Hispolon inhibits breast cancer cell migration by reversal of epithelial-to-mesenchymal transition via suppressing the ROS/ERK/Slug/E-cadherin pathway

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Abstract. Hispolon has been shown to have anticancer effects on various tumors. However, whether hispolon exerts anti-migration activity in breast cancer cells and the underlying mechanisms, have not been elucidated yet. In the present study, our data demonstrated that hispolon inhibited TPA-induced breast cancer MCF-7 cell migration at sub-toxic concentrations in vitro. Hispolon decreased the level of cellular ROS significantly and repressed TPA-induced phosphorylation of extracellular signal-regulated kinase (ERK), accompanied by upregulation of E-cadherin and downregulation of the transcriptional repressor Slug. Furthermore, N-acetyl-cysteine, an antioxidant agent, markedly suppressed TPA-induced epithelial-to-mesenchymal transition, cell migration and activation of ERK. Taken together, our results indicated that hispolon suppressed the migration of breast cancer cells via suppressing the ROS/ERK/Slug/E-cadherin pathway. Hispolon may be developed as a potential antimetastasis agent to breast cancer.

Introduction

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer-related deaths among women worldwide (1). The high breast cancer mortality rates are mainly caused by the metastasis of tumor cells (2), which can induce radiation therapy and chemotherapy resistance in advanced breast cancer. Therefore, the therapeutic strategies including the development of effective antimetastatic agents have potential benefits for breast cancer treatment.

Growing evidence indicates that the epithelial mesenchymal transition (EMT), a developmental process which involves loss of cell-cell junctions and re-organization of the actin cytoskeleton, resulting in loss of apical-basal polarity and acquisition of a spindle-like mesenchymal morphology, plays an important role in breast cancer metastasis (3,4). E-cadherin, a key factor included in EMT, has been associated with the metastasis process in breast cancer. The loss function of E-cadherin elicits active signals that induce tumor cell migration, invasion and metastatic dissemination (5,6). Moreover, levels of E-cadherin have also been found to correlate with enhanced metastasis and associate with poor prognosis and relapse in breast cancer patients (7). Transcription of E-cadherin is repressed by zinc finger proteins of the Slug/Snail family and Smad-interacting protein (8,9). To date, numerous clinicopathological studies have shown positive correlations between the expressions of the transcription factors Snail and Slug, which are the key inducible factors of EMT, and poor clinical outcomes in breast, ovary, colorectal and lung cancer (10).

Reactive oxygen species (ROS), continuously generated from mitochondrial respiratory chain during intracellular metabolism, are a family of molecules in response to environmental stimuli, including superoxide radical (O2·−), hydrogen peroxide (H2O2), hydroxyl radical (·OH) and singlet oxygen. Accumulating evidence suggests that ROS are emerging as critical signaling stimuli that mediate a variety cellular functions including cell cycle progression, apoptosis and motility (11-13). ROS signaling pathway has been reported to be intimately involved with EMT in tumor progression (14,15). Various factors such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-I and interleukin-1β (IL-1β) induce EMT in the progression of tumor cells through ROS generation (16-19). It is also suggested that ROS play an essential role in TPA-induced sustained PKC-ERK activation which is responsible for EMT and migration of HepG2 (20). Another study showed that long-term oxidative stress may induce invasive potential of mammary epithelial cells (21).

Hispolon, a poly-phenol compound, which was first isolated from Inonotus hispidus and has since been isolated from many...
tropical mushrooms (22,23). Previous studies have shown that hispolon inhibits tumor growth and metastasis through various signal pathways (22,24-26). Also we have shown that hispolon-induced apoptosis in human gastric cancer cells through a ROS-mediated mitochondrial pathway (22,24). However, whether hispolon could exert anti-migration activity in breast cancer cells as well as the underlying mechanisms have not been elucidated. In the present study, we found that hispolon inhibited TPA-induced migration of MCF-7 cells by recovering E-cadherin activity. In addition, this effect was dependent on the ROS/p-ERK/slug/E-cadherin related signaling pathway.

Materials and methods

Materials and cell culture. Transwell chambers were purchased from Corning Costar (USA). Antibodies specific for ERK, phospho-ERK, JNK, phospho-JNK, p38, phospho-p38 MAPK, AKT, phospho-AKT, Snail, Slug and E-cadherin were all purchased from Cell Signaling Technology (Beverly, MA, USA). Human breast cancer MCF-7 cells were grown in 96-well microtiter plates for drug treatment.

Chemicals and inhibitors. Hispolon was synthesized as previously described and its purity was established on the basis of the spectral (1H, 13C NMR and mass) data. Hispolon stocks (20 mM) were prepared in dimethylsulphoxide (DMSO) and stored at -20˚C. TPA and NAC were purchased from Sigma (St. Louis, MO, USA). Human breast cancer MCF-7 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and streptomycin 100 mg/ml, and were incubated at 37˚C in a humidified atmosphere of 95% air and 5% CO₂.

Cell viability assay. The effect of hispolon on the viability of MCF-7 cells was evaluated using MTT method. Briefly, cells were grown in 96-well microtiter plates for drug treatment. Following incubation for the indicated times, cells were incubated with MTT (0.5 mg/ml) for 4 h. The formazan precipitate was dissolved in 150 µl DMSO, and the absorbance was detected at 490 nm with a Model ELX800 microplate reader (Bio-Tek Instruments). Each test was performed in triplicate experiments.

Cell migration assay. The cell migration assay was conducted using Transwell chambers according to the manufacturer’s instructions. Briefly, 5x10⁴ MCF-7 cells suspended in 500 µl of serum-free medium and seeded into the upper chamber of the inserts. After treatment with different concentrations of hispolon, 750 µl of serum-free medium containing 100 ng/ml of TPA was added to the bottom wells as a chemoattractant. The chambers were incubated at 37°C for 24 h. After incubation, the filter inserts were removed from the wells and the cells on the upper side of the filter were removed using cotton swabs. Cells that had invaded to the underside of the filter were first fixed with methanol (15 min), and then stained with 2% ethanol containing 0.2% crystal violet powder (15 min). After being dried, the stained cells were enumerated under light microscope at x10 objective.

Intracellular ROS detection. DCFH-DA is a cell-permeable fluorescent dye specific for ROS and is used to detect intracellular ROS levels. Cells were seeded in 12-well plates and were cultured in serum-free medium for 24 h, and then treated with 100 ng/ml TPA for different periods of time. At the designated time points, cells were washed twice with phosphate-buffered saline (PBS) and fixed with 10% form-aldehyde for 10 min. Intracellular ROS levels were assessed by staining with DCFH-DA (5 µM) for 30 min at 37°C. Fluorescent images were captured using an inverted fluorescence microscope (Nikon Eclipse TE300; Nikon, Melville, NY, USA).

Western blotting. Total cell extracts were prepared using an M-PER mammalian protein extraction reagent kit (Pierce) according to the manufacturer's instructions. The protein concentration of each extract was determined by the Bradford assay. Cell extracts were separated by electrophoresis on 6-15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and then transferred to nitrocellulose membranes. The membranes were probed with a primary antibody followed by a secondary antibody conjugated to horseradish peroxidase. Protein bands on the membranes were detected by enhanced chemiluminescence [Western Lightning (Perkin-Elmer Boston, MA, USA) or SuperSignal West Femto (Pierce, Rockford, IL, USA)].

Statistical analysis. Numerical data are presented as means ± SD of different determinations. Statistical significance between treatment and control groups was analyzed using Student’s t-test. Values of p<0.05 were considered to indicate a statistically significant result.

Results

TPA downregulates E-cadherin expression through the production of ROS in MCF-7 cells. 12-0-Tetradecanoylphorbol-13-acetate (TPA) is one of the most utilized agents for studying the mechanisms of carcinogenesis. To characterize the effect of TPA on cell morphology in human breast cancer MCF-7 cells, we treated cells with TPA (100 ng/ml) for 24 h. As shown in Fig. 1A, treatment with TPA-induced an obvious morphological change, from a cobblestone-like morphology to fibroblastic-spindle shape, which is a typical morphology change in EMT. To further confirm the presence of EMT and investigate the molecular mechanism by which TPA-induced the morphological change, we investigated the expression of E-cadherin which plays a key role during EMT. Western blot analysis showed that TPA downregulated total E-cadherin protein levels in a dose- and time-dependent manner in MCF-7 cells (Fig. 1B).

DCFH-DA was used to examine the effect of TPA treatment on intracellular ROS production. TPA treatment induced ROS production in a time-dependent manner in MCF-7 cells (Fig. 2A). To confirm the relation of ROS production and E-cadherin expression, cells were pretreated with NAC, an antioxidant reagent. As shown in Fig. 2B, downregulation of E-cadherin induced by TPA was inhibited by pretreating NAC. To further confirm the effect of ROS on E-cadherin expression, MCF-7 cells were treated with exogenous H₂O₂. Notably, H₂O₂ treatment downregulated E-cadherin protein

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levels in a dose-dependent manner (Fig. 2C). These results suggested that the effect of TPA on E-cadherin in MCF-7 cells was closely related with the production of intracellular ROS.

Slug expression is upregulated by TPA-induced ROS production. To investigate whether TPA downregulated E-cadherin expression by modulating the transcriptional regulation of E-cadherin, we examined the protein levels of E-cadherin transcriptional repressors, Snail and Slug. Treatment with TPA significantly increased Snail and Slug expression in a time-dependent manner (Fig. 3A). To determine whether ROS production was involved in TPA-induced increasing levels of Snail and Slug proteins, cells were pretreated with NAC in the presence of TPA. As shown in Fig. 3B, NAC pretreatment diminished TPA-stimulated Slug levels, whereas the increase in Snail levels were not affected. Notably, exogenous H$_2$O$_2$ increased both Snail and Slug levels in a different manner compared to TPA (Fig. 3C).

Hispolon inhibits TPA-induced EMT. Hispolon is a natural polyphenol compound isolated from medical fungus. We first evaluated the effect of hispolon on the viability of breast cancer MCF-7 cells. At the concentrations tested, between 5 and 20 µM, for duration of 24 h, hispolon demonstrated negligible antiproliferative effects on the cells (Fig. 4A). To ascertain that any possible anti-migration effects of hispolon observed was not due to their antiproliferative activities, non-lethal concentrations (≤20 µM) were used for the following experiments.
Figure 3. TPA induces Snail and Slug expression through ROS production. (A) Cells were treated with 100 ng/ml TPA for various times, and the protein levels of Snail and Slug were analyzed by western blotting. (B) Cells were pretreated for 3 h with NAC, followed by treatment for 3 h (Snail) or 12 h (Slug) with 100 ng/ml TPA, and protein levels were analyzed by western blotting. (C) Cells were treated with H2O2 for different times and protein levels of Snail and Slug were examined by western blotting.

Figure 4. Hispolon inhibits TPA-induced EMT by reducing cellular ROS production at sublethal doses. (A) Effect of hispolon on the viability of MCF-7 cells. Following 24 h treatment with different doses of hispolon in the presence or absence of 100 ng/ml TPA, viability of MCF-7 cells was determined using MTT. Data represent mean ± SD of three independent experiments. (B) TPA-mediated morphological changes were abolished by hispolon. Cells were pretreated with hispolon (20 µM) or NAC (40 mM) for 3 h, followed by treatment with 100 ng/ml TPA for 24 h, and the resultant morphology was examined microscopically. (C) TPA-induced intracellular ROS production was inhibited by hispolon. MCF-7 cells were serum starved for 24 h and were pretreated with hispolon or NAC for 3 h, followed by treatment with 100 ng/ml TPA for 5 min. Cells were washed twice with PBS and fixed with 10% formaldehyde. Intracellular ROS levels were measured by staining with DCFH-DA (10 µM), and images were captured using a fluorescence microscope. (D) TPA-mediated downregulation of E-cadherin was abolished by hispolon. Cells were exposed to different doses of hispolon for 3 h and then were stimulated with 100 ng/ml of TPA for 24 h. E-cadherin protein levels were examined by western blotting. (E) Cells were pretreated for 3 h with hispolon, followed by treatment for 3 h (Snail) or 12 h (Slug) with 100 ng/ml TPA, and protein levels were analyzed by western blotting.
From the morphological aspect, hispolon markedly inhibited the TPA-induced morphologic change. Following treatment with TPA, MCF-7 cells acquired spindle-like cell morphology. Notably, pretreatment with 20 µM hispolon significantly inhibited TPA-induced changes in cell morphology (Fig. 4B). To show whether hispolon inhibited the change of cell morphology by expression of E-cadherin, we examined the protein level of E-cadherin by western blotting in dose-response analysis. As shown in Fig. 4D, downregulation of E-cadherin was clearly abolished by hispolon in a dose-dependent manner. Since Snail and Slug are considered to be master regulators of E-cadherin, the protein expression levels of transcription factors were evaluated by western blotting. The Slug expression level was upregulated by TPA stimulation in MCF-7 cells, which was abolished by hispolon in a dose-dependent manner. However, the expression of Snail was not affected by hispolon (Fig. 4E).

To determine whether ROS production was involved in hispolon against TPA-induced EMT, we examined hispolon-mediated cellular ROS level changes. The cells were pretreated with the antioxidant reagent NAC followed by treatment with TPA. Strikingly, the cellular ROS levels were reduced after pretreatment with hispolon (Fig. 4C). As shown in Fig. 4B and C, NAC decreased the intracellular ROS levels and inhibited the morphological change which was induced by treatment of TPA in MCF-7 cells. These results demonstrated that hispolon inhibited TPA-induced EMT through repressing the ROS production.

**Hispolon inhibits TPA-induced EMT by activation of ERK.** Numerous studies have demonstrated that tumor metastasis originates from abnormal regulation of the activation of intracellular signal molecules such as mitogen-activated protein kinase and tyrosine kinase (MAPK), including ERK, p38, c-Jun N-terminal kinase (JNK) and AKT kinase which are important downstream signaling cascades involved in tumor cell migration. Herein, we examined the expressions of phospho-AKT, AKT, phospho-MAPK and MAPK expression in MCF-7 cells. The phosphorylation levels of AKT and MAPK were increased by TPA time-dependently. A time-course analysis showed that the phosphorylation levels of AKT and MAPK peaked at 5-30 min after TPA stimulation and gradually recovered to the baseline values 3 h later (Fig. 5A). These results indicated that AKT and MAPK signaling pathways were actually capable of being activated in response to TPA in MCF-7 cells.

To assess whether MAPK and AKT activation were involved in TPA-induced EMT, cells were pretreated with
special kinase inhibitors. Notably, we found that the AKT inhibitor LY294002 and the ERK inhibitor U0126 diminished the inhibitory effects of TPA on E-cadherin protein levels, but the p38 MAPK and JNK inhibitor did not (Fig. 5B). Furthermore, as shown in Fig. 5C, treatment with the ERK inhibitor U0126 diminished the upregulation of TPA-induced Slug. Treatment with the AKT inhibitor LY294002 diminished the upregulation of TPA-induced Slug. To some extent, ERK and AKT kinases were involved in TPA-induced EMT.

To investigate whether ROS production, ERK and AKT activation, and the subsequent upregulation of transcription factors were involved in hispolon inhibiting TPA-induced EMT, cells were pretreated with hispolon. Notably, we found that hispolon markedly diminished TPA-induced ERK phosphorylation but did not affect TPA-induced phosphorylation of p38 MAPK, JNK and AKT (Fig. 6). Notably, treatment with antioxidant NAC showed similar phenomenon (Fig. 6). Taken together, these results suggested that hispolon down-regulated Slug expression via ERK signaling by inhibiting the production of intracellular ROS.

Hispolon inhibits TPA-induced cell migration. Since hispolon inhibited the TPA-induced EMT in MCF-7 cells, we next examined whether the inhibitory effect of hispolon on EMT led to an inhibition of cellular migration in MCF-7 cells. As shown in Fig. 7, migration assay revealed that hispolon reduced TPA-mediated cellular migration. U0126 and NAC inhibited TPA-induced cell migration in MCF-7 cells and to the same extent by hispolon. These results suggested that hispolon actually inhibits the cellular migration phenotype of the EMT in MCF-7 cells. It is assumed that the inhibitory activity of hispolon on the cellular migration phenotype is due to its inhibitory effect of ROS-ERK signaling pathway.

Taken together, these results indicated that hispolon inhibits the TPA-induced EMT, and is characterized by E-cadherin downregulation, morphologic changes and an increase in the cellular migrating phenotype by inhibiting ROS/ERK/Slug/E-cadherin pathway in MCF-7 cells.

Discussion

Metastasis is a major obstacle for cancer therapy and is a primary cause of mortality in many cancer types, including breast cancer. TPA is a well-known tumor promoter and exhibits many biological effects by altering gene expression. Previous studies revealed that TPA was a potential inducer of tumor invasion/migration in breast cancer cells and EMT in human prostate cancer ARCaP cells (27,28). Additionally, TPA has been shown to downregulate E-cadherin expression in human cancer cells, resulting in tumor metastasis (29,30). Moreover, studies showed that ROS are essential mediators of TPA-induced cell migration and invasion (20,31). However, the specific role of ROS in the downregulation of E-cadherin expression caused by TPA signaling remains to be elucidated. In the present study, we reported that ROS-mediated TPA-induced E-cadherin downregulation and cell migration. ROS exerted their effects via activating ERK and increasing Slug expression. In addition, our studies suggested that hispolon, a natural polyphenol compound, could inhibit TPA-induced cell migration through ROS/ERK/Slug/E-cadherin signaling pathway.

ROS generation is induced by various growth factors, cytokines and tumor promoters (17,18,32). The contribution of an elevation in ROS to carcinogenesis was detected in several different types of cancer cells, and the ROS-mediated signaling cascade in tumor metastasis was highlighted.
However, the importance of ROS generation in TPA-induced migration of breast cancer cells is still undefined. In the present study, increasing ROS generation followed by cell migration, MAPK activation and loss of E-cadherin expression were observed in TPA-treated MCF-7 breast cells, and they were blocked by addition of the antioxidant NAC through reduction in ROS. These results suggest that the production of ROS may be a critical mediator in TPA-induced EMT, and TPA-induced ROS generation was an initial event in the progression of breast cancer metastasis. Notably, the time course of exogenous H$_2$O$_2$-induced Slug expression was significantly different from that of TPA. Moreover, NAC abolished TPA-induced expression of Slug in MCF-7 cells, but treatment with exogenous H$_2$O$_2$ increased both expression of Snail and Slug. These results indicated that the functional consequences of TPA-induced endogenous ROS signaling differed from those of exogenous H$_2$O$_2$ treatment. Previous research showed that NF-$\kappa$B rather than ROS play a more important role in the TNF-$\alpha$-induced EMT of MCF-7 cells (33). However, our present study suggested EMT transition was mainly due to the production of cellular ROS stimulated by TPA.

Loss of E-cadherin gene expression, which is the hallmark of epithelial-mesenchymal transition (EMT), is mainly due to upregulation of E-cadherin repressors such as Snail and Slug. Indeed, ectopic expression of Snail or Slug in SKOV3 cells results in EMT associated enhanced motility, invasiveness and tumorigenicity (34). In the present study, we found that TPA could induce Snail and Slug expression by producing intracellular ROS. However, treatment with the antioxidant agent NAC totally blocked ROS production but only partially abolished the TPA-induced EMT and cell migration and did not inhibit TPA-induced Snail expression. Thus, we predicted that other ROS independent pathways may contribute to Snail induction and mediate TPA-induced E-cadherin downregulation. Additionally, we also found hispolon and NAC abolished TPA-induced expression of Slug, but not Snail in MCF-7 cells. Although Slug is thought to function in a redundant manner with Snail, several recent studies suggested unique functions of Slug. i) Slug was essential for Notch1:Jagged 1-mediated EMT, tumor growth and metastasis (35); ii) Slug expression was correlated with poor breast cancer prognosis (36); iii) Slug, but not Snail or Twist was expressed in ES cells and was part of the ES cell signature activated in several cancer cells (37); iv) Slug but not Snail expression, was linked to ductal development in the breast including tubule maintenance or growth within invasive ductal carcinoma (38). These observations along with the results of the present study highlighted critical role of Slug in breast cancer metastasis.

MAPK and AKT cascades are major signaling pathways which can drive the metastasis of tumor cells (39). It was demonstrated that increased ROS levels may enhance MAPK activities in the malignant progression of cancer cells (17,19). However, the role of ROS in MAPK activation induced cell migration is not clear. In the present study, TPA treatment significantly increased ERK, p38 and JNK expression in breast cancer cells. However, only TPA-induced activation of ERK was inhibited by NAC treatment. In addition, E-cadherin inhibition by U0126 significantly reduced TPA-induced breast cancer cell migration. Taken together, our results indicated that ROS-dependent ERK activation was involved in TPA-induced E-cadherin downregulation and cell invasion in breast cancer cells. AKT kinase is a convergence point for multiple extracellular and other upstream signals, functioning as a master switch to generate a plethora of intracellular signals and responses. Downstream targets of AKT are thought to be involved in survival, growth, metastasis and metabolic-related pathways (40,41). Activation of AKT signaling has been detected in cells undergoing EMT (42). In the present study, phosphorylation of AKT was not inhibited by pretreatment with hispolon. The AKT kinase inhibitor LY294002 prevented TPA-induced upregulation of Snail or Slug, or downregulation of E-cadherin, suggesting the AKT pathway was not involved in the antemetastasis effect of hispolon. These results indicated that a different signal pathway may be involved in the EMT progression, and ERK pathway was the major mediator in MCF-7 cells treated by hispolon.

For centuries, people have been harnessing the power of nature to provide medicinal solutions to various diseases. In particular, rich sources of natural compounds isolated from plants have potential anticancer activities. Various well-known naturally occurring agents, such as resveratrol (43), quercetin (44), curcumin (45) and gengerol (46,47) have been demonstrated to exert inhibitory effects on cancer cell invasion and metastasis. Hispolon, isolated from medical fungus, is a natural polyphenol compound with low toxicity, have antiviral, antiproliferative and immunomodulatory activities (24,25,48). Moreover, numerous studies have shown the antiproliferation activity of hispolon in many types of human cancers. Our previous research showed hispolon could inhibit TPA-induced migration and invasion of MDA-MB-231 cells by reducing MMP-9 secretion and expression, mainly through the NF-$\kappa$B signaling pathway (49). In the present study, we confirmed that hispolon can inhibit the migration of MCF-7, breast cancer cells by a completely different mechanism. Previous literature show ER signaling may globally regulate the EMT program (50) and loss of ER may change the expression profile of specific matrix macromolecules (51). Moreover, hispolon inhibited cell growth through modulation of ER in estrogen-positive breast cancer cells (52). Regarding different ER status of the two breast cancer cell types involved in our research, the different mechanism of anti-migration of hispolon may depend on the different ER status. Although further studies are needed to establish the exact signal pathway, our results taken together strengthen the potential of hispolon as a multitarget drug in anticancer therapy.

In the present study, we demonstrated that hispolon inhibited migration of breast cancer MCF-7 cells through E-cadherin. Hispolon could downregulate Slug by inhibiting ERK signaling, thereby inhibiting the TPA-mediated EMT in breast cancer cells. Our results provided new insight into the mechanisms of hispolon inhibition of cancer cell metastasis and suggested that hispolon could be a potential agent for treatment of breast cancers.

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