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Heme oxygenase-1 inhibits breast cancer invasion via suppressing the expression of matrix metalloproteinase-9

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Abstract

In the present study, we investigated the antitumor effects of the invasiveness and migration of heme oxygenase 1 (HO-1) in human breast carcinoma cells. 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced matrix metalloproteinase-9 (MMP-9) enzyme activity and gene expression at both protein and mRNA levels were examined in human breast carcinoma cells (MCF-7 and MDA-MB-231), and the addition of the MMP-9 inhibitor, SB3CT, significantly suppressed TPA-induced invasion and migration according to the *in vitro* Transwell assay. Elevation of HO-1 gene expression by ferric protoporphyrin IX inhibited TPA-induced invasion of MCF-7 cells, which was blocked by adding the heme oxygenase inhibitor, tin protoporphyrin IX, or transfection of cells with HO-1 short hairpin RNA. MCF-7 cells overexpressing HO-1 (MCF-7/HO-1) were established in the present study, and TPA-induced MMP-9 gene expression, tumor invasion, and colony formation were significantly reduced in MCF-7/HO-1 cells, compared with those in Neo-transfected cells. Activation of protein kinase C α /extracellular signal-regulated kinases/AP-1 with stimulation of reactive oxygen species production was involved in TPA-induced invasion of MCF-7 cells, which was attenuated by HO-1 protein induced by ferric protoporphyrin IX or transfection of HO-1 expression vectors. Additionally, the addition of carbon monoxide, but not ferric ions, biliverdin, or bilirubin, inhibited TPA-induced invasion through suppress-

ing MMP-9, extracellular signal-regulated kinases, and AP-1 activation stimulated by TPA. The beneficial role of HO-1 in blocking tumor invasion was first identified in this study. [Mol Cancer Ther 2008;7(5):1195–206]

Introduction

Tumor metastasis is the leading cause of cancer mortality and occurs by degradation of the cellular basement membrane and spreads to distant organs resulting in the formation of secondary tumors. Proteolytic degradation of the extracellular matrix by tumor cell-secreted proteases, including plasminogen activators, serine proteinases, and matrix metalloproteinases (MMP), is important for tumor angiogenesis, invasion, and metastasis (1, 2). Increased expressions of MMPs, particularly MMP-2 (gelatinase A; 72-kDa type IV collagenase) and MMP-9 (gelatinase B; 92-kDa type IV collagenase), have been intensely correlated with the malignancy of tumors and poor survival in patients, especially with breast cancer (3, 4). MMP-2 is commonly constitutively present in tissues, whereas MMP-9 is stimulated by growth factors, inflammatory cytokines, and phorbol ester (5–7). Induction of MMP-9 by amphiregulin and heregulin contributes to the invasiveness of breast cancers (8, 9), and increases in the secretion of MMP-9 by CXCR4 and CXCL12 were concomitant with enhanced cell migration and invasion in colorectal and prostate cancers (10, 11). Therefore, agents with ability to block MMP-9 activation may reserve the therapeutic potential to treatment human cancers.

Heme oxygenase (EC 1.14.99.3) is the rate-limiting enzyme in heme metabolism by catalyzing degradation of the heme group into carbon monoxide (CO), free iron, and biliverdin. At least three mammalian heme oxygenase isoforms have been identified. Heme oxygenase 2 and heme oxygenase 3 are constitutively expressed in selected tissues, whereas heme oxygenase 1 (HO-1) is a stress-responsive protein induced by heat, heavy metals, oxidants, and heme. Induction of the HO-1 protein has been reported to protect against a variety of stress conditions such as hydrogen peroxide, cisplatin, UV irradiation, and inflammatory cytokine-mediated cell damage (12–15). HO-1 has also been shown to inhibit the proliferation and induce apoptosis in several cancer cells such as breast carcinoma cells (16). Induction of HO-1 by the chemopreventive agent sulforaphane contributes to suppression of tumor cell growth by increasing the antioxidant response genes of hepatoma and breast cancer cells (17, 18). Although the antitumor effect of HO-1 has been investigated, the roles of HO-1 in the invasion and migration of breast carcinoma cells are still undefined.

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Reactive oxygen species (ROS) is an important secondary messenger in the regulation of intracellular signaling cascades. An increase in intracellular ROS level was detected in tumors, and stimulation of the proliferation and invasion of tumor cells with inducing MMP-9 activation by ROS has been identified (19, 20). HO-1 induction has been reported to reduce intracellular ROS level (14, 16, 21). Our previous study indicated that HO-1 induction protected macrophages and glioma cells from hydrogen peroxide-induced apoptosis (14, 21). However, the role of ROS in the action of HO-1 on the invasion of breast carcinoma cells is still undefined. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has been shown to induce carcinogenesis through activation of intracellular signaling cascades including protein kinase C (PKC), mitogen-activated protein kinase, and ROS *in vitro* and *in vivo* (19, 22–24). In the present study, the effect of HO-1 induction on TPA-induced MMP-9 expression and invasiveness in relation to activation of PKC/ROS/extracellular signal-regulated kinases (ERK) cascade in human breast cancer cells was investigated.

Materials and Methods

Chemicals

Antibodies against HO-1 and ERK were from Santa Cruz Biotechnology (1:1,000). Antibodies against MMP-9, MMP-2, and α -tubulin were from Lab Vision (1:500). The phosphospecific ERK antibody was from Cell Signaling Technology (1:1,000). Antibodies against PKC α and c-Jun were obtained from Transduction Laboratories (1:1,000). Ferric protoporphyrin IX (FePP) and tin protoporphyrin IX (SnPP) were purchased from Porphyrin Product. Specific inhibitors of phosphatidylinositol 3-kinase (LY294002), the mitogen-activated protein kinase family (PD98059, SP600125, and SB203580), and MMP-9 (SB3CT) were purchased from Calbiochem. Inhibitors of PKC (GF109203X and H7) were purchased from Tocris Cookson. [Ru(CO)₃Cl₂]₂ (RuCO) and RuCl₃ were purchased from Sigma-Aldrich. All other chemicals were purchased from Sigma unless indicated otherwise.

Cell Culture

The human MCF-7 and MDA-MB-231 breast carcinoma cell lines were obtained from the American Type Culture Collection. Cells were maintained in MEM supplemented with 5% heat-inactivated fetal bovine serum, 100 units penicillin-streptomycin, 1 mmol/L sodium pyruvate, and 0.1 mmol/L nonessential amino acid at 37°C in a humidified incubator containing 5% CO₂. All culture reagents were purchased from Life Technologies.

Transfection

The dominant-negative MEKK and the pcDNA3 control vector were transiently transfected into MCF-7 cells using the LipofectAMINE 2000 transfection reagent (Life Technology) according to the manufacturer's instructions. After 24 h, transfected cells were used for further experiments. Cells were transfected with the expression vector, pCMV-neo or pCMV-hHO-1, encoding full-length

human HO-1 cDNA by LipofectAMINE 2000. After 24 h, cells were placed in MEM containing 10% fetal bovine serum and 1 mg/mL G418 (Life Technologies). G418-resistant cells were selected and expanded. For the HO-1 gene knockdown, cells were transfected with a pSM2 retroviral vector containing a short hairpin small interfering RNA against HO-1 gene (HO-1 shRNA-1, GenBank accession no. NM_012580; HO-1 shRNA-2, GenBank accession no. NM_002133) or a control nonspecific vector (con-shRNA) expressed under the control of the U6 promoter (Open Biosystems). The stable cell lines were selected with 0.8 μ g/mL puromycin and screened for HO-1 protein expression.

Reporter Gene Assay

MCF-7 cells were grown in a 10-cm culture dish, subconfluent cells were replaced with serum-free Opti-MEM (Life Technologies), and cells were transfected with the pAP-1-Luc reporter construct using LipofectAMINE 2000. After transfection, the medium was replaced, and cells were cultured for an additional 24 h. Cells were plated in 24-well plates and treated with TPA (50 ng/mL) for 8 h. Luciferase activities were measured by a luminometer (Berthold Detection Systems) using the dual-luciferase Reporter Assay (Promega) according to the manufacturer's instructions. To normalize the transfected efficiency, cells were cotransfected with the *Renilla* luciferase vector (pRL-TK) as the internal control. Luciferase activity was measured by firefly luciferase activity/*Renilla* luciferase. The fold induction was calculated by dividing by the control group. All transient transfections were repeated in three independent experiments.

Measurement of ROS Production

Intracellular ROS production was monitored by flow cytometry using an oxidant-sensitive 2',7'-dichlorodihydrofluorescein diacetate probe (Molecular Probes). This dye is a stable compound that rapidly diffuses into cells and is hydrolyzed by intracellular esterase to yield 2',7'-dichlorodihydrofluorescein, which is trapped within cells. Hydrogen peroxide and low-molecular-weight peroxide produced by cells oxidize 2',7'-dichlorodihydrofluorescein to the highly fluorescent compound, 2',7'-dichlorofluorescein. Thus, the fluorescence intensity is proportional to the amount of peroxide produced by cells. In the present study, MCF-7 cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate (100 μ mol/L) for 30 min in the dark followed by TPA treatment in the presence of *N*-acetylcysteine (NAC) or diphenylene iodonium (DPI) for 30 min. After incubation, cells were resuspended in ice-cold PBS and detected using a 525-nm (FL1-H) band-pass filter by FACScan flow cytometry (Becton Dickinson).

Western Blotting

Cells lysates were prepared by suspending cells in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 1% NP-40, 150 mmol/L NaCl, 1 mmol/L EGTA, 0.025% sodium deoxycholate, 1 mmol/L NaF, 1 mmol/L sodium orthovanadate, and 1 mmol/L phenylmethylsulfonyl fluoride], and equal amounts of protein were prepared, separated on 8%

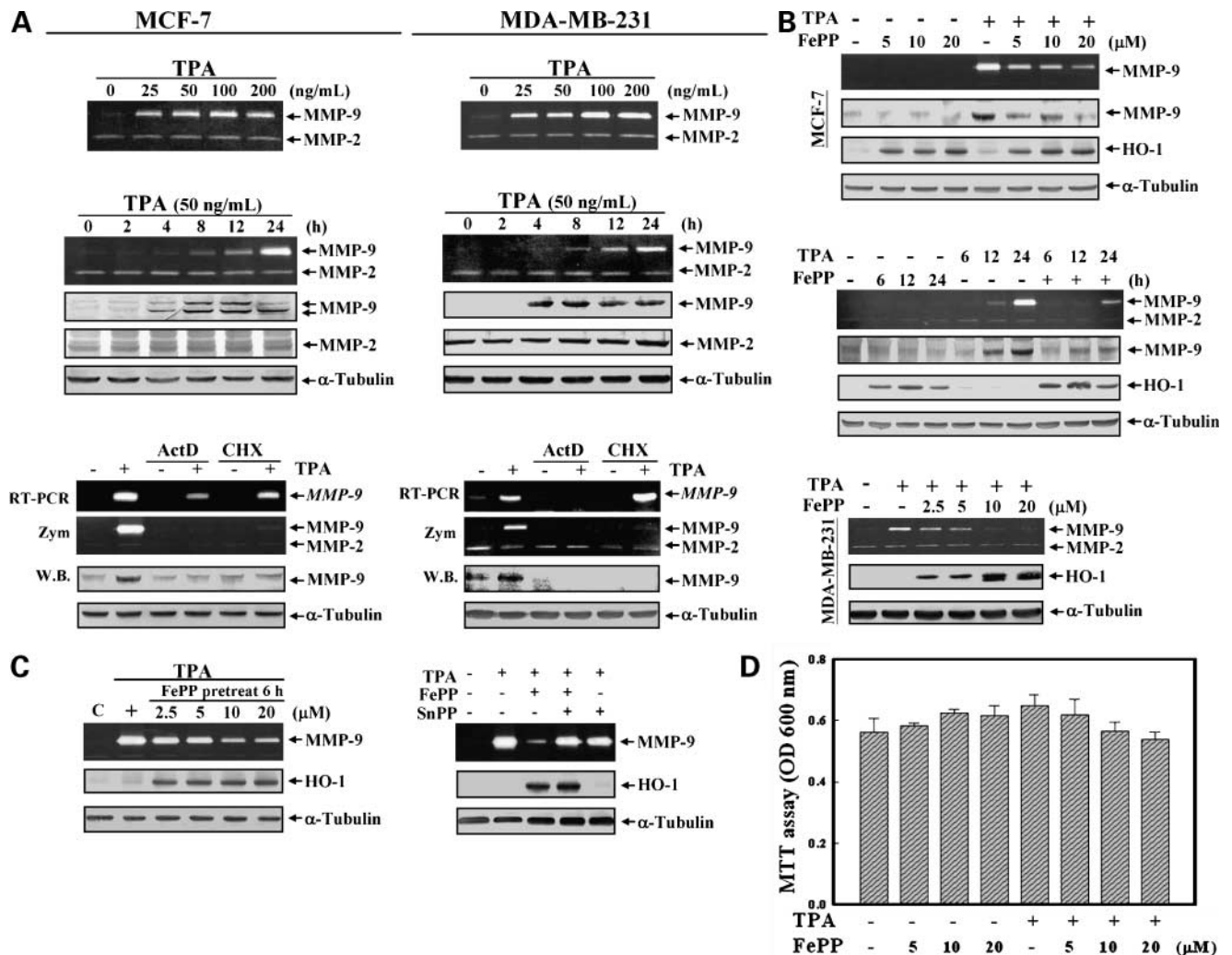


Figure 1. Induction of HO-1 by FePP inhibits TPA-induced MMP-9 expression. **A**, effect of TPA on the secretions of MMP-9 and MMP-2 in MCF-7 and MDA-MB-231 cells. MCF-7 and MDA-MB-231 cells were treated with different concentrations of TPA (25, 50, 100, and 200 ng/mL) for 24 h, and the conditioned medium was collected and analyzed by gelatin zymography (*top*). Cells were incubated with TPA (50 ng/mL) for 0 to 24 h, and the expressions of MMP-2 and -9 protein and enzyme activities were analyzed by Western blotting and gelatin zymography, respectively (*middle*). Cells were treated with TPA with or without actinomycin D (*ActD*; 1 μg/mL) and cyclohexamide (*CHX*; 5 μg/mL); the expressions of MMP-9 mRNA, protein, and enzyme activity were analyzed by reverse transcription-PCR, Western blotting, and gelatin zymography, respectively (*bottom*). **B**, FePP inhibition of TPA-induced MMP-9 expression. MCF-7 cells were treated with TPA in the presence or absence of FePP (5, 10, and 20 μmol/L) or treated with TPA plus FePP (20 μmol/L) for different time intervals, and the cell lysates or conditioned medium were analyzed by Western blotting or gelatin zymography (*top*). Expressions of HO-1 and MMP-9 in MDA-MB-231 cells were treated as described above (*bottom*). **C**, cells were pretreated with FePP (2.5, 5, 10, and 20 μmol/L) for 6 h and then washed without serum, the serum-free medium was replaced, and TPA was added for a further 24 h. The cell lysates or conditioned medium were analyzed by Western blotting or gelatin zymography (*left*). Cells were treated with TPA in the presence or absence of FePP (10 μmol/L) or the HO-1 inhibitor, SnPP (8 μmol/L), for 24 h, and the expressions of HO-1 and MMP-9 were analyzed by Western blotting and gelatin zymography, respectively (*right*). **D**, cells were treated under the same conditions described in **B**, and the viability of cells was measured by the MTT assay.

SDS-polyacrylamide gels, and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). The membrane was blocked with 1% bovine serum albumin at room temperature for 1 h and then incubated with specific indicated antibodies for a further 3 h. Protein expression was visualized by incubation with the colorimetric substrates, nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. The band intensity was quantitated by Alpha Imager 2200 analysis system (Alpha Innotech) and expressed as folds of control group.

Reverse Transcription-PCR Analysis

Total RNA was isolated with a RNA extraction kit (Amersham Bioscience), and the concentration of total RNA was measured with a spectrophotometer. RNA (2 μg) was converted to cDNA by a Reverse Transcription-PCR Beads kit (Amersham Bioscience) according to manufacturer's protocol. The PCR product of each sample was analyzed by electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining. Oligonucleotide primer sequences used were as follows: MMP-9

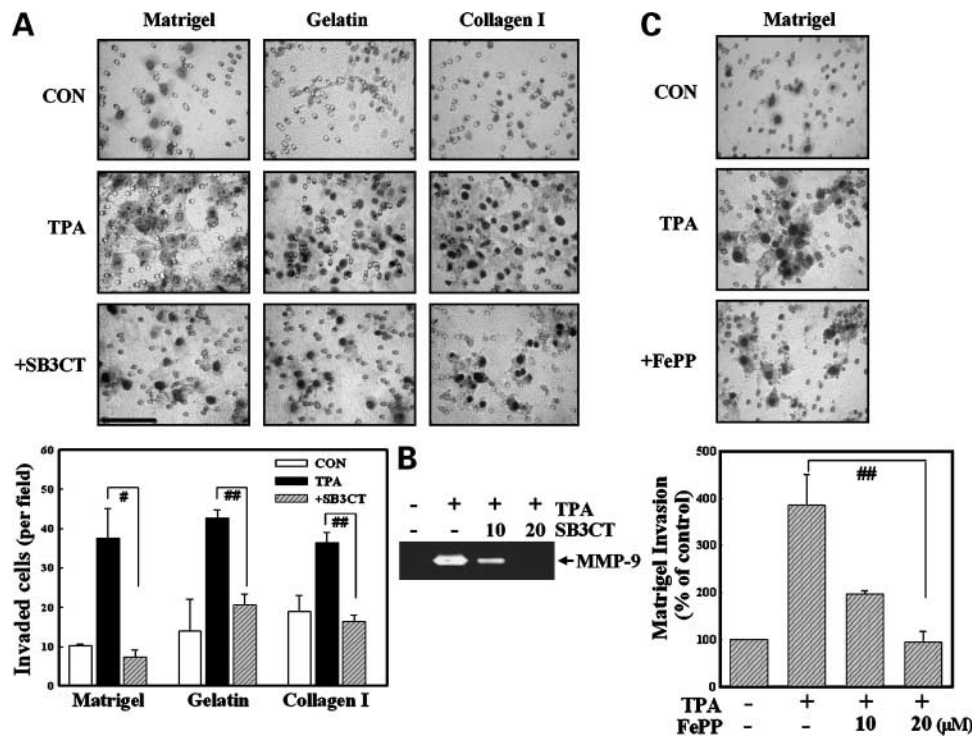


Figure 2. FePP suppression of TPA-induced invasiveness of human breast cancer cells. **A**, MMP-9 expression is crucial for the invasive ability of human breast cancer cells. TPA induction of the invasive capability in MCF-7 cells was assessed by counting the number of cells that passed through the Transwell coated with different matrix components as described in Materials and Methods. Photomicrographs of the invaded cells were observed at $\times 400$ magnification (bar, 100 μm). The invading cells were stained and counted in at least three fields per filter. **B**, cells were treated with SB3CT (10 and 20 $\mu\text{mol/L}$) for 1 h followed by the addition of TPA (50 ng/mL), and the conditioned medium was analyzed by gelatin zymography. **C**, FePP suppression of the invasive ability of MCF-7 cells. The Matrigel invasion assay of MCF-7 cells was described in Materials and Methods. Cells were exposed to TPA in the presence or absence of FePP (10 and 20 $\mu\text{mol/L}$) for 48 h, and the invading cells were stained and counted in at least three fields per filter as described in **A**. Data were repeated at least three times from three independent experiments and expressed as the mean \pm SE. The results were statistically analyzed by Student's *t* test. #, $P < 0.05$, compared with the TPA-treated group.

5-CACTGTCCACCCCTCAGAGC-3 (sense) and 5-GCCA-CTTGTCGGCGATAAGG-3 (antisense), HO-1 5-CAGGCA-GAGAATGCTGAGTTC-3 (sense) and 5-GATGTTGAGC-AGGAACGCAGT-3 (antisense), and glyceraldehyde-3-phosphate dehydrogenase 5-TGAAGGTCGGTGTGAACG-GATTTGGC-3 (sense) and 5-CATGTAGGCCATGAGGTC-CACCAC-3 (antisense). The annealing temperatures for MMP-9, HO-1, and glyceraldehyde-3-phosphate dehydrogenase were 58°C, 60°C, and 60°C, respectively.

Gelatin Zymography

The enzymatic activities of MMP-2 and MMP-9 were determined by gel zymography as described previously (25). Briefly, cells were seeded and allowed to grow to confluence for 24 h and then maintained in serum-free medium. The conditioned medium was collected 24 h after stimulation, mixed with nonreducing sample buffer, and subjected to electrophoresis in a 10% polyacrylamide gel containing 0.1% (w/v) gelatin. The gel was washed with washing buffer containing 2.5% Triton X-100, 50 mmol/L Tris-HCl (pH 7.5), and incubated at 37°C for 24 h in 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L CaCl₂, 1 mmol/L ZnCl₂, and 40 mmol/L NaN₃. The gel was stained with 0.25% (w/v) Coomassie brilliant blue in 45% (v/v) methanol and 1% (v/v) acetic acid.

Direct MMP-9 Enzyme Activity Assay

Purified MMP-9 (100 ng) from human fibroblast was incubated with 0, 12.5, 25, 50, and 100 $\mu\text{mol/L}$ RuCO or RuCl₃ in 50 μL Tris buffer (50 mmol/L, pH 7.4) at 37°C for 30 min. Incubated buffer (20 μL) was analyzed for its gelatinolytic activity as described as above.

In vitro Invasion Assay

The *in vitro* invasion assay was carried out to examine tumor invasiveness as described previously (25) with some modifications. Briefly, a 24-well Transwell unit with 8 μm polycarbonate Nucleopore filters (Corning) were coated with 60 μL of 0.8 mg/mL Englebreth-Holm-Swarm sarcoma tumor extract (EHS Matrigel), 0.1 mg/mL gelatin, or 5 mg/mL type I collagen at room temperature for 2 h to form a genuine reconstituted basement membrane. MCF-7 cells (1×10^5) were placed in the upper compartment, and the medium containing 10% fetal bovine serum was added to the lower compartment. The Transwell plates were incubated at 37°C for 48 h. Cells invaded to the lower surface of the membrane were stained with Giemsa staining and observed using light microscope.

Soft-Agar Assay

Colony formation of MCF-7 cells was done as described previously (20). In brief, cells were plated in 1 mL MEM

containing 0.35% agarose and 5% fetal bovine serum overlaid with 1 mL of 0.7% agarose. Cultures were maintained for 3 weeks, refreshed with 1 mL MEM supplemented with 5% fetal bovine serum, and subjected to the indicated treatment twice per week. Colonies were observed and photographed using a light microscope, and the number of colonies in each well was measured. Each value was derived from three independent experiments, and the results are expressed as the mean \pm SE.

Statistical Analysis

All data presented in the present study have been repeated at least three times from three independent experiments and are expressed as the mean \pm SE. The significance of the difference from the respective group for each experimental test condition was assayed using

Student's *t* test for each paired experiment. $P < 0.01$ or $P < 0.05$ was regarded as indicating a significant difference.

Results

FePP Down-regulates TPA-Induced MMP-9 Secretion via Induction of HO-1 Gene Expression in Human Breast Cancer Cells

We first studied the effect of TPA on the expressions of MMP-2 and MMP-9 in the nonmetastatic MCF-7 and highly metastatic MDA-MB-231 cell lines. TPA at the doses of 25, 50, 100, and 200 ng/mL induced the enzyme activity of MMP-9, but not of MMP-2, in both MCF-7 and MDA-MB-231 cells according to gelatin zymography. Similarly, TPA (50 ng/mL) time-dependently induced MMP-9

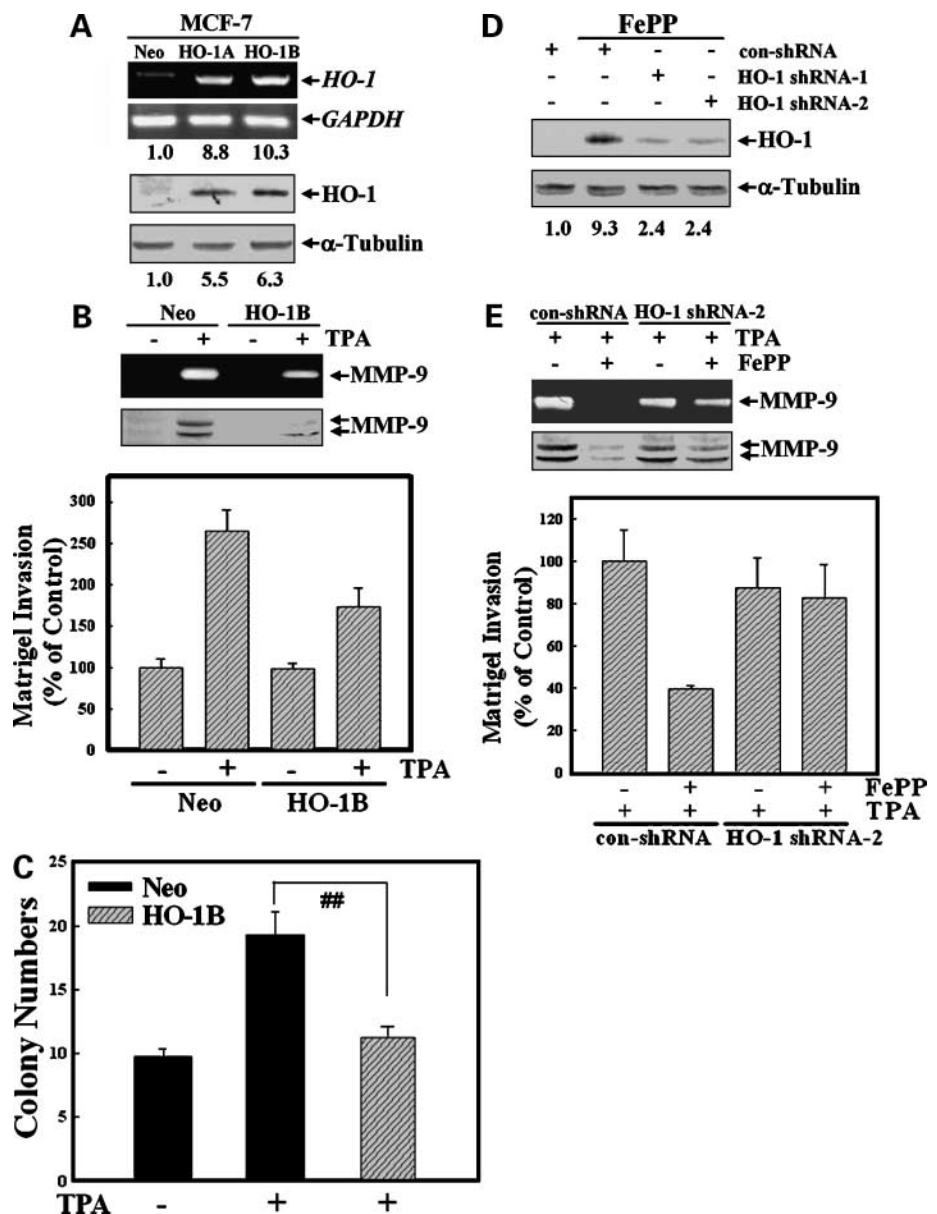


Figure 3. HO-1 down-regulates TPA-induced MMP-9 expression, tumor invasion, and colony formation. **A**, expression of HO-1 decreased TPA-induced invasiveness. The level of HO-1 mRNA and protein in MCF-7/Neo, MCF-7/HO-1A, and MCF-7/HO-1B cells was evaluated by reverse transcription-PCR and Western blot analysis. **B**, MMP-9 expression levels in MCF-7/Neo and MCF-7/HO-1B cells in the presence or absence of TPA (50 ng/mL) were analyzed by gelatin zymography and Western blotting (*top*). The invasive ability was evaluated by a Matrigel-coated Transwell assay (*bottom*). **C**, MCF-7/Neo and MCF-7/HO-1B cells were incubated with TPA for 21 days, and the numbers of colonies were assessed by a soft-agar assay as described in Materials and Methods. Data derived from three independent experiments were statistically analyzed by Student's *t* test. ##, $P < 0.01$, significantly different from the indicated groups. **D**, HO-1 shRNA attenuated the inhibitory effects of HO-1. Cells were stably transfected with shRNA against the *HO-1* gene (MCF-7/HO-1 shRNA-1 and MCF-7/HO-1 shRNA-2) or the control vector (con-shRNA) as described in Materials and Methods. Cells were treated with FePP (10 μ M/L) for 24 h; the expression of HO-1 protein was evaluated by Western blotting. **E**, MMP-9 expression levels in MCF-7/con-shRNA and MCF-7/HO-1 shRNA-2 cells in the presence or absence of TPA (50 ng/mL) and FePP (10 μ M/L) were analyzed by gelatin zymography and Western blotting, respectively (*top*). The invasive ability was assessed by a Matrigel-coated Transwell assay (*bottom*).

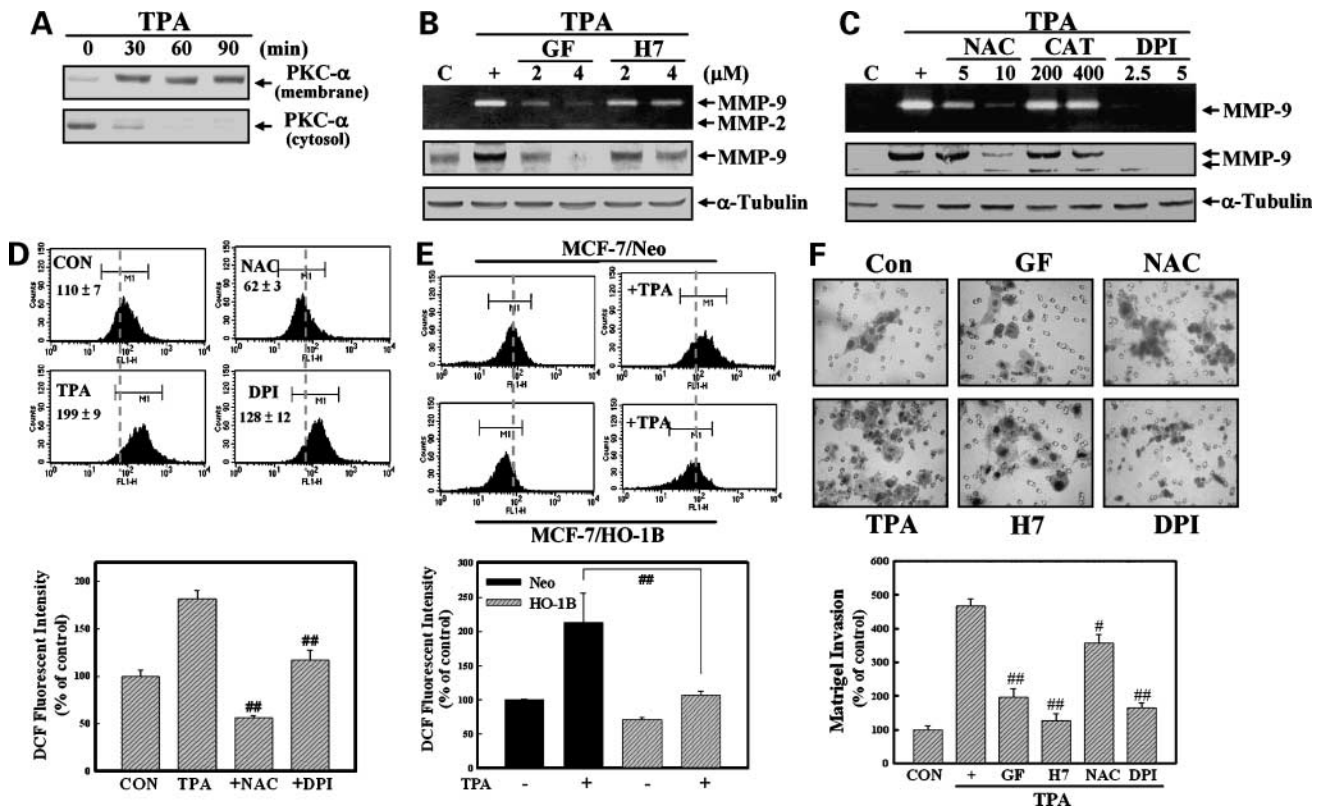


Figure 4. Suppression of PKC α activation and ROS production inhibit MMP-9 expression. **A**, MCF-7 cells were treated with TPA for different periods, and the expression of PKC α in different subcellular fractions was determined. **B**, cells were incubated with GF109203X (GF; 2 and 4 μ M) or H7 (2 and 4 μ M) for 30 min followed by TPA treatment for 24 h, and the conditioned medium and cell extracts were analyzed by gelatin zymography and Western blotting. **C**, cells were treated with NAC (5 and 10 mmol/L), catalase (CAT; 200, 400 U/mL), or DPI (2.5 and 5 μ M) in the presence of TPA for 24 h, and the MMP-9 levels were determined. **D**, cells were pretreated with NAC (10 mmol/L) and DPI (5 μ M) followed by TPA treatment for 2 h. Intracellular peroxide levels were measured by flow cytometric analysis. Data were repeated at least three times from three independent experiments and were expressed as the mean \pm SE. The results were statistically analyzed by Student's *t* test. ##, $P < 0.01$, compared with the TPA-treated group. **E**, subconfluent MCF-7/Neo or MCF-7/HO-1B cells were replaced with medium without serum for 6 h, cells were treated with TPA for an additional 2 h, and the peroxide levels were analyzed by flow cytometric analysis. Data were derived from three independent experiments and analyzed by Student's *t* test. ##, $P < 0.01$, compared to the TPA-treated group. **F**, MCF-7 cells were treated with GF109203X (4 μ M), H7 (5 μ M), NAC (10 mmol/L), and DPI (10 μ M) in the presence of TPA for 24 h, and the invasive ability was assessed by a Matrigel-coated Transwell assay. The results were statistically analyzed by Student's *t* test. #, $P < 0.05$; ##, $P < 0.01$, compared with the TPA-treated group.

enzyme activity and protein expression but had no effect on MMP-2 protein expression. The induction of MMP-9 enzyme and gene expression at both mRNA and protein levels was attenuated by adding the transcriptional inhibitor, actinomycin D, or translational inhibitor, cyclohexamide (Fig. 1A). Interestingly, treatment of cells with the HO-1 chemical inducer, FePP, dose- and time-dependently induced HO-1 protein expression with suppression of MMP-9 protein expression and enzyme activity induced by TPA in both MCF-7 and MDA-MB-231 cells (Fig. 1B). To exclude the possibility that FePP-inhibited TPA-induced MMP-9 occurs through an interaction with TPA, MCF-7 cells were treated with different doses of FePP for 6 h followed by removing any additional FePP from the medium, and TPA was then added for a further 24 h. Results showed an increase in HO-1 protein expression in accordance with a decrease in MMP-9 enzyme activity. In the presence of the HO-1 inhibitor,

SnPP, the inhibition of TPA-induced MMP-9 enzyme activity by FePP were blocked (Fig. 1C). Additionally, data from the MTT reduction of TPA-induced MMP-9 expression might not be mediated by its cytotoxic effect on MCF-7 cells (Fig. 1D).

FePP Suppresses TPA-Induced Invasion of MCF-7 Breast Cancer Cells

An *in vitro* Transwell assay was applied to examine the invasion and migration of MCF-7 cells under different treatments in the present study, and three cellular matrix components, Matrigel, gelatin, and type I collagen, were used. As shown in Fig. 2A, TPA treatment significantly induced MCF-7 cell invasion through separate Matrigel-coated, gelatin-coated, and collagen I-coated wells, and TPA-induced invasion and MMP-9 enzyme activation were attenuated by adding the specific MMP-9 inhibitor, SB3CT (Fig. 2B), which suggests that MMP-9 activation was involved in TPA-induced invasion of MCF-7 cells. We

further examined the effect of the HO-1 inducer, FePP, on TPA-induced invasion of MCF-7 cells. Figure 2C shows that a significant decrease in the number of invaded cells stimulated by TPA was detected in the presence of FePP.

HO-1 Overexpression Inhibits TPA-Induced Invasion of MCF-7 Breast Cancer Cells

To provide direct scientific evidence to support the inhibitory effect of HO-1 against TPA-induced invasion,

MCF-7 cells were transfected with a HO-1-overexpressing vector, and two stable clones of HO-1-overexpressing MCF-7 cells (MCF-7/HO-1A and MCF-7/HO-1B) were established. Data from the reverse transcription-PCR and Western blot analysis showed that the levels of HO-1 mRNA and protein were elevated in MCF-7/HO-1A and MCF-7/HO-1B cells compared with those in Neo-transfected MCF-7 cells (MCF-7/Neo; Fig. 3A). In the

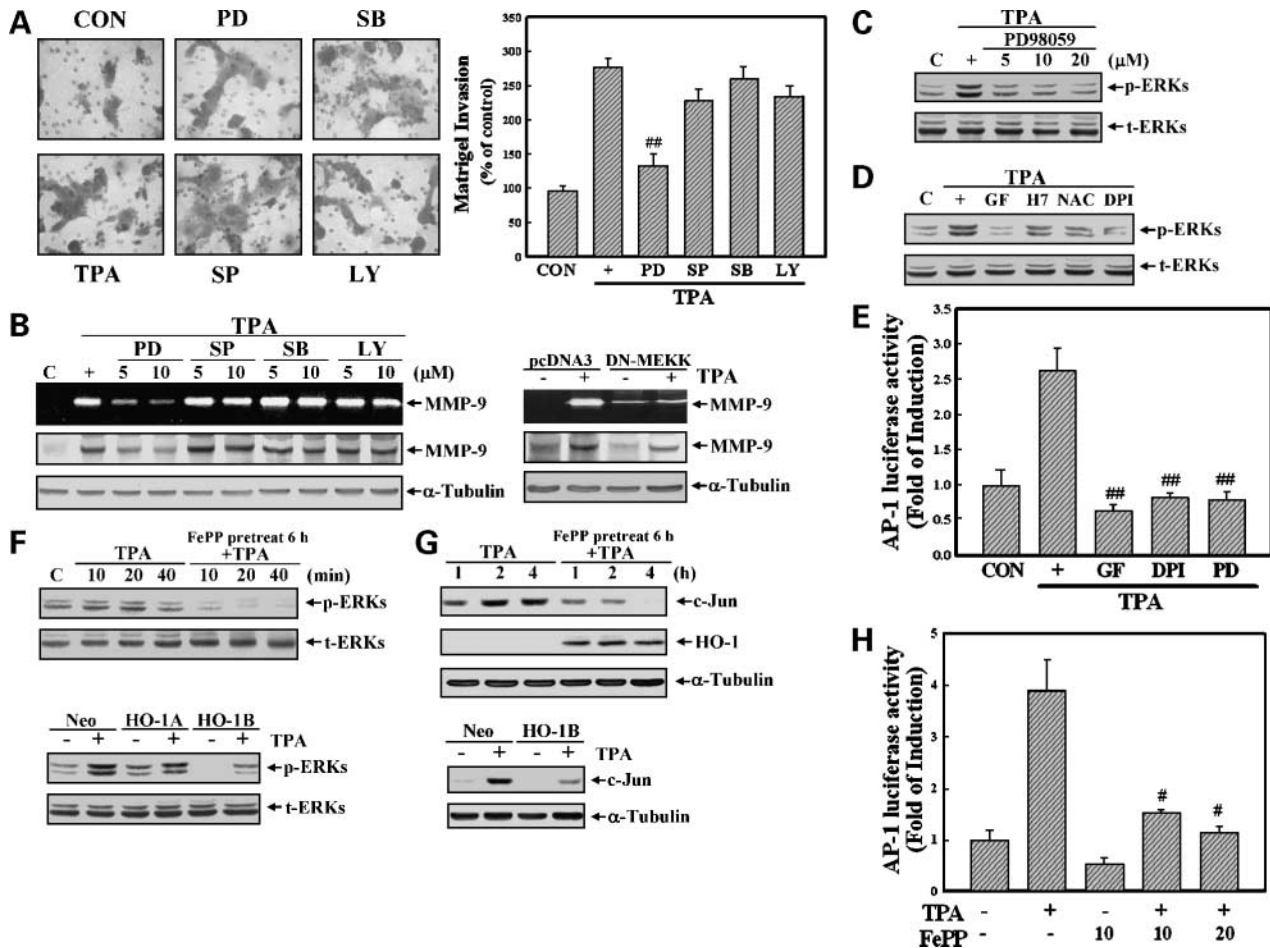


Figure 5. HO-1 suppression of TPA-induced ERK/AP-1 activation. **A**, the ERK inhibitor, PD98059, inhibited TPA-induced invasiveness of MCF-7 cells. Cells were treated with PD98059 (PD), SP600125 (SP), SB203580 (SB), or LY294002 (LY) (10 μ M/L) in the presence of TPA for 48 h, and the invasive ability was determined by a Matrigel Transwell assay. **B**, activation of ERK is required for MMP-9 expression. Cells were treated with PD98059, SP600125, SB203580, or LY294002 (5 and 10 μ M/L) for 30 min followed by TPA treatment for 24 h, and cell lysates and conditioned medium were subjected to Western blotting and gelatin zymography, respectively (left). Cells were transiently transfected with the dominant-negative MEKK (DN-MEKK) plasmid or the control vector (pcDNA3) followed by TPA treatment for 24 h, and the MMP-9 level was evaluated (right). **C**, cells were pretreated with PD98059 (5, 10, and 20 μ M/L) for 30 min followed by TPA treatment, and the phosphorylation of ERK was detected by Western blotting using specific anti-phosphorylated ERK antibodies. **D**, activation of ERK is located downstream of the PKC-ROS pathway. Cells were pretreated with GF103902X (4 μ M/L), H7 (4 μ M/L), NAC (10 mmol/L), or DPI (5 μ M/L) for 30 min followed by the addition of TPA for 30 min, and the expressions of phosphorylated and total ERK were analyzed by Western blotting. **E**, MCF-7 cells were transiently transfected with pAP-1-Luc for 24 h; cells were then treated with GF103902X (4 μ M/L), PD98059 (10 μ M/L), and DPI (5 μ M/L) in the presence of TPA for 8 h; and AP-1 promoter activity was measured with a dual-luciferase assay kit. **F**, HO-1 attenuates TPA-induced ERK activation. Cells were pretreated with FePP (10 μ M/L) for 6 h, washed twice with serum-free medium, and then treated with TPA for different time intervals (top). The media of MCF-7/HO-1A and MCF-7/HO-1B cells were replaced with serum-free medium for 6 h and treated with TPA for 30 min (bottom), and the expressions of phosphorylated and total ERK were analyzed by Western blotting. **G**, induction of HO-1 down-regulates TPA-induced AP-1 activation. Cells were treated under the same conditions as described in F; the expressions of c-Jun and HO-1 protein were analyzed by Western blotting (top). MCF-7/Neo and MCF-7/HO-1B cells were treated as described above, and the expression of c-Jun protein was analyzed by Western blotting (bottom). **H**, cells were transiently transfected with the pAP-1-Luc plasmid for 24 h followed by treatment with FePP and TPA as described above, and the AP-1 promoter activity was measured by a dual-luciferase assay. Data were repeated at least three times from three independent experiments and were expressed as the mean \pm SE. The results were statistically analyzed by Student's *t* test. #, $P < 0.05$; ##, $P < 0.01$, compared with the TPA-treated group.

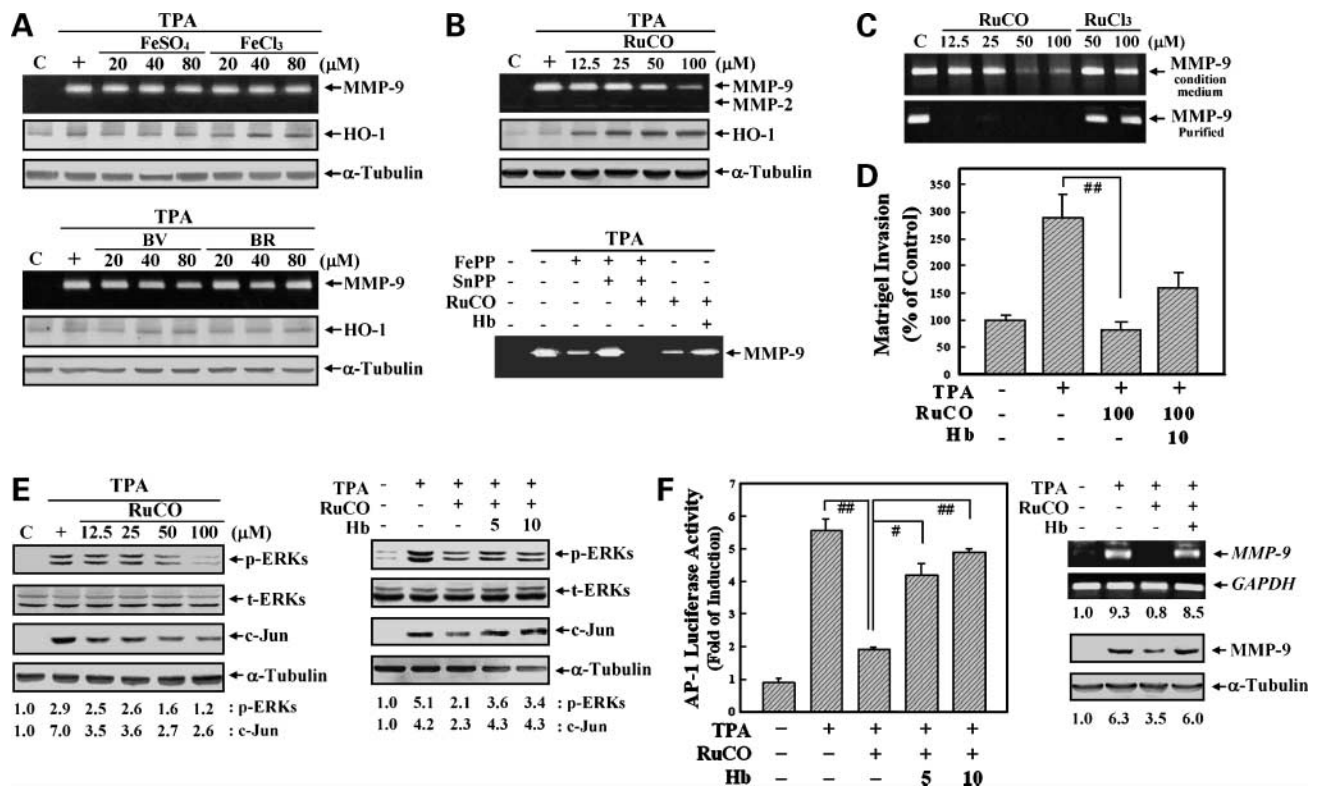


Figure 6. CO mediates the inhibitory effect of HO-1 via suppressing ERK/AP-1 activation. **A**, neither FeSO₄, FeCl₃, biliverdin, nor bilirubin inhibited MMP-9 secretion. Cells were treated with biliverdin, bilirubin, FeSO₄, or FeCl₃ (20, 40, and 80 μmol/L) followed by TPA addition for 24 h, and the secretion of MMP-9 was analyzed by Western blotting or gelatin zymography. **B**, CO inhibits TPA-induced MMP-9 protein expression, enzyme activity, and tumor invasion. Cells were treated with RuCO (12.5, 25, 50, and 100 μmol/L) in the presence of TPA for 24 h, and the expressions of HO-1 and MMP-9 were analyzed by Western blotting or gelatin zymography (*top*). Cells were treated with TPA in the presence or absence of FePP (10 μmol/L), SnPP (8 μmol/L), RuCO (100 μmol/L), or hemoglobin (*Hb*; 10 μg/mL) for 24 h, and the conditioned medium was analyzed by gelatin zymography (*bottom*). **C**, purified MMP-9 protein (100 ng) from human fibroblasts (*bottom*) or TPA-treated conditioned medium (*top*) was incubated with different concentrations (12.5, 25, 50, and 100 μmol/L) of RuCO or RuCl₃ (50 and 100 μmol/L) for 30 min at 37°C. The activity of the MMP-9 enzyme in each sample was analyzed by gelatin zymography. **D**, MCF-7 cells were treated with TPA in the presence or absence of RuCO (100 μmol/L) and hemoglobin (10 μg/mL) for 48 h, and the invasive ability of cells was assessed by a Matrigel invasion assay. **E**, CO inhibited MMP-9 expression through blocking ERK/AP-1 activation. Cells were treated with RuCO in the absence (*left*) or presence (*right*) of hemoglobin (5 and 10 μg/mL) for 30 min followed by TPA treatment, and cell extracts were subjected to Western blotting. The quantification of phosphorylated ERK and c-Jun by densitometry was represented as folds of induction relative to the control. **F**, CO suppression of AP-1 transcriptional activity down-regulates *MMP-9* gene expression. Cells were transiently transfected with the pAP-1-Luc plasmid for 24 h and then treated with TPA in the presence or absence of RuCO and hemoglobin for 8 h. AP-1 promoter activity was measured by a dual-luciferase assay (*left*). Cells were treated under the same conditions as described in **E**; MMP-9 mRNA and protein levels were analyzed by reverse transcription-PCR and Western blotting, respectively (*right*). Data were repeated at least three times from three independent experiments and were expressed as the mean ± SE. The results were statistically analyzed by Student's *t* test. #, *P* < 0.05; ##, *P* < 0.01, compared with the TPA-treated group.

presence of TPA treatment, the induced levels of MMP-9 enzyme and MMP-9 protein were reduced in MCF-7/HO-1B cells with a decrease in the percentage of invasion compared with that in MCF-7/Neo cells (Fig. 3B). Furthermore, data from the colony formation assay showed that TPA treatment induced colony formation in MCF-7/Neo cells, and the number of colonies was significantly reduced in MCF-7/HO-1B cells (Fig. 3C). Additionally, the role of the HO-1 gene in FePP-inhibited invasion elicited by TPA was investigated via transfection of MCF-7 cells with HO-1 small hairpin RNA (shRNA). As shown in Fig. 3D, transfection of MCF-7 cells with HO-1 shRNA significantly reduced HO-1 protein expression stimulated by FePP, and the levels of TPA-induced MMP-9 enzyme and protein expressions by FePP in MCF-7 cells transfected with control

nonspecific shRNA (con-shRNA) were attenuated by transfection of cells with HO-1 shRNA. Attenuation of the inhibitory effect of FePP against TPA-induced invasion by HO-1 shRNA transfection was identified via an *in vitro* invasion assay (Fig. 3E).

Induction of PKC α Protein Translocation and ROS Production in TPA-Induced MMP-9 Activation and Invasion

The possibility whether HO-1 inhibition of TPA-induced invasion occurring via suppression of PKC α activation and ROS production was investigated. As shown in Fig. 4A, an increase in the protein level of PKC α in the membrane fraction was identified in TPA-treated MCF-7 cells, and treatment of MCF-7 cells with the PKC α inhibitors, GF103902X and H7, significantly decreased MMP-9 enzyme activation and protein expression (Fig. 4B). Also,

the addition of the ROS scavengers, NAC and DPI, but not catalase, inhibited TPA-induced MMP-9 enzyme activation and protein expression in MCF-7 cells in accordance with a significant decrease in the peroxide level elicited by TPA via the 2',7'-dichlorodihydrofluorescein diacetate assay, and the inhibitory percentages of NAC and DPI against TPA-induced peroxide production were 65% and 36%, respectively (Fig. 4C and D). Furthermore, HO-1 overexpression was able to reduce the peroxide production induced by TPA in MCF-7/HO-1B cells compared with MCF-7/Neo cells (Fig. 4E). Inhibition of TPA-induced invasion by GF109203X, H7, NAC, and DPI was identified in MCF-7 cells by the Matrigel-Transwell assay (Fig. 4F).

ERK Activation, Downstream Events of PKC α , and ROS Production Participate in TPA-Induced Invasion

Pharmacologic studies were done to examine the roles of intracellular kinases in TPA-induced invasiveness of MCF-7 cells using specific chemical inhibitors including PD98059 (an ERK inhibitor), SB203580 (a p38 inhibitor), SP600125 (a JNK inhibitor), and LY294002 (an Akt inhibitor) in the present study. As shown in Fig. 5A, application of PD98059, but not the others, significantly inhibited TPA-induced invasiveness according to the Matrigel-Transwell assay. Data from gelatin zymography and Western blotting also showed that PD98059, but not the others, reduced MMP-9 enzyme activity and protein expression with a reduction in ERK protein phosphorylation stimulated by TPA (Fig. 5B, *left*, and C), and MMP-9 enzyme activity and protein expression were inhibited by transfection of cells with dominant-negative MEKK (Fig. 5B, *right*). In addition, pretreatment of MCF-7 cells with the antioxidants, NAC and DPI, or the PKC α inhibitors, GF103902X and H7, blocked ERK protein phosphorylation (Fig. 5D) and AP-1 promoter activity (Fig. 5E), suggesting that activation of the ERK/AP-1 signaling pathway, which is located downstream of PKC and ROS, is crucial for MMP-9 expression and invasiveness of MCF-7 cells. Moreover, inhibition of TPA-induced ERK protein phosphorylation was observed in FePP-treated cells and HO-1-overexpressing cells (MCF-7/HO-1B; Fig. 5F), and a decrease in TPA-induced c-Jun protein expression and AP-1 promoter activity by FePP treatment and HO-1 overexpression (MCF-7/HO-1B) were detected with increasing HO-1 protein expression (Fig. 5G and H).

CO May Contribute to HO-1 Inhibition of TPA-Induced MMP-9 Activation and Invasion

HO-1 is a key enzyme catalyzing the metabolism of heme, through which process ferric ions, biliverdin, and CO are produced. Therefore, we investigated if ferric ions, biliverdin, and CO participate in HO-1 inhibition of TPA-induced invasion and MMP-9 activation. As shown in Fig. 6A, neither FeSO₄, FeCl₃, bilirubin, nor biliverdin had any obvious effects on TPA-induced MMP-9 enzyme activity or HO-1 protein expression in MCF-7 cells. Importantly, the addition of the chemical CO donor, RuCO, increased HO-1 protein expression accompanied by a reduction in MMP-9 enzyme activity (Fig. 6B, *top*). Data from the pharmacologic studies showed that RuCO

addition was able to potentiate the inhibitory effect of FePP against TPA-induced MMP-9 enzyme activity, which was blocked by SnPP. The inhibitory effect of RuCO against TPA-induced MMP-9 enzyme activity was blocked by adding the CO scavenger, hemoglobin (Fig. 6B, *bottom*). To further identify if CO can directly affect MMP-9 enzyme activity, conditioned medium derived from TPA-treated MCF-7 cells or from the purified MMP-9 enzyme was used. As illustrated in Fig. 6C, *in vitro* incubation of RuCO with conditioned medium or the purified MMP-9 enzyme significantly inhibited MMP-9 enzyme activity according to gelatin zymography; however, RuCl₃, an analogue of RuCO without a CO-releasing effect, did not. Inhibition of TPA-induced MCF-7 cell invasion by RuCO was detected by a Matrigel-Transwell assay, and it was attenuated by hemoglobin (Fig. 6D). Furthermore, we examined the effect of RuCO on TPA-induced invasive signaling cascade including phosphorylated ERK, c-Jun, and AP-1 activations. Figure 6E shows that RuCO dose-dependently inhibited TPA-induced ERK protein phosphorylation with suppression of c-Jun protein expression, and the inhibitory effect of RuCO was blocked by hemoglobin. Furthermore, RuCO significantly inhibited TPA-induced AP-1 transcriptional activity (Fig. 6F, *left*), and MMP-9 gene expression at both the mRNA and protein levels was also identified; this was attenuated by adding hemoglobin (Fig. 6F, *right*).

Discussion

The anti-invasiveness of HO-1 against human breast carcinoma cells was first investigated in the present study. Increasing intracellular HO-1 protein levels by FePP or by transfecting cells with the HO-1 expression vector significantly inhibited TPA-induced invasion in accordance with suppression of ROS production and PKC, ERK, and AP-1 activations. In addition, the anti-invasiveness of CO through blocking MMP-9 and ERK/AP-1 activations was identified. This suggested that HO-1 inducers may play roles as agents that inhibit tumor invasion, with the potential for further therapeutic development.

TPA has been used as a tumor promoter in chemical-induced carcinogenesis *in vitro* and *in vivo*. Previous studies showed that TPA treatment is able to promote tumor migration and invasion by stimulating MMP-2 or MMP-9 expression in glioma, colon, and hepatoma cells (26–28). However, the mechanism by which TPA induces breast cancer cell invasion has not been well delineated. In the present study, TPA induced the invasion of MCF-7 and MDA-MB-231 breast carcinoma cells with increases in MMP-9 gene expression at both protein and mRNA levels, and the invasion of MCF-7 cells elicited by TPA was blocked by adding SB3CT, a specific MMP-9 inhibitor. This suggests that TPA may induce the invasion of human breast cancer cells through activation of the MMP-9 enzyme.

The intracellular signal pathway activated by TPA has been extensively reported. Activation of PKC by TPA is critical for tumor invasion and MMP-9 induction (7, 29). Our previous study showed that activation of PKC α by

TPA induced the proliferation and secretion of MMP-2 in fibroblasts and colorectal carcinoma cells (25, 30). In the present study, activation of PKC α was detected in TPA-treated MCF-7 cells, and TPA-induced invasion and MMP-9 activation were blocked by the addition of the PKC inhibitors, GF109203X and H7. This suggests that activation of PKC is involved in TPA-induced invasion of breast carcinoma cells. Furthermore, TPA activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt has been shown in several cell lines. Nomura et al. indicated that TPA induced migration of glioma cells via activation of p38 mitogen-activated protein kinase (31). Taylor et al. reported that the stimulation of ERK is critical for TPA-induced angiogenesis (32). However, it is still unclear if mitogen-activated protein kinase or phosphatidylinositol 3-kinase/Akt activation is indeed involved in TPA-induced MMP-9 gene expression and invasion by human breast carcinoma cells. Pharmacologic studies using specific inhibitors of ERK, (PD98059), JNK (SP600125), p38 (SB203580), and Akt (LY294002) showed that incubation of MCF-7 cells with the ERK inhibitor, PD98059, but not the others, and inhibiting ERK by transfection of the dominant-negative MEKK plasmid significantly inhibited TPA-induced invasion accompanied by a reduction in MMP-9 enzyme activity. TPA induction of ERK protein phosphorylation and AP-1 luciferase activity was blocked by the ERK inhibitor, PD98059, and the PKC inhibitors, GF109203X and H7. This indicates that activation of ERK/AP-1 is located downstream of PKC activation in TPA-induced invasiveness of breast carcinoma cells.

Growing evidence supports the cytoprotective and anti-inflammatory actions of HO-1 *in vitro* and *in vivo*. However, studies regarding to the role of HO-1 in tumor progression are still controversial. Induction of HO-1 by cobalt protoporphyrin or Ad-HO-1 infection inhibited the proliferation of breast carcinoma cells (16). In human colorectal and oral carcinomas, HO-1 induction may contribute to lowering the risk of lymph node metastasis (33, 34). Moreover, down-regulation of HO-1 has been identified in association with increasing of malignant progression of hepatocellular carcinoma (35). However, several studies have also indicated that HO-1 induction promotes tumor angiogenesis and induces tumor resistance to chemotherapy (36–38). In a TPA-induced invasive model, induction of the HO-1 protein significantly prevented the invasion and activation of MMP-9 elicited by TPA, and the anti-invasiveness of HO-1 was attenuated by adding its inhibitor, SnPP, or HO-1 shRNA. A decrease in ERK/c-Jun/AP-1 activation induced by TPA was identified in HO-1-overexpressing cells. This suggests that HO-1 induction contributes to the anti-invasiveness, and a reduction in MMP-9 activation via blocking ERK/c-Jun/AP-1 activation is involved.

HO-1 conversion of heme to ferric ions, CO, and biliverdin, which is reduced to bilirubin by biliverdin reductase, and several biological functions of ferric ions, CO, biliverdin, and bilirubin have been reported. Both biliverdin and bilirubin are potent antioxidants, and the

inhibition of prothrombotic molecular expression by bilirubin via decreasing intracellular ROS level has been observed (39). CO is a secondary messenger with multiple biological activities. Inhibition of MMP-1, MMP-2, MMP-7, and MMP-9 by CO has been reported (40–42). Graham et al. (43) identified CO reduction of tumor invasion via suppression of urokinase activity in MDA-MB-231 cells. However, there is a lack of evidence for the anti-invasiveness of CO in relation to MMP-9 activation. CO-induced HO-1 protein expression, consistent with the blocking of MMP-9 expression and cell invasion, was observed in TPA-treated MCF-7 cells, and CO addition may reverse the inhibitory effect of SnPP against FePP-inhibited MMP-9 enzyme activity stimulated by TPA. Hemoglobin, a CO scavenger, was able to attenuate the inhibitory effect of CO against TPA-induced ERK, c-Jun, and AP-1 luciferase activities. The role of HO-1 induction on CO inhibition of MMP-9 enzyme activity is still unclear. SnPP addition was able to attenuate the inhibitory effect of RuCO against TPA-induced MMP-9 enzyme activity (data not shown). In the present study, SnPP suppression of FePP-inhibited MMP-9 enzyme activity stimulated by TPA was reversed by RuCO addition. It suggests that CO may directly or indirectly participates in HO-1 inhibition of MMP-9 enzyme activity elicited by TPA. Furthermore, RuCO, but not its respective compound RuCl₃, possessed the ability to inhibit MMP-9 enzyme activity in a condition with or without cells. Previous studies indicated that CO reserved the ability to interact with several proteins including NADPH oxidase, iNOS, and cytochrome *c* reductase (40, 44). This suggests that (a) metal component (Ru) is not involved in RuCO inhibition of tumor invasion and MMP-9 activation induced by TPA, (b) CO participates in HO-1 inhibition of TPA-induced tumor invasion of breast carcinoma cells via suppressing ERK/c-Jun/AP-1 activation, and (c) CO interaction with the MMP-9 protein may contribute to its inhibition on MMP-9 enzyme activity *in vitro*.

ROS are secondary messengers that modulate intracellular signal transduction, and our previous study indicated that increasing ROS production is an initial event in TPA-induced cellular proliferation. In epidermal keratinocytes, TPA induces MMP-9 secretion through activation of NADPH oxidase (45), and in hepatoma cells, TPA increases tumor migration via ROS production (19). These data suggest that ROS elevation may contribute to tumor progression. Data from the present study also support ROS participation in TPA-induced invasion, activation of the MMP-9, and ERK/AP-1 cascades. HO-1 has also been reported to possess an antioxidant capacity, and our previous study showed that HO-1 overexpression reduced ROS production elicited by hydrogen peroxide in macrophages (14). Turkseven et al. indicated that the antioxidant effect of HO-1 is mediated by stimulating SOD enzyme activity in cells (46). Taillé et al. showed that HO-1 and CO are capable of inhibiting NADPH oxidase activity and consequently suppression of superoxide anion production (47, 48). Free heme depletion has been shown in HO-1

inhibition of ROS via decreasing NADPH oxidase activity. Data derived from detection of intracellular heme level show that FePP-induced intracellular heme level in HO-1-overexpressing MCF-7 is lower than that in parental MCF-7 cells (Data not shown). In the present study, TPA-induced ROS production and MMP-9 activation was abolished by overexpression of HO-1 protein in MCF-7 cells. These data support the notion that the antioxidant activity plays an important role in HO-1 against the invasion of breast carcinoma cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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