Induction of heme oxygenase-1 and inhibition of TPA-induced matrix metalloproteinase-9 expression by andrographolide in MCF-7 human breast cancer cells

Che-Yi Chao1,*, Chong-Kuei Li1,2,*, Ya-Ting Hsu2, Chia-Yang Lu1, Kai-Li Liu1,†, Chien-Chun Li1,‡ and Haw-Wen Chen1,†

1Department of Health and Nutrition Biotechnology, Asia University, Taichung 413, Taiwan, 2Department of Nutrition, China Medical University, Taichung 404, Taiwan, 3School of Nutrition, Chung Shan Medical University, Taichung 402, Taiwan and 4Department of Nutrition, Chung Shan Medical University Hospital, Taichung 402, Taiwan

*To whom correspondence should be addressed. Tel: +886 4 22053666 ext. 7520; Fax: +886 4 2206 2891; Email: chenhw@mail.cmu.edu.tw

†Correspondence may also be addressed to Chien-Chun Li. Tel: +886 4 24730022 ext. 7520; Fax: +886 4 2206 2891; Email: chienchien367@gmail.com

Matrix metalloproteinase-9 (MMP-9) plays a critical role in cancer metastasis. Andrographolide (AP) is a diterpene lactone in the leaves and stem of Andrographis paniculata (Burm. f) Ness that has been reported to possess anticancer activity. In this study, we investigated the effect of AP on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced MMP-9 expression and invasion in MCF-7 breast cancer cells and the possible mechanisms involved. The results showed that AP dose-dependently inhibited TPA-induced MMP-9 protein expression, enzyme activity, migration and invasion. In addition, AP significantly induced heme oxygenase-1 (HO-1) messenger RNA (mRNA) and protein expression. Transfection with HO-1 small interfering RNA knocked down the HO-1 expression and reversed the inhibition of MMP-9 expression by AP. HO-1 end products, such as carbon monoxide, free iron and bilirubin, suppressed the TPA-induced MMP-9 mRNA and protein expression, enzyme activity, migration and invasion in MCF-7 cells. Furthermore, TPA-induced extracellular signal-regulated kinase (ERK) 1/2 and Akt phosphorylation and the DNA binding activity of activator protein-1 (AP-1) and nuclear factor-kappa B (NF-kB) were attenuated by pretreatment with AP and HO-1 end products. In conclusion, these results suggest that AP inhibits TPA-induced cell migration and invasion by reducing MMP-9 activation, which is mediated mainly by inhibition of the ERK1/2 and phosphatidylinositol 3-kinase/Akt signaling pathways and subsequent AP-1 and NF-kB transactivation. Additionally, induction of HO-1 expression is at least partially involved in the inhibition of TPA-induced MMP-9 activation and cell migration in MCF-7 cells by AP.

Introduction

Cancer is the second leading cause of death in economically developed countries and the third leading cause of death in developing countries (1). Breast cancer is the most commonly diagnosed cancer among women worldwide. Ninety percent of human cancer death is attributed to tumor metastasis. Metastasis is an extremely complicated process that occurs by degradation of the cellular basement membrane and tumor spread to distant organs leading to the formation of secondary tumors (2). Metastasis proceeds through a series of sequential steps that include cancer cell proliferation, adhesion, invasion, migration and angiogenesis (3).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that comprise four subclasses based on substrate specificity: collagenases, gelatinases, stromelysins and membrane-associated MMPs (4). Because of their role in the degradation of environmental barriers, such as the extracellular matrix and the basement membrane, MMPs have been considered as potential diagnostic and prognostic biomarkers in many types and stages of cancer (5). Among the MMPs, MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are strongly correlated with tumor invasion and metastasis in breast cancer cells (6). In general, MMP-2 is constitutively expressed in highly metastatic tumors, whereas MMP-9 is highly inducible by a large variety of stimuli, including growth factors (7), inflammatory cytokines (8) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (9). TPA acts as a tumor promoter that induces MMP-9 expression by modulating the activation of transcription factors such as activator protein-1 (AP-1) and nuclear factor-kappa B (NF-kB) through protein kinase C, mitogen-activated protein kinases and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways (10,11). Therefore, these upstream molecules that regulate MMP-9 expression or enzymatic activity are critical in treating cancer metastasis. It has been shown that plant-derived compounds with chemopreventive potential inhibit the invasiveness of several types of cancer by modifying MMP-9 expression (11,12).

Andrographis paniculata (Burm. f) Ness is an herbal plant in the Acanthaceae family. It is widely cultivated in India, Thailand and China and has been used as a traditional medicine in the treatment of various diseases (13,14). Andrographolide (AP) is the most abundant diterpene lactone in the leaves and stem of A. paniculata and possesses several bioactive properties, including anticancer (15), anti-inflammation (16), hepatoprotective (17) and anti-infection (18) properties. Regarding its anticancer effect, AP was reported to inhibit MMP-7 expression via downregulation of the PI3K/Akt signaling pathway, which results in reduced invasiveness of human non-small cell lung cancer A549 cells (19). In addition, AP has been shown to inhibit human colorectal carcinoma Lovo cell growth by G1-S phase arrest and increased expression of p53, p21 and p16 (20). AP was also shown to induce apoptosis in B16-F10 melanoma cells by inhibiting NF-kB-induced bcl-2 activation and modulating p53-induced caspase-3 gene expression (21).

Heme oxygenase (HO) is an inducible enzyme that catalyzes the rate-limiting step in the degradation of heme and produces carbon monoxide, free iron and bilirubin, which is further catabolized into bilirubin by biliverdin reductase (22). Thus far, three mammalian HO isoforms have been identified. Among them, HO-2 and HO-3 are constitutively expressed in selected tissues, whereas HO-1 can be induced by a variety of stress-related stimuli, such as its substrate heme, heavy metals, oxidants, UV irradiation and inflammatory cytokines (23). A growing body of evidence suggests that HO-1 may play a role in the pathogenesis and progression of several types of malignancies (24). Induction of HO-1 potently inhibits the growth and spread of tumors (25,26).

The anticancer effect of AP has been studied in oral squamous cell carcinoma by inhibition of 7,12-dimethyl-1,2-benzanthracene-induced aberrant NF-kB activation (15) and in MCF-7 cells through induction of cell cycle inhibitory protein p27 and decreased expression of cyclin-dependent kinase 4, which results in cell cycle arrest at the G1/S phase (27). The antiangiogenic role of HO-1 and subsequent inhibition of prostate carcinogenesis by HO-1 overexpression have also been demonstrated (25). In our previous study, we corroborated that AP can

Abbreviations: AP, andrographolide; AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; HO, heme oxygenase; MMP, matrix metalloproteinase; mRNA, messenger RNA; NF-kB, nuclear factor-kappa B; PI3K, phosphatidylinositol 3-kinase; shRNA, small hairpin RNA; siRNA, small interfering RNA; TPA, 12-O-tetradecanoylphorbol-13-acetate.

†These authors contributed equally to this work.
induce HO-1 (28). In this study, therefore, we investigated the effect of AP on TPA-induced MMP-9 expression and invasion in MCF-7 breast cancer cells and the possible mechanisms involved and the role of HO-1 in the action of AP.

Materials and methods

Chemicals

Dubelco’s modified Eagle’s medium, medium 199, OPTI-modified Eagle’s medium, 25% trypsin-ethylenediaminetetraacetic acid and penicillin-streptomycin solution were from Gibco-BRL (Grand Island, NY); endothelial cell growth supplement was from Upstate Biotechnology (Lake Placid, NY); fetal bovine serum (FBS) was from HyClone (Logan, UT); collagen was from Collaborative Biomedical Products (Bedford, MA); AP and antibody against HO-1 were from Calbiochem (Darmstadt, Germany); sodium bicarbonate, heparin, gelatin, TPA, tricarbonyldichlororuthenium(II) dimer (RuCo), bilirubin and antibody against β-actin were from Sigma–Aldrich (St Louis, MO); ferric chloride (FeCl₃) was from Merck Chemical Company (Darmstadt, Germany); antibody against MMP-9 was from Millipore (Billerica, MA) and antibodies against extracellular signal-regulated kinase (ERK) 1/2, phospho-ERK1/2, Akt and phospho-Akt (S473) were from Cell Signaling Technology (Danvers, MA).

Cell culture

The human breast cancer cell line MCF-7 was cultured on collagen-coated cell culture dishes in Dubelco’s modified Eagle’s medium (pH 7.2) supplemented with 1.5 g/l NaHCO₃, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO₂ humidified incubator. The HUVEC was obtained from Clonetics (San Diego, CA) and was cultured on gelatin-coated cell culture dishes in medium 199 supplemented with 0.1 g/l heparin, 37.5 mg/l endothelial cell growth supplement, 20% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO₂ humidified incubator.

Cell viability assay

Cell viability was assessed by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay. MCF-7 cells were grown to 70–80% confluence in 24 well culture dishes in Dulbecco’s modified Eagle’s medium supplemented with 1.5 g/l sodium bicarbonate, 10% FBS and 2% penicillin-streptomycin. The molecular structure of AP is shown in Figure 1. AP was purchased from National RNAi Core Facility Platform, Taipei, Taiwan. The RNA interference was performed according to our previous study (29). Two different sequences targeting human HO-1 mRNA were chosen and purchased from National RNAi Core Facility Platform, Taipei, Taiwan.

Gelatin zymography assay

The enzyme activity of MMP-9 was analyzed by gelatin zymography as described previously (30). After treatment, the conditioned media were collected, mixed with loading buffer and subjected to electrophoresis on 8% sodium dodecyl sulfate–polyacrylamide gels containing 0.1% (wt/vol) gelatin. Electrophoresis was performed at 120V for 2h. Gels were then washed twice with washing buffer (2.5% Triton X-100) at room temperature to remove sodium dodecyl sulfate, followed by incubation at 37°C for 12–16h in reaction buffer (40 mM Tris–HCl, pH 8.0, 10 mM CaCl₂, and 0.02% NaN₃) and then stained with Coomassie blue R-250 (0.125% Coomassie blue R-250, 0.1% amino black, 50% methanol and 10% acetic acid) for 1h and destained with destaining solution (20% methanol and 10% acetic acid) for 30min. MMP-9 gelatinolytic activity was detected as clear bands in a dark blue background, which were scanned by an image analyzer (Fujifilm LAS-4000, Japan). The bands were quantified with ImageGauge (Fujifilm, Tokyo).

RANA interference by small interfering RNA of HO-1

Knockdown of the HO-1 gene in MCF-7 cells was performed according to our previous study (29).

RANA interference by small hairpin RNA of HO-1

Lentiviral infection was performed according to the method of a previous study (31). Two different sequences targeting human HO-1 mRNA were chosen and purchased from National RNAi Core Facility Platform, Taipei, Taiwan. The RNAi clones were identified by their unique number assigned by the RNAi Consortium (TRCN) as follows: TRCN0000290436 (responding sequence: CAGTGTGGCTAGAGGCTTAT) was used for shHO-1(1) targeted to HO-1, TRCN0000290436 (responding sequence: GCTGTAGATCGAGAGACTT) was used for shHO-1(2) targeted to HO-1 and the TRCN00000772246 (responding sequence: CCAATCACAGAATCGTCGTAT) was used for vector control lacZ. Biotinylated oligonucleotides (forward: 5'-GGGACTTTCCCAGGCCAAGG-3') and 2.5-μg/ml streptomycin and 10% FBS. After 24h of attachment, the cells were infected with packaged lentiviruses for another 24h. The following day, the medium was removed, and the cells were selected by using 2 μg/ml puromycin for 2 days. The cells were then passaged to 10 cm plastic culture dishes and were ready for assay.

Statistical analysis

Data were analyzed by using analysis of variance (SAS Institute, Cary, NC). The significance of the difference among mean values was determined by one-way analysis of variance followed by Tukey’s test, and the difference between mean values was determined by Student’s t-test. P values of <0.05 were considered to be statistically significant.

Results

AP inhibits TPA-induced MCF-7 cell migration and invasion in a concentration-dependent manner

The molecular structure of AP is shown in Figure 1A. We first determined the effect of AP on MCF-7 cell viability in the presence or absence of TPA and confirmed the inhibition of MCF-7 cell migration and invasion by AP. As measured by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay, the cell viabilities of MCF-7 cells treated with 50 μg/ml TPA were 60°C for 30 s. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. The relative quantification of mRNA expression was calculated with the 2^[-ΔΔCt] method (30) after normalization for expression of glyceraldehyde-3-phosphate dehydrogenase.

Nuclear extract preparation, western blotting analysis and electrophoretic mobility shift assay

Nuclear protein preparation was performed as described previously (31), and western blotting analysis as well as electrophoretic mobility shift assay were performed according to our previous study (29). Synthetic biotin-labeled double-stranded AP-1 consensus oligonucleotides (forward: 5'-GGCTTCAGCTGTGTTATGGATAAATGGCCCAAGG-3'; reverse: 5'-AAAGGGCCCAAGCAGCCAGG-3') were used to measure AP-1 nuclear protein DNA binding activity and synthetic biotin-labeled double-stranded NF-kB consensus oligonucleotides (forward: 5'-AGTATGAGGACCTTGGCGAGCAAG-3'; reverse: 5'-GCTGAGGAAAGTTCAGCCTTCAACT-3') were used to measure NF-kB nuclear protein DNA binding activity. Unlabeled and mutant double-stranded AP-1 oligonucleotide (5'-AGTGGGACTTGGCCAGCAAG-3') or NF-kB oligonucleotide (5'-AGTTTACCGCAGCTTCCAGGCCG-3') were used to confirm the protein-binding specificity, respectively.

Gelatin zymography assay

The enzyme activity of MMP-9 was analyzed by gelatin zymography as described previously (30). After treatment, the conditioned media were collected, mixed with loading buffer and subjected to electrophoresis on 8% sodium dodecyl sulfate–polyacrylamide gels containing 0.1% (wt/vol) gelatin. Electrophoresis was performed at 120V for 2h. Gels were then washed twice with washing buffer (2.5% Triton X-100) at room temperature to remove sodium dodecyl sulfate, followed by incubation at 37°C for 12–16h in reaction buffer (40 mM Tris–HCl, pH 8.0, 10 mM CaCl₂, and 0.02% NaN₃) and then stained with Coomassie blue R-250 (0.125% Coomassie blue R-250, 0.1% amino black, 50% methanol and 10% acetic acid) for 1h and destained with destaining solution (20% methanol and 10% acetic acid) for 30min. MMP-9 gelatinolytic activity was detected as clear bands in a dark blue background, which were scanned by an image analyzer (Fujifilm LAS-4000, Japan). The bands were quantified with ImageGauge (Fujifilm, Tokyo).

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The molecular structure of AP is shown in Figure 1A. We first determined the effect of AP on MCF-7 cell viability in the presence or absence of TPA and confirmed the inhibition of MCF-7 cell migration and invasion by AP. As measured by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay, the cell viabilities of MCF-7 cells treated with 50 μg/ml TPA
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alone; 1, 2, 5, 10 or 20 μM AP; or TPA and 1, 2, 5, 10 or 20 μM AP were 112.7 ± 3.2%, 97.6 ± 2.1%, 95.1 ± 3.5%, 94.3 ± 3.4%, 93.7 ± 2.3%, 93.0 ± 3.1%, 108.5 ± 4.3%, 107.7 ± 3.6%, 107.3 ± 3.6%, 106.8 ± 2.2% and 105.8 ± 2.6%, respectively, compared with the unstimulated controls (100%). Thus, there were no adverse effects on the growth of cells up to a concentration of 20 μM AP in the presence or absence of 50ng/ml of TPA, which was used to induce migration and invasion of MCF-7 cells. The highest concentration of AP used in this study was 20 μM.

In preliminary screening experiments, we determined that 50ng/ml TPA was the dosage needed to induce MMP-9 enzyme activity in MCF-7 cells (Supplementary Figure 1S, available at Carcinogenesis Online). TPA (50ng/ml) significantly induced MMP-9 enzyme activity in MCF-7 cells (P < 0.05; Figure 1). As shown in Figure 2A, pretreatment of MCF-7 cells with AP reduced TPA-induced MMP-9 mRNA expression in a dose-dependent manner. AP similarly inhibited the TPA-induced MMP-9 protein level (Figure 2B) and enzyme activity (Figure 2C).

**Fig. 1.** (A) Molecular structure of AP. (B and C) AP inhibited TPA-induced MCF-7 cell migration and invasion, respectively, in a concentration-dependent manner. Cells were pretreated with 0–20 μM AP for 16h and were then treated with or without 50ng/ml TPA for an additional 48h. Migration and invasion assays were performed using Boyden chamber as described in Materials and methods. Representative photomicrographs of the membrane-associated cells (blue part) were assayed by Liu’s stain, and the number of migrated and invasive cells was counted in five fields of view from each filter. Results were repeated at least three times from three independent experiments and presented as mean ± SD, n = 3. Results not sharing the same letter are significantly different (P < 0.05).

The inhibitory effect of AP on cell migration and invasion is associated with the inhibition of TPA-induced MMP-9 expression and induction of HO-1 expression

Tumor migration and invasion are the initial steps in metastasis, and MMP-9 is recognized to play an important role in the metastasis of cancer cells (6). To examine whether the inhibitory effect of AP on the migration and invasion of the cells was associated with inhibition of TPA-induced MMP-9 expression, we determined mRNA and protein levels of MMP-9 and MMP-9 enzyme activity in the various treatment groups by use of quantitative real-time PCR, western blot analysis and gelatin zymography assay, respectively. TPA (50ng/ml) significantly induced the expression of MMP-9 mRNA and protein and MMP-9 enzyme activity in MCF-7 cells compared with untreated cells (P < 0.05; Figure 2). As shown in Figure 2A, pretreatment of MCF-7 cells with AP reduced TPA-induced MMP-9 mRNA expression in a dose-dependent manner. AP similarly inhibited the TPA-induced MMP-9 protein level (Figure 2B) and enzyme activity (Figure 2C).
Because of the inhibitory effect of HO-1 on tumor invasion and metastasis, agents that can induce HO-1 may be candidates for chemoprevention. As shown in Figure 2D, AP dose-dependently induced HO-1 mRNA expression in the presence of TPA in MCF-7 cells. We found the same pattern for HO-1 protein expression (Figure 2E).

Small interfering RNA or small hairpin RNA knockdown of HO-1 attenuates AP inhibition of TPA-induced MMP-9 expression, enzyme activity and cell migration

We further verified the role of HO-1 in MMP-9 expression and cell migration by use of HO-1 small interfering RNA (siRNA) and small hairpin RNA (shRNA). The efficiency of the siRNA SMARTpool system in knocking down HO-1 was assayed by western blot (Figure 3A). Transfection of MCF-7 cells with HO-1 siRNA resulted in significant recovery of AP inhibition of TPA-induced MMP-9 protein expression ($P < 0.05$; Figure 3A, lane 6 versus 3) and enzyme activity ($P < 0.05$; Figure 3B, lane 6 versus 3). Accompanied by the silencing of HO-1 expression by transfecting with shRNA, AP inhibition of TPA-induced cell migration was partially reversed (Figure 3C).

HO-1 end products inhibit TPA-induced MMP-9 protein expression and enzyme activity, as well as cell migration and invasion

HO-1 is a rate-limiting enzyme that catalyzes the degradation of heme to carbon monoxide, free iron and biliverdin, the last of which is subsequently catabolized into bilirubin by biliverdin reductase (22). Accordingly, we used RuCO, FeCl$_3$ and bilirubin to determine the individual role of HO-1 end products in the TPA-induced MMP-9 expression, cell migration and invasion. As shown in Figure 4A, pretreatment with RuCO (a chemical carbon monoxide donor), FeCl$_3$ and bilirubin dose-dependently inhibited TPA-induced MMP-9 mRNA expression, and this inhibition was independent of cell damage caused by these compounds in the presence of TPA (Supplementary Figure 2S, available at Carcinogenesis Online). A similar inhibitory effect of these chemicals was found on TPA-induced MMP-9 protein expression (Figure 4B) and enzyme activity (Figure 4C). In addition, pretreatment with these chemicals significantly inhibited TPA-induced cell migration ($P < 0.05$; Figure 4D) and cell invasion ($P < 0.05$; Figure 4E) in a dose-dependent manner.

Fig. 2. AP inhibited TPA-induced MMP-9 expression and induced HO-1 expression. (A–C) Dose-dependent inhibitory effect of AP on TPA-induced MMP-9 mRNA and protein expression as well as enzyme activity, respectively, in MCF-7 cells. Cells were pretreated with 0–20 μM AP for 16h and were then stimulated with 50ng/ml of TPA for an additional 24h. (D and E) AP induced HO-1 mRNA and protein expression in a dose-dependent manner in the presence of TPA. Results are presented as mean ± SD, n = 3. Results not sharing the same letter are significantly different ($P < 0.05$).
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AP and HO-1 end products downregulate TPA-induced ERK and PI3K/Akt signaling pathways and activation of AP-1 and NF-κB transcription factors

Because TPA has been shown to activate the mitogen-activated protein kinases and PI3K/Akt pathways in several cell lines (34,35), we investigated the signaling pathways by which AP inhibited TPA-induced MMP-9 expression and subsequent cell migration and invasion. TPA (50 ng/ml) significantly induced phosphorylation of ERK and Akt 15 min after treatment, and the induction was sustained until 60 min (Supplementary Figure 3S, available at Carcinogenesis Online). Pretreatment of cells with 10 μM AP for 16 h significantly inhibited TPA-induced ERK and Akt activation (P < 0.05; Figure 5A). Similar inhibition was observed with the HO-1 end products (P < 0.05; Figure 5B). There are AP-1 and NF-κB response elements in the MMP-9 promoter (36), and AP pretreatment significantly suppressed AP-1 (Figure 5C) and NF-κB (Figure 5D) binding to these response elements. A similar inhibitory effect of the HO-1 end products was found on TPA-induced AP-1 (Figure 5E) and NF-κB (Figure 5F) DNA binding activity.

Discussion

Phytochemicals are chemical compounds that occur naturally in plants and that have been used to offer medicinal benefits for a variety of diseases for centuries. Naturally occurring agents such as resveratrol (37), quercetin (38) and AP (39) are known to possess anticarcinogenic activities. However, the effects of AP on tumor progression have not been well delineated. Several possible mechanisms for the anticancer capacity of AP have been suggested, including inhibition of cancer cell growth (40), promotion of cell cycle arrest (20) and reduction of metastasis (41) and angiogenesis (42) of cancer cells. In this study, we explored the effects of AP on breast cancer...
We demonstrated that AP effectively inhibited TPA-induced MCF-7 cell invasion and that this suppression was likely associated with an upregulation of HO-1 expression and a downregulation of MMP-9 expression. TPA is a well-known inflammatory stimulus and tumor promoter that dramatically induces the invasiveness of MCF-7 human breast cancer cells (43). A previous study indicated that TPA treatment can promote tumor metastasis by stimulating MMP-9 expression in cancer cells (44). In addition, the correlation of MMP-9 with cancer malignancy was further evidenced in a study showing that levels of the proteolytic enzyme were higher in the serum of cancer patients than in the serum of a patient group with benign tumors (45). Agents that downregulate MMP-9 have been demonstrated to inhibit cancer cell migration and invasion (11,12). In this study, TPA induced the migration (Figure 1B) and invasion (Figure 1C) of MCF-7 breast cancer cells with increases in MMP-9 gene expression and a downregulation of MMP9 expression at both the mRNA (Figure 2A) and protein (Figure 2B) levels and in MMP-9 enzyme activity (Figure 2C), and these were inhibited by AP pretreatment. This suggests that MMP-9 plays an important role in the TPA-induced migration and invasion of human breast cancer cells.

In addition to the cytoprotective and anti-inflammatory activities of HO-1 (46), HO-1 has been shown to correlate with tumor progression (26). However, some controversies remain about the role of HO-1 in tumor progression, especially in different types of tumors. A previous study indicated that induction of HO-1 may increase survival of colorectal cancer patients, and it was thought that this was through a lowering of risk of lymph node metastasis (47). Downregulation of HO-1 expression has been suggested...
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Fig. 5. AP and HO-1 end products downregulated TPA-induced ERK and PI3K/Akt signaling pathways and the activation of AP-1 and NF-κB transcription factors. (A) AP downregulated TPA-induced ERK and PI3K/Akt signaling pathways. Cells were pretreated with 10 μM AP for 16 h followed by incubation with or without 50 ng/ml of TPA for 30 min. (B) HO-1 end products downregulated TPA-induced ERK and PI3K/Akt signaling pathways. Cells were pretreated with 100 μM RuCO, 50 μM FeCl₃, and 25 μM bilirubin for 1 h followed by incubation with or without 50 ng/ml of TPA for 30 min. Fold activation is shown as an increase in the normalized phosphorylation in the treated cells relative to the control. Results are presented as mean ± SD, n = 3. Results not sharing the same letter are significantly different (P < 0.05). (C and D) AP inhibited TPA-induced binding of AP-1 and NF-κB transcription factors, respectively, to the response elements in target gene promoter. Cells were pretreated with 10 μM RuCO, 50 μM FeCl₃, and 25 μM bilirubin for 1 h followed by incubation with 50 ng/ml of TPA for 4 h. Aliquots of nuclear extracts were used for electrophoretic mobility shift assay.
to be associated with an increase in malignant progression of hepatocellular carcinoma (48). Moreover, the beneficial role of HO-1 in blocking breast cancer invasion has been identified (22). In contrast, induction of HO-1 by osteopontin has been demonstrated to enhance the migration and invasion of glioma cells (49). Osteopontin-induced increase in glioma cell migration was counteracted by HO-1 inhibitor or HO-1 siRNA. The TRC8 gene, encoding for an endoplasmic reticulum-resident E3 ligase, is shown to exhibit a tumor-suppressive effect (50). TRC8 targets HO-1, an antioxidant enzyme highly expressed in various cancers, for ubiquitination and degradation. HO-1 overexpression increased the migration and invasion of Hela cells, and these effects were abolished by TRC8 overexpression (50). Therefore, investigations of the role of HO-1 seem to be important not only for a better understanding of tumor growth regulation but also for clinical practice. In this study, we demonstrated that HO-1 mRNA (Figure 2D) and protein (Figure 2E) expression were dose-dependently induced by AP. Knockdown of the HO-1 gene by siRNA reversed the inhibition by AP of TPA-induced MMP-9 protein expression (Figure 3A) and enzyme activity (Figure 3B). These results indicate the importance of HO-1 in the inhibition of TPA-induced MMP-9 expression by AP.

Carbon monoxide, free iron and bilirubin are released as the end products of HO-1-catalyzed heme degradation, and several biological functions of these end products have been reported, such as anti-inflammatory, anti-apoptosis and antioxidation effects (51,52). However, evidence is lacking for the anti-invasiveness of HO-1 end products in relation to MMP-9 activation. In this study, we used RuCl₃, FeCl₃ and bilirubin to verify the role of HO-1 end products in the TPA-induced MMP-9 expression and cell invasion. We found that these end products dose-dependently inhibit TPA-induced MMP-9 mRNA (Figure 4A) and protein expression (Figure 4B) as well as enzyme activity (Figure 4C). In addition, a similar inhibitory effect of these end products was found on TPA-induced cell migration (Figure 4D) and cell invasion (Figure 4E). Thus, these results provide evidence that the end products of HO-1 participate in the HO-1 inhibition of TPA-induced tumor invasion by suppressing MMP-9 expression.

The transcriptional regulation of MMP-9 by TPA is reported to be mediated by the mitogen-activated protein kinases and PI3K/Akt pathways (10). In this study, we showed that TPA significantly induced ERK and Akt phosphorylation and that this induction was attenuated by AP (Figure 5A). These results were confirmed by Park et al. (9), who showed that the ERK1/2 and PI3K/Akt signaling pathways are involved in the induction of MMP-9 expression by TPA in MCF-7 cells. In addition, studies have suggested that the transcription factors AP-1 and NF-κB play a critical role in TPA-induced MMP-9 transactivation (53). Consistent with previous findings, we showed that TPA increased the DNA binding activity of AP-1 and NF-κB and that AP inhibited TPA-induced AP-1 (Figure 5C) and NF-κB (Figure 5D) DNA binding complex formation. These results imply that the inhibition of TPA-induced MMP-9 expression and enzyme activity by AP is mainly mediated by AP-1 and NF-κB activation via the ERK1/2 and PI3K/Akt signaling pathways, which leads to suppression of MCF-7 breast cancer cell migration and invasion.

Conclusion

In conclusion, AP inhibits TPA-induced MMP-9 expression, enzyme activity, cell migration and invasion, at least in part, via the induction of HO-1. Moreover, AP also acts to prevent TPA-induced cell migration and invasion by reducing MMP-9 activation through an inhibition of the ERK1/2 and PI3K/Akt signaling pathways and a reduction of AP-1 and NF-κB DNA binding activity. Taken together, our findings provide new insights into the molecular mechanisms involved in the prevention of progression of MCF-7 breast cancer cells by AP. Our results suggest that AP may be a promising therapeutic against breast cancer invasion and metastasis.

Supplementary material

Supplementary Figures 1S–3S can be found at http://carcin.oxfordjournals.org/

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References

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