12-Deoxyphorbol 13-palmitate inhibits the expression of VEGF and HIF-1α in MCF-7 cells by blocking the PI3K/Akt/mTOR signaling pathway

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Abstract. Vascular endothelial growth factor (VEGF) is an essential component for angiogenesis, and hypoxia-inducible factor-1α (HIF-1α), which controls the switch of glycolytic and oxidative metabolism, activates the transcription of VEGF. 12-Deoxyphorbol 13-palmitate (DP) is a compound isolated from the roots of *Euphorbia fischeriana*, and has been revealed to possess anticancer activity. In the present study, we found that DP is an effective inhibitor of VEGF and HIF-1α in MCF-7 cells. DP markedly reduced cell viability as determined by MTT assay. ELISA, western blotting and RT-qPCR assays indicated that DP significantly decreased the protein and mRNA expression of VEGF and the protein expression of HIF-1α, while HIF-1α mRNA remained unchanged. In addition, the entrance of HIF-1α into the nucleus was blocked after DP treatment as detected by immunofluorescence analysis. In a further study, we proved that the effects mentioned above were associated with constitutive interference of the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway. DP effectively inhibited the phosphorylation of PI3K and its downstream factors p-Akt and p-mTOR, oppositely enhanced the expression of TSC1 (hamartin) and TSC2 (tuberin), which could be reversed by the co-treatment with the PI3K inhibitor wortmannin. Moreover, the addition of wortmanin further downregulated the protein levels of VEGF and HIF-1α. The results revealed that DP inhibited the expression of VEGF and HIF-1α through the PI3K/Akt/mTOR signaling pathway, confirming that DP may be a potential therapeutic candidate for breast cancer.

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Introduction

Breast cancer is a heterogeneous disease with various subtypes exhibiting distinct biological features, resulting in differences in response patterns to various clinical strategies and treatment modalities (1). Currently, the research of breast cancer in regards to molecular techniques and gene signatures has been a central issue for the development of novel therapeutics (2). Angiogenesis, one of the established approaches for targeting tumors, is a complex procedure containing a succession of genetic alterations, of which VEGF is a key mediator. In accordance with the results of various preclinical studies, significant therapeutic effects of VEGF blockers have been demonstrated in various types of cancers, even in some progressive cancers (3). Our previous study on 12-deoxyphorbol 13-palmitate (DP) showed that it possesses an anti-angiogenesis effect *in vitro* and *in vivo* through the VEGFR-2-signaling pathway (4). In the present study, we explored the ability of DP to inhibit the expression of VEGF and hypoxia inducible factor-1α (HIF-1α) via the PI3K/Akt/mTOR pathway.

VEGF is an endothelial cell-specific mitogen and VEGF-mediated related signaling contributes to key aspects of tumor angiogenesis and vascular permeability (5). The upstream gene of VEGF, HIF-1α, is overexpressed in the early stages of mammary carcinogenesis, and is correlated with diagnostic and prognostic indicators, making HIF-1α a potential target for new approaches to inhibit angiogenesis (6). HIF-1α, under hypoxic stress, binds with HIF-1β, and controls the expression of multiple downstream target genes by combining with hypoxia response elements (HREs) of the target genes inside the nucleus to promote further responses (7). The expression of HIF-1α is controlled by multiple mechanisms, in which protein ubiquitination is primarily involved. In addition, research has found that HIF-1α accumulation depends on *de novo* protein synthesis (9), and some pathways participate in the regulation of HIF-1α, such as the PI3K/Akt/mTOR pathway (10).

PI3K/Akt/mTOR, a crucial intracellular pathway, has been well established to play a significant role in tumor cell proliferation and growth (11). Activation of PI3K transforms phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3), and then triggers Akt phosphorylation on Thr 308. Phosphorylated Akt unleashes mTOR through inactivation of TSC1/TSC2 (12). mTOR, a protein kinase, is a therapeutic target for cancer

treatment which is ubiquitously expressed in cells (13). As a tumor-suppressor, the TSC-TSC2 complex emerges as a critical negative regulator of signaling downstream of Akt and upstream of mTOR (14). Accumulated evidence from cancer and genetic biology indicates the benefit of targeting the PI3K/Akt/mTOR signaling pathway to inhibit tumor angiogenesis. Collectively, we hypothesized that DP possesses anticancer activity in breast cancer by impacting tumor angiogenesis.

Materials and methods

Materials. DP was isolated from *Euphorbia fischeriana* and its purity was >99% as characterized by gas chromatography. The Valukine Human VEGF Immunoassay kit was purchased from R&D Systems (Minneapolis, MN, USA). Wortmanin was purchased from Cayman (Denver, CO, USA). Cell culture reagents were purchased from HyClone (Logan, UT, USA). MTT, dimethylsulfoxide (DMSO) and $CoCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). RNAiso Plus, SYBR® Premix *Ex* Taq™ and PrimeScript® RT reagent kit (Perfect Real Time) were purchased from Takara (Dalian, Liaoning, China).

Cell culture. The human breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were maintained at 37˚C in a CO_2 incubator with an atmosphere composed of 5% CO_2 and 95% air. To mimic a hypoxic condition, the cells were treated with 150 μM $CoCl_2$ as the hypoxic control group.

Cell viability assay. The cells were divided into normoxic and hypoxic control groups and treated with various concentrations (10-40 μM) of DP. Cell viability was determined by the MTT assay. MCF-7 cells were seeded in 96-well plates at a density of $3x10^3$ cells/well and incubated for 6, 12 and 24 h after a 12-h adherence. Then, 20 μl of MTT solution (5 mg/ml) was added into each well for another 4-h incubation at 37˚C. After 4 h, the medium was removed and the crystals were dissolved using 200 μl DMSO before constant shaking for 10 min. The absorbance values were analyzed by an enzyme-labeling instrument at 570 nm. Three replicate were conducted for the MTT assay.

Enzyme-linked immunosorbent assay (ELISA) for VEGF. For quantification of VEGF excreted by the MCF-7 cells, the cells were incubated overnight in 6-well dishes with $2x10^6$ cells/well. Following DP treatments for 12 h, the medium was collected and examined by a Valukine Human VEGF Immunoassay kit following the manufacturer's instructions.

RT-qPCR. After DP treatments, total RNA was isolated from the cells using total RNA extraction reagent RNAiso Plus according to the product manual. Total RNA was reverse-transcribed to cDNA with the PrimeScript® RT reagent kit following the manufacturer's instructions. The amplification of cDNA was performed in triplicate using SYBR® Premix *Ex* Taq™ kit under conditions of 95˚C for 30 sec, 95˚C for 5 sec and 60˚C for 31 sec. The VEGF, HIF-1α, TSC1 and TSC2 mRNA were analyzed and the final data were presented using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used for real-time PCR were: VEGF F, 5'-GTCCAACTTCTGGGCTGTTCT-3' and R, 5'-CCCTCTCCTCTTCCTTCTCTTC-3'; HIF-1α F, 5'-TAGCCGAGGAAGAACTATGAAC3' and R, 5'-CACACT GAGGTTGGTTACTGTTG3'; TSC1 F, 5'-GCACTCTTT CATCGCCTTTATG-3' and R, 5'-ATCATTGGCTTGAC CACTTCTT-3'; TSC2 F, 5'-CAGACAATGGGAGACACAT CAC-3' and R, 5'-CtAAGTTCACCAGCACCAGAAG-3'.

Western blotting. MCF-7 cells were incubated overnight before treatment with various concentrations of DP and wortmannin for 12 h. After washing with cold PBS twice, the cells were lysed with RIPA lysis buffer (Beyotime, Shanghai, China), and total proteins were quantitated with a BCA protein assay kit (CWBio, Beijing, China). Equal protein (30 μg) of each sample was separated by SDS-polyacrylamide gels (SDS-PAGE) and then blotted onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with the primary mouse antibodies against GAPDH (CWBio), HIF-1α (BD Biosciences, Minneapolis, MN, USA), TSC1 (Cell Signaling Technology, Danvers, MA, USA) and rabbit antibodies against VEGF (Abcam, Cambridge, UK), TSC2, p-Akt, Akt, p-PI3K, PI3K, mTOR and p-mTOR at 4˚C overnight followed by HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG antibody (both from Cell Signaling Technology) incubated for 2 h at room temperature. Blots were then visualized using the West Pico Chemiluminescent Substrate (Pierce, Woburn, MA, USA). GAPDH was used as the internal reference.

Immunofluorescence. MCF-7 cells were plated in 6-well plates, then treated with DP (0 and 20 μM) for 12 h. After incubation overnight at 4˚C with a 1:100 dilution of mouse anti-HIF-1α, the cells were washed three times with PBS and then incubated with an anti-mouse FITC-labeled secondary antibody for 1 h, and then cells were stained with DAPI for 5 min after washing three times. The immunofluorescence was observed with a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Statistical analysis. All data are presented as the mean ± SD. Student's t-tests were conducted to analyze the differences between experimental groups and control groups using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). A P-value of ≤ 0.05 was considered to indicate a statistically significant result.

Results

DP inhibits the proliferation of MCF-7 cells. In this experiment, we tested the effect of DP on the proliferation of MCF-7 cells using MTT assay. Results of the MTT assays showed that following treatment with 150 μM $CoCl_2$, the cell viability did not exhibit significant differences compared with the normoxic control (Fig. 1). DP decreased the viability of MCF-7 cells in a time- and dose-dependent manner. DP reduced the viability slightly at 6 h without any statistical differences following treatments at 10, 20 and 40 μM. Treatment at 10 μM showed no inhibition when compared with the hypoxic control at 6, 12 and 24 h. Treatments at 20 and 40 μM inhibited the cell viability which ranged between 26 and 75% at 12 and 24 h.

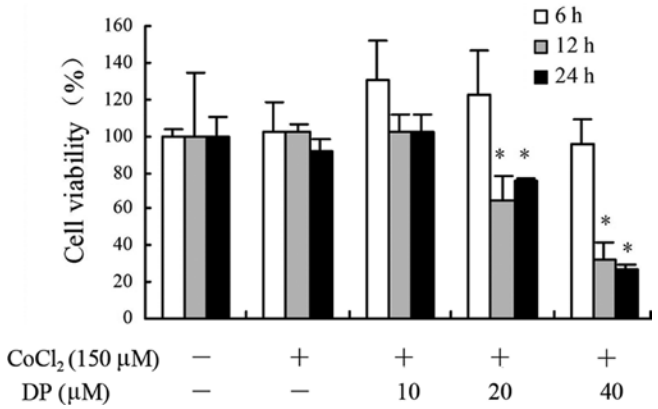

Figure 1. DP decreases the viability of MCF-7 cells. Results are expressed as the percentage of cell viability compared to the normoxic control. Values are represented as the mean ± SD from three independent experiments. *p<0.05 compared to the normoxic control group. DP, 12-deoxyphorbol 13-palmitate.

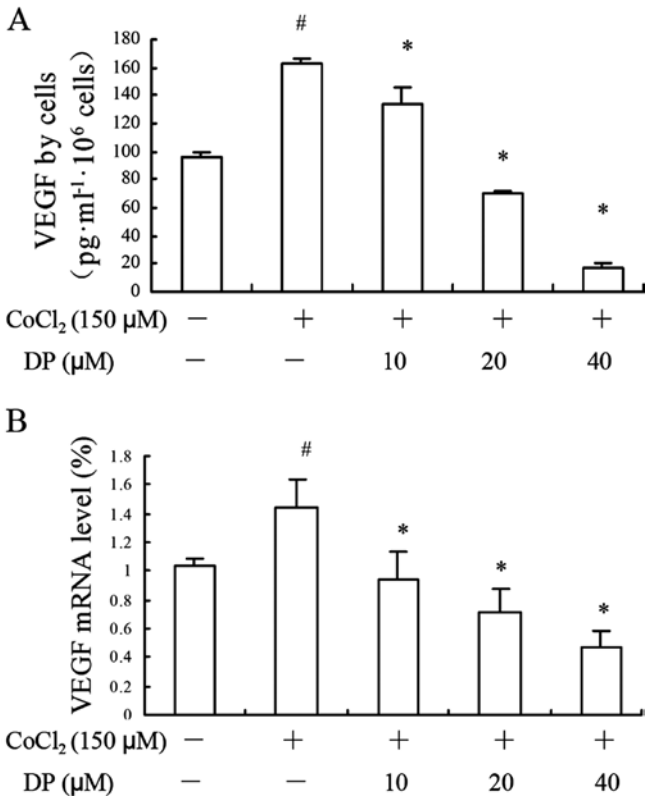

Figure 2. DP decreases the excretion and transcription of VEGF induced by hypoxia. (A) ELISA results are expressed as normalized data. (B) Effect of DP on the VEGF mRNA level in MCF-7 cells by RT-qPCR assay using the $2^{-\Delta\Delta Ct}$ method. The results are showed as mean ± SD from three independent experiments. #p<0.05 compared to the normoxic control, *p<0.05 compared to the hypoxic control group. DP, 12-deoxyphorbol 13-palmitate; VEGF, vascular endothelial growth factor.

DP reduces the transcription and excretion of VEGF. VEGF is activated not only in normal human tissues yet is also associated with oncogenesis of breast cancer (15). As an important pro-angiogenic factor, VEGF excreted from tumor cells promotes the development of nearby vessels. In the present study, we investigated the impact of DP on VEGF protein and mRNA levels by ELISA and RT-qPCR assays, respectively. In the ELISA assay, the VEGF accumulation is presented as normalized data based on the cell number. A notable increase in VEGF level was detected when cells were exposed to a hypoxia condition, and DP downregulated VEGF at both the protein (Fig. 2A) and mRNA (Fig. 2B) levels. The results indicated that DP reversed the expression and transcription of VEGF induced by hypoxia, which confirms it to be a potential inhibitor for reducing neovascularity.

DP re-distributes the location of HIF-1α. An increase and stabilization of nuclear translocation of HIF-1α is a key factor to VEGF gene expression and angiogenesis (16). To explore the effect of DP on the location of HIF-1α, immunofluorescence was used. As shown in Fig. 3, in the presence of DP, the HIF-1α fluorescent intensity became weaker. Simultaneously, the localization of HIF-1α was re-distributed in the cells. The expression of HIF-1α was obviously concentrated in and around the nucleus in the hypoxic control, yet this accumulation decreased after the addition of DP (20 μM), which confirmed that DP interrupts nuclear HIF-1α translocation.

DP inhibits the expression of HIF-1α without affecting its transcriptional activity. Hypoxia is involved in the process of angiogenesis, and a growing number of studies have focused on regulating the HIF family to alter the expression of VEGF (17). To further confirm the results above, western blotting and RT-qPCR were performed to examine the total HIF-1α expression in the cells. As shown in Fig. 4A, MCF-7 cells were treated with different concentrations of DP under a hypoxia condition for 12 h. The protein level of HIF-1α under a hypoxia condition was markedly increased when compared with the level under a normoxic condition, and this increase was abolished by DP in a concentration-dependent manner. However, the protein level was downregulated while the mRNA level of HIF-1α remained unchanged (Fig. 4B), suggesting that DP may downregulate the expression of HIF-1α without affecting it at the transcriptional level in the MCF-7 cells.

DP inhibits the PI3K/Akt/HIF-1α signaling pathway in MCF-7 cells. The PI3K/Akt/HIF-1α pathway is important in the majority of human types of cancers. Research has found that the PI3K/Akt pathway is involved in tumor angiogenesis by an HIF-1-dependent mechanism (18). To evaluate the effect of DP on the PI3K/Akt/HIF-1α pathway, we detected the phosphorylation of PI3K, Akt and mTOR. The MCF-7 cells were treated with DP at various concentrations under a hypoxic condition for 12 h. Treatment of DP resulted in a decrease in the expression of p-PI3K, which was correlated with its effectors p-Akt and p-mTOR, whereas DP did not affect the total protein levels of these proteins (Fig. 5).

DP regulates HIF-1α and VEGF in a PI3K and TSC1/TSC2-dependent manner. TSC1 and TSC2 are tumor-suppressor genes, and important negative regulators of mTOR, with responsiveness to the growth factors related to PI3K and Akt (19). DP enhanced the protein levels of TSC1 and TSC2 in the MCF-7 cells in a concentration-dependent manner, and this enhancement by DP required the activity of PI3K, as the addition of the PI3K inhibitor wortmannin (100 nM)

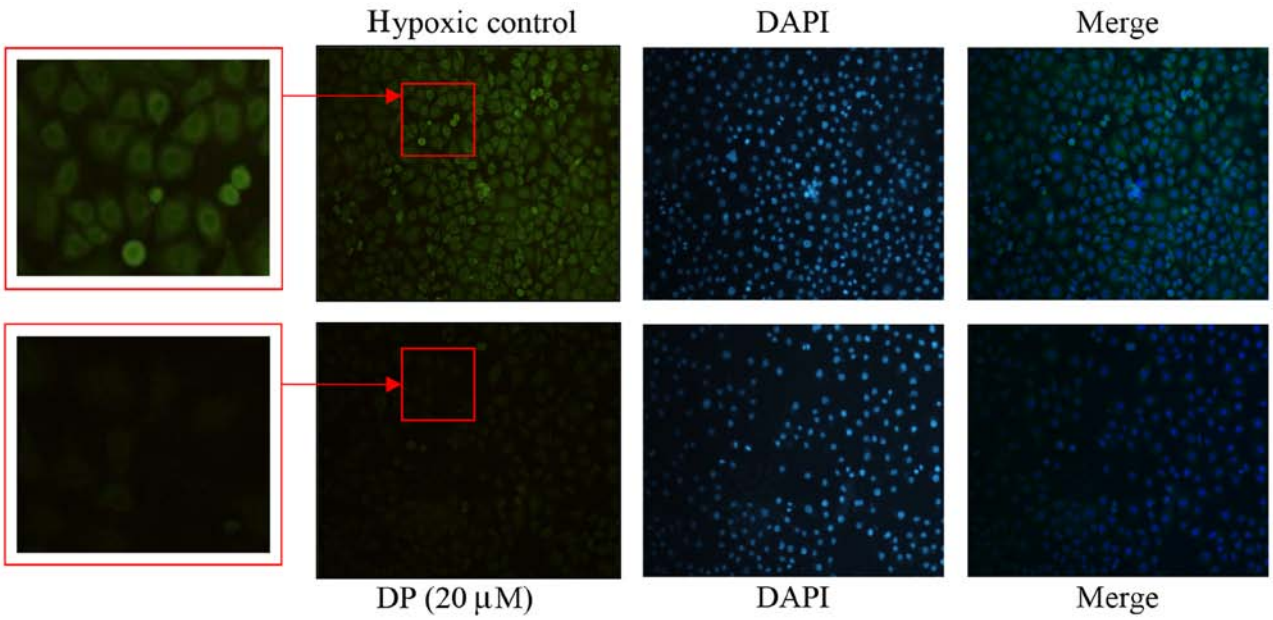

Figure 3. DP re-distributes the location of HIF-1α. Anti-mouse FITC antibody against HIF-1α was used, DAPI was used to counterstain the nuclei. Magnification, x400 and x200. DP, 12-deoxyphorbol 13-palmitate; HIF-1α, hypoxia inducible factor-1α.

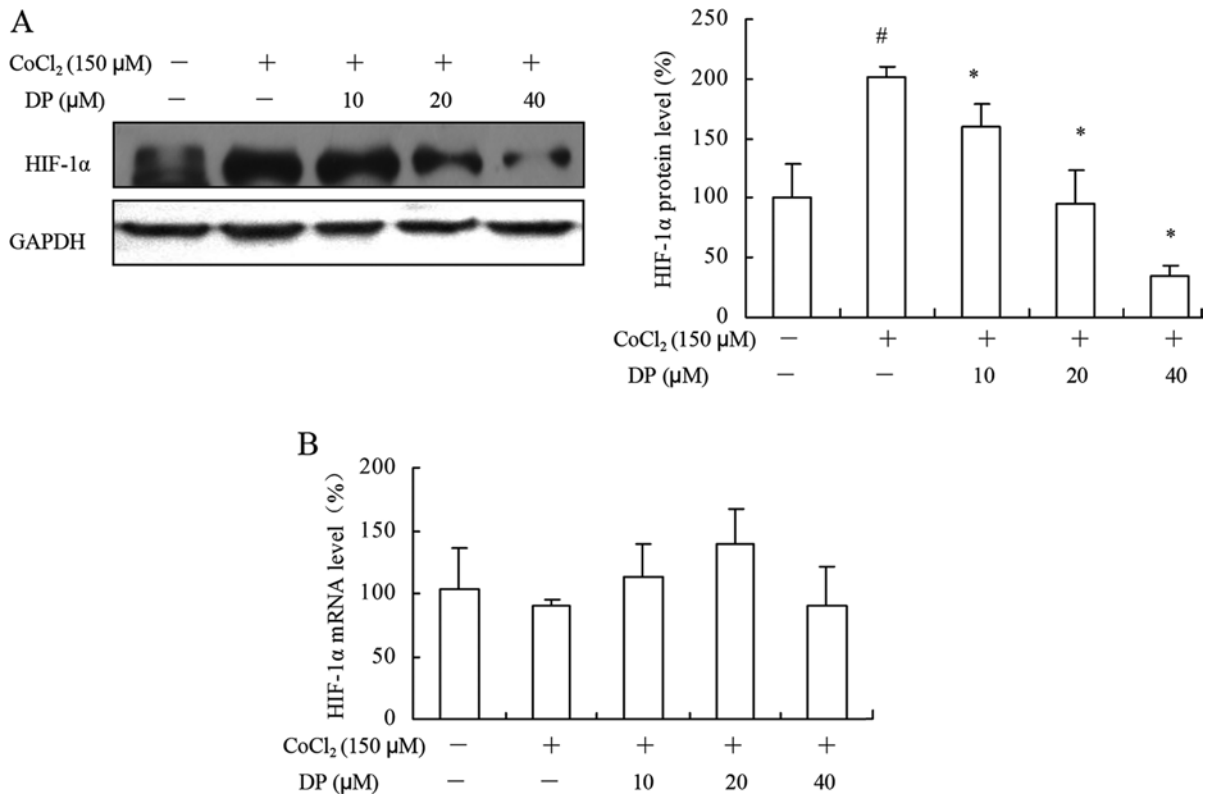

Figure 4. DP inhibits the expression of HIF-1α without affecting its transcriptional activity. (A) Western blotting results of HIF-1α were normalized by GAPDH and are relative to the control group. (B) The HIF-1α mRNA level was detected by RT-qPCR and analyzed by the $2^{-\Delta\Delta Ct}$ method. The results are shown as mean ± SD. $^{\#}p<0.05$ compared to the normoxic control, $^{*}p<0.05$ compared to the hypoxic control group. DP, 12-deoxyphorbol 13-palmitate; HIF-1α, hypoxia inducible factor-1α.

significantly reversed this effect. Next, we found that co-treatment of wortmannin with DP (40 μM) downregulated the expression of HIF-1α and VEGF slightly by western blotting (Fig. 6A). Furthermore, the RT-qPCR results exhibited the same tendency of TSC1 and TSC2 mRNA with the protein levels (Fig. 6B and C). These results collectively suggest that DP inhibited VEGF and HIF-1α through a PI3K-related signaling pathway.

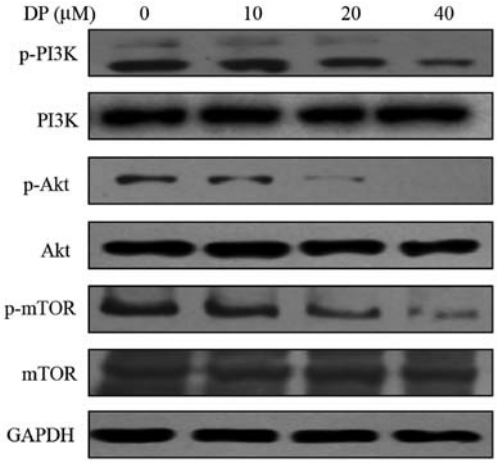

Figure 5. Effect of DP on the PI3K/Akt/mTOR pathway. Expression levels of p-PI3K, p-Akt and p-mTOR protein in MCF-7 cells were measured by western blotting and normalized to GAPDH. DP, 12-deoxyphorbol 13-palmitate.

Discussion

Breast cancer is the leading cause of cancer-related death in females. It is a heterogeneous disease and is commonly associated with epigenetic and multiple genetic abnormalities (20,21). Breast cancer is angiogenesis-dependent, which explains its vulnerability to various angiogenesis-related factors such as vascular endothelial growth factor (VEGF) in preclinical and clinical research (22). Reich *et al* found that the blockade of VEGF by small interfering RNA (siRNA) inhibited neovascularization in a mouse model (23). Considering the central importance of VEGF emphasized by clinical trials (24), bevacizumab, a humanized murine monoclonal antibody with strong affinity for VEGF, is the most popular anti-angiogenic agent used in cancer therapy (25). Therefore, in the present study, we found that 12-deoxyphorbol 13-palmitate (DP) suppressed the mRNA level and excretion of VEGF in MCF-7 cells. Thus, we speculated that DP influences the growth of blood vessel in breast tumors.

Hypoxia-inducible factor-1α (HIF-1α) is a strong stimulator of neovascularization and excretion of VEGF at the transcription level (26-29). We identified that DP has the potential to inhibit VEGF expression in MCF-7 cells. Thus, we next detected the effect of DP on HIF-1α. HIF-1α is sensitively regulated by hypoxia, and mediates the adaptation of cells to low oxygen states (30,31). Thus, in our experiment, $CoCl_2$ was used to mimic hypoxia, at least in part (32). The hypoxia-induced increase in HIF-1α was blocked by the addition of DP at the protein level but not at the mRNA level, which is in accordance with previous studies (33,34), and which is presumably linked to increasing HIF-1α translation (35). Given the findings, it is concluded that DP suppressed HIF-1α-mediated VEGF expression.

The PI3K/Akt/mTOR signaling pathway plays a critical role in regulating cellular properties, such as proliferation, motility, survival and angiogenesis (36). The activation of the PI3K/Akt/mTOR pathway in tumor cells increases the excretion of VEGF by both HIF-1-dependent and -independent mechanisms, and the induction of HIF-1α inhibits mTOR activation and stimulates the VEGF-VEGFR1 angiogenic pathway,

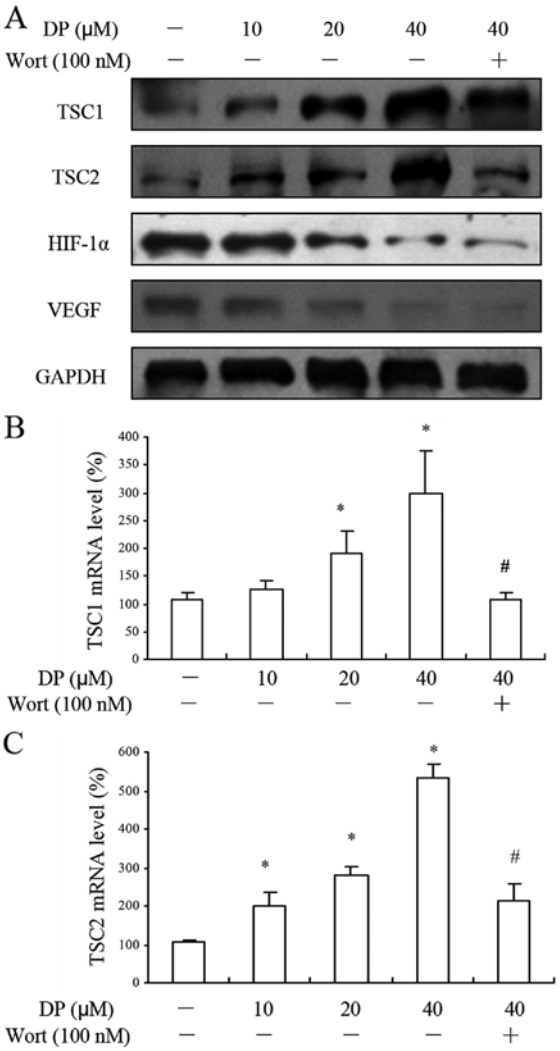

Figure 6. Effect of DP and wortmannin on TSC1, TSC2, HIF-1α and VEGF expression. (A) The protein levels of TSC1, TSC2, HIF-1α and VEGF after DP and wortmannin (Wort) treatment in the MCF-7 cells. (B) TSC1 and (C) TSC2 mRNA levels were altered after DP and wortmannin treatment. The results are shown as mean ± SD. $^{\#}p<0.05$ compared to DP 40 μM group, $^{*}p<0.05$ compared to the hypoxic control group. DP, 12-deoxyphorbol 13-palmitate; VEGF, vascular endothelial growth factor.

which is turned off as hypoxia subsides (37,38). In contrast, the inhibition of this pathway by LY294002 and mTOR inhibitor rapamycin was found to reduce the accumulation of HIF-1α in MCF-7 and prostate cancer cell lines (39). We identified that DP interrupted the PI3K/Akt/mTOR cascade. Moreover, it has been reported that phosphorylated Akt unleashes mTORC1 by inhibiting activation of TSC1 and TSC2 (40). Indeed, in the present study, the DP-induced increase in TSC1 and TSC2 was blocked by addition of wortmannin, which together suggest that DP downregulates VEGF and HIF-1α by interrupting the PI3K pathway.

Concerning clinical development, the complexity of the PI3K/Akt/mTOR pathway and its extensive crosstalk with other kinase cascades, will result in challenges in identifying patients who will benefit most from breast cancer treatments (41,42). DP may be a potential anti-VEGF and -HIF-1α drug for breast cancer. Yet, its potential side-effects require careful investigation.

Acknowledgements

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