus vehicle control cells. Data represent the mean ± SE of three independent experiments.

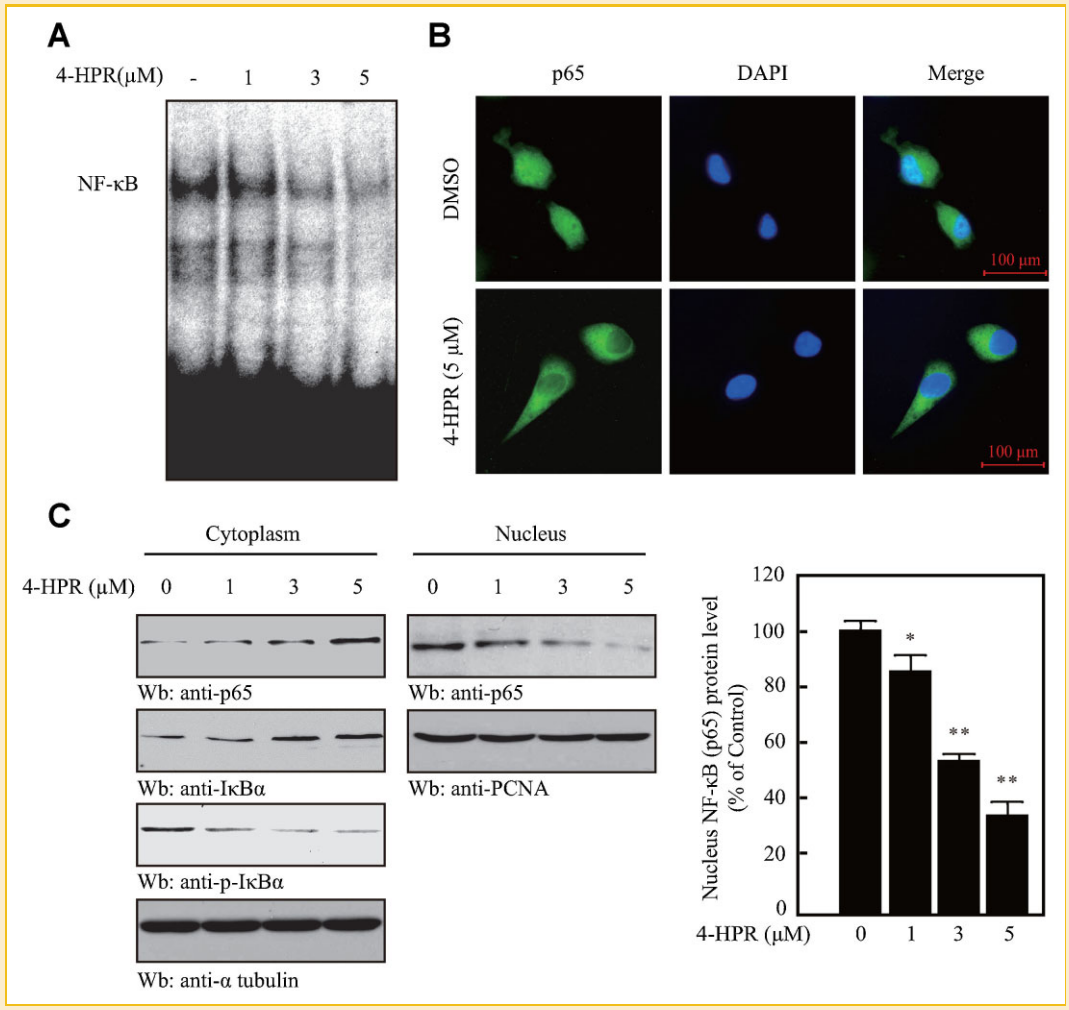

Fig. 6. 4-HPR inhibits the basal NF-κB activity in MDA-MB-231 cells. A: MDA-MB-231 cells were treated with the indicated concentration of 4-HPR for 4 h. Then the nuclear extract was assayed by EMSA. (B) MDA-MB-231 cells were treated with or without 5 μM 4-HPR for 4 h and then the cells were fixed with paraformaldehyde, incubated with polyclonal anti-p65 antibody for 1 h, and incubated with FITC-conjugated anti-rabbit antibody for 45 min. After mounting, fluorescent images were taken by OLYMPUS IX71 fluorescence microscope. C: MDA-MB-231 cells were treated with the indicated concentration of 4-HPR for 4 h. The cells were harvested and fractionated into the cytoplasm and the nucleus. Lysates were then separated on a 10% SDS-polyacrylamide gel and subjected to Western blotting with anti-p65, anti-p-IκBα, and anti-IκBα antibodies. The analysis was repeated in three times, and α-tubulin and PCNA were used as markers for the cytoplasmic and nuclear fractions. The NF-κB protein level that was translocated to the nucleus was quantified by densitometric analyses. The bars represent the mean ± SD. One-way ANOVA was performed to determine statistical significance ($^{*}P < 0.05$, $^{**}P < 0.001$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

DISCUSSION

4-HPR has potent chemo-preventive and anti-metastatic effects in several animal models [Green et al., 1999; Shaker et al., 2000; Hursting et al., 2001; Boya et al., 2003] and is currently under clinical trials for use as a preventive agent for ovarian carcinoma, lung carcinoma, and breast neoplasia [Cai and Jones, 1998; Levi et al., 2001]. In addition, 4-HPR can exert chemotherapeutic effects and kill cancer cells in variety of in vitro and in vivo models through the induction of apoptosis [Wu et al., 2001; Fontana and Rishi, 2002]. In our study, we found that 4-HPR had little effect on MCF-7 cell viability, but it did affect cell invasion (Fig. 1). This finding prompted us to survey the relationship between 4-HPR and the expression of MMP-9, a protease that has been implicated in tumor invasion in a number of settings. Our results indicate that 4-HPR is a potent inhibitor of PMA-induced MMP-9 expression in two breast cancer cells lines, namely, MCF-7 and MDA-MB-231, suggesting that the ability of 4-HPR to inhibit MMP-9 expression may be a general phenomenon.

Using Western blot, RT–PCR, and luciferase reporter gene assay, we determined that the ultimate target of the inhibitory effect of 4-HPR was MMP-9 gene transcription. The expression of proteases such as MMP-9 is regulated by diverse growth factors, cytokines, and xenobiotics such as PMA. PMA has been used as a tumor promoter in chemically induced carcinogenesis in vitro and in vivo. Previous our study showed that PMA is able to promote tumor migration and invasion by stimulating MMP-9 expression in breast cancer cells [Kim et al., 2011]. The MMP-9 promoter region contains

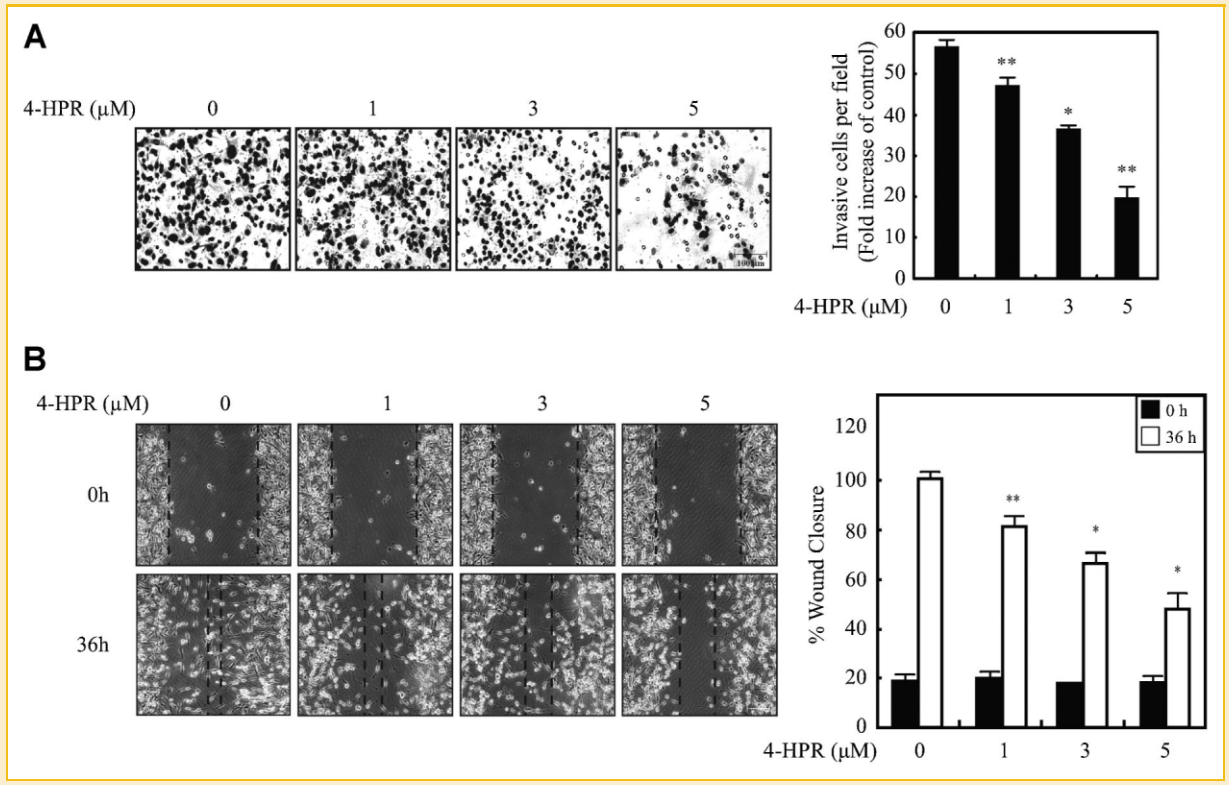

Fig. 7. 4-HPR inhibits the migration and invasion of MDA-MB-231 cells. A: MDA-MB-231 cells were treated with the indicated concentration of 4-HPR for 36 h. The invasive ability of cells was determined using a matrigel invasion assay. Migrated cells were stained with Diff-Quik and counted for quantitative analysis. Original magnification was 200×. Scale bars, 100 μm. The quantification of penetrated cells is represented as the mean of three independent experiments. $^{*}P < 0.01$, $^{**}P < 0.001$ versus vehicle alone-treated cells. B: MDA-MB-231 cells were scratched with a pipette tip and incubated with the indicated concentration of 4-HPR for 36 h. The wound area was quantified by measuring the cell-free area. Original magnification was 100×. Scale bars, 100 μm. Cell migration into the wound area is represented as the percentage of recovery relative to 0 h. $^{*}P < 0.01$, $^{**}P < 0.001$ versus vehicle alone-treated cells.

the cis-regulatory element, including one NF-κB- and two AP-1-binding sites, which are absent in the promoter region of MMP-2 [Sato and Seiki, 1993; Kim et al., 2011]. In this study, we found that the NF-κB-binding site is necessary for the inhibition of MMP-9 expression by 4-HPR in both PMA-treated MCF-7 cells as well as in MDA-MB-231 cells, which have constitutively activated NF-κB. NF-κB plays a central role in promoting cancer cell motility and invasion [Baldwin, 2001], and cancer progression in multiple cancers, including melanomas and breast, prostate, colorectal, and ovarian cancers, and specific forms of leukemia and lymphoma, is correlated with a significant elevation in NF-κB expression [Shattuck-Brandt and Richmond, 1997; Liang et al., 2009]. Moreover, NF-κB is involved in the regulation of production of inflammatory cytokines and inflammation [Barnes and Karin, 1997]. Here, we demonstrated that 4-HPR suppressed PMA-induced MMP-9 expression through the inhibition of NF-κB activity. Shimada et al. [2002] also demonstrated that 4-HPR inhibits NF-κB activation in prostate cancer cell lines. These data suggest that 4-HPR is a potent inhibitor of MMP-9 transcription, and that 4-HPR suppresses the expression of NF-κB-regulated genes, which, in turn, affects various biological events such as carcinogenesis and inflammation. Our results are supported by a recent study, which showed that 4-HPR reduces the invasion of the metastatic breast cancer cell line MDA-MB-231 [Simeone et al., 2006]. In conclusion, our findings suggest that 4-HPR inhibits NF-κB activity via the IκB pathway, leading to reduction in the expression of MMP-9 and suppression of PMA-induced invasion in MCF-7 and MDA-MB-231 breast cancer cells. Therefore, 4-HPR is a potential chemotherapeutic candidate with a broad spectrum of anticancer activities.

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