

Dihydroartemisinin inhibits endothelial cell proliferation through the suppression of the ERK signaling pathway

FENGYUN DONG^{1*}, HU TIAN^{2*}, SUHUA YAN³, LIQUN LI¹, XIAOFENG DONG², FUHAI WANG², JIE LI², CHANGSHENG LI⁴, ZHIQUN CAO⁵, XIAOCHUN LIU⁶ and JU LIU¹

¹Laboratory of Microvascular Medicine, Medical Research Center; Departments of ²General Surgery, ³Cardiology and ⁴Traditional Chinese Medicine, Shandong Provincial Qianfoshan Hospital, Shandong University; ⁵Department of Medicine, Hospital of Shandong Traditional Chinese Medicine, Jinan, Shandong 250014, P.R. China; ⁶Department of Investigational Cancer Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

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Abstract. Disrupting tumor angiogenesis serves as an important strategy for cancer therapy. Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin, has exhibited potent anti-angiogenic activity. However, the molecular mechanisms underlying this effect have not been fully understood. The present study aimed to investigate the role of DHA on endothelial cell proliferation, the essential process in angiogenesis. Human umbilical vein endothelial cells (HUVECs) treated with DHA were examined for proliferation, apoptosis and activation of the extracellular signal-regulated kinase (ERK) signaling pathway. Proliferation of HUVECs was inhibited by 20 μ M DHA without induction of apoptosis. DHA also reduced the phosphorylation of ERK1/2, and downregulated the mRNA and protein expression of ERK1/2 in HUVECs. In addition, DHA suppressed the transcription and protein expression of ERK1/2 downstream effectors c-Fos and c-Myc. Electrical cell-substrate impedance sensing real-time analysis demonstrated that ERK signaling inhibitor PD98059 comprises the anti-proliferative effects of DHA. Thus, DHA inhibits endothelial cell proliferation by suppressing the ERK signaling pathway. The present study strengthened the potential of DHA as an angiogenesis inhibitor for cancer treatment.

Introduction

Angiogenesis refers to the formation of new blood vessels from existing vessels (1). As tissues require blood vessels to supply nutrients and oxygen, angiogenesis is required for solid tumor

growth and metastasis. Tumor angiogenesis is initiated when tumor cells secrete growth factors, such as vascular endothelial growth factor (VEGF), which bind to the receptors on endothelial cells (2,3). Once activated, the endothelial cells go through a process involving proliferation, migration, reorganization of the extracellular matrix, tube formation and maturation, and eventually form a functional vascular plexus (2,4). Endothelial cell proliferation is the key initial step of angiogenesis and is tightly regulated by a number of intersecting pathways (5). The essential components of these signaling pathways may offer targets for anti-angiogenesis therapeutic intervention (6).

Mitogen-activated protein kinase (MAPK) signaling cascades participate in the regulation of numerous fundamental cellular processes (7). In mammals, MAPKs have three major subfamilies: Extracellular signal-regulated kinase (ERK1/2 or p44/42 MAPK), p38 MAPK and c-Jun N-terminal kinase (JNK) (8). The ERK signaling pathway regulates cell proliferation and survival. In endothelial cells, the ERK pathway is initiated by the binding of VEGF or basic fibroblast growth factor (bFGF) to their receptors, which leads to the activation of Ras proteins anchored in the plasma membrane (9). Subsequently, the Ras proteins exchange their bound guanosine diphosphate for guanosine triphosphate, which triggers a conformational change in Ras and activation of Raf. Raf subsequently phosphorylates MAPK/ERK1/2 (MEK1/2), which in turn activates ERK1/2 through phosphorylation of a threonine and a tyrosine residue, such as Thr202/Tyr204 of ERK1 and Thr183/Tyr185 of ERK2. Activated ERK1/2 subsequently phosphorylates the serine/threonine residues of >50 downstream substrates, leading to modification of gene expression profiles and an increase in endothelial cell proliferation and survival (9).

Artemisinin is an antimalarial drug isolated from *Artemisia annua* L. (10). Artemisinin and its derivatives have also been found to inhibit the growth of lung, prostate, breast, colon and pancreatic cancer (11). Dihydroartemisinin (DHA), a semi-synthetic derivative of artemisinin, has exhibited potent anticancer and anti-angiogenesis activities (12). For example, DHA inhibited angiogenesis in a rat embryo development model (13) and a chicken chorioallantoic membrane model (12). Through *in vitro* assays, DHA decreased endothelial cell

Correspondence to: Professor Ju Liu, Laboratory of Microvascular Medicine, Medical Research Center, Shandong Provincial Qianfoshan Hospital, Shandong University, 16766 Jingshi Road, Jinan, Shandong 250014, P.R. China
E-mail: ju.liu@sdu.edu.cn

*Contributed equally

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proliferation, migration and tube formation (14–16). Despite the emergence of DHA as novel component of cancer chemotherapies, the mechanisms of its anti-angiogenic effects have not been fully understood.

Previous studies reported that MAPK signaling cascades are differentially regulated by artemisinin and its derivatives depending on the cell type and experimental settings. Artemisinin upregulates phosphorylation of ERK1/2 and p38 MAPK to promote neurite outgrowth in p12 cells (17). In human monocytic THP-1 cells, artemisinin blocked the phosphorylation of JNK, ERK1/2 and p38 MAPK (18). In HL-60 leukemia cells, DHA induced apoptosis through activation of p38 MAPK, but not JNK or ERK1/2 (19). In endothelial cells cultured with VEGF, DHA failed to increase the activation of p38 pathway (20). Thus far, the effects of the artemisinin family of drugs on the ERK signaling pathway have not been studied in endothelial cells.

In the present study, the role of the ERK signaling pathway in the DHA-induced reduction of endothelial cell proliferation was investigated. At a concentration of 20 μ M, DHA inhibits proliferation of human umbilical vein endothelial cells (HUVECs), and suppresses the expression and phosphorylation of ERK1/2, as well as their downstream effectors, c-Fos and c-Myc. In addition, the ERK pathway inhibitor PD98059 comprises the anti-proliferative effects of DHA. Thus, DHA inhibits endothelial cell proliferation by suppressing the ERK signaling pathway.

Materials and methods

Cell culture. HUVECs were purchased from Lonza (Basel, Switzerland) and were cultured in endothelial basal cell medium-2 supplemented using the EGM-2-MV bullet kit (Lonza) and antibiotics (100 IU/ml penicillin and 100 μ g/ml streptomycin). The cells were cultured in humidified air at 37°C with 5% CO₂. DHA (Sigma-Aldrich, St. Louis, MO, USA) and PD98059 (Cell Signaling Technology, Beverly, MA, USA) were dissolved in dimethyl sulfoxide.

Cell proliferation assay. Cell proliferation was evaluated with the MTT assay kit (Cayman Chemical, Ann Arbor, MI, USA). Briefly, the cells were seeded at a density of 5×10^3 cells/well in a 96-well plate. The following day, DHA (0, 1, 5, 10, 20, 50 and 100 μ M) was added to the culture media. After 24 h, the cells were washed with phosphate-buffered saline (PBS) and MTT solution (10 μ l of 5 mg/ml) was added to each well for 2 h. Formazan crystals were solubilized and colorimetric intensity was analyzed using a 96-well plate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 570 nm.

Cell viability assay. Cell viability was assessed at different time points after DHA treatment. Cultures were washed and incubated in 0.05% trypsin for 2 min at 37°C. Following disaggregation, cell suspensions were diluted 1:1 in 0.4% trypan blue (w/v in 0.9% NaCl) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and the percentage of dye-free cells was calculated.

Annexin V-fluorescein isocyanate (FITC)/propidium iodide (PI) analyses. The apoptosis of HUVECs treated with DHA was detected by Annexin V-FITC and PI staining according

to the manufacturer's instructions (NeoBiosciences, Shenzhen, China). Briefly, cells were trypsinized, pelleted, washed with PBS and resuspended in binding buffer containing Annexin V-FITC (0.25%) and PI (1 μ g/ml). Detection of positive staining from 1×10^5 cells was obtained using a FACSaria II flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using the FACSDiva acquisition and analysis software.

Western blotting. Cells were washed with PBS and lysed in radioimmunoprecipitation assay buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 50 mM NaF, 1% nonidet P-40, 0.1% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 1 μ g/ml leupeptin]. Protein concentrations of cleared lysates were determined using the bicinchoninic assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated by SDS-polyacrylamide gel (8% polyacrylamide gel) and transferred to a polyvinylidene fluoride (PVDF) membrane. PVDF membranes were blocked with 5% skimmed milk and incubated overnight with the primary antibody in PBS-Tween at 4°C. Immunoreactivity was detected with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. The primary antibodies included proliferating cell nuclear antigen (PCNA; PC10), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (D13.14.4E), p44/42 MAPK (ERK1/2) (137F5), c-Fos and c-Myc antibodies (Cell Signaling Technology) and anti- β -actin antibody (Sigma-Aldrich). β -actin levels were used as controls for protein loading.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA isolation and cDNA synthesis were performed using the RNeasy mini kit (Qiagen, Hilden, Germany) and High Capacity RNA-to-cDNA Master mix (Applied Biosystems, Foster City, CA, USA), respectively. RT-qPCR was performed using a ViiA 7 Real-Time PCR system (Life Technologies, Carlsbad, CA, USA). All the PCR reactions were repeated in triplicate. Relative expression was calculated using β -actin as an endogenous internal control. The primer sequences were summarized in Table I.

Electric cell-substrate impedance sensing (ECIS) analysis. The transcellular resistance across a monolayer of endothelial cells was measured using the ECIS technique (ECIS model 1600; Applied BioPhysics, Troy, NY, USA). Briefly, HUVECs were plated in ECIS arrays and allowed to grow into 50% confluence. DHA and/or PD98059 were added to the wells and the resistance across the EC layer was acquired every 8 sec. Data plots are representative of triplicate experiments, with each graph showing impedance readings from a separate well, at 40 distinct electrodes per well.

Results

Dose-response effects of DHA on HUVEC proliferation. Endothelial cell proliferation is an essential process for angiogenesis (6). To examine the effects of DHA on endothelial cell proliferation, the MTT assay was performed for HUVECs treated with different concentrations of DHA. DHA significantly reduced HUVEC growth at a concentration of ≥ 20 μ M

Table I. RT-qPCR primer sequences.

Gene	Sequence	Size, bp	Tm, °C
ERK1 (<i>MAPK3</i>)			
Forward	CTACACGCAGTTGCAGTACAT	157	58.66
Reverse	CAGCAGGATCTGGATCTCCC		59.31
ERK2 (<i>MAPK1</i>)			
Forward	TACACCAACCTCTCGTACATCG	169	59.58
Reverse	CATGTCTGAAGCGCAGTAAGATT		59.38
<i>c-fos</i>			
Forward	TAGTTAGTAGCATGTTGAGCCAGG	333	60.14
Reverse	ACCACCTCAACAATGCATGA		57.71
<i>c-myc</i>			
Forward	AATGAAAAGGCCCCCAAGGTAGTTATCC	112	64.87
Reverse	GTCGTTTCCGCAACAAGTCCTCTTC		64.41
β -actin			
Forward	GGCACCACACCTTCTACAATG	352	59.19
Reverse	GTGGTGGTGAAGCTGTAGCC		60.96

All sequences are in the 5' to 3' orientation. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; bp, base pairs; Tm, temperature; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase.

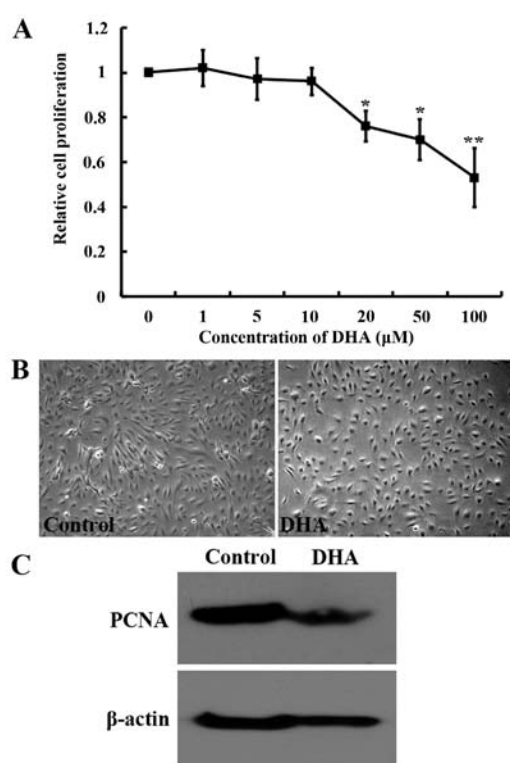


Figure 1. Dihydroartemisinin (DHA) inhibits proliferation of human umbilical vein endothelial cells (HUVECs). (A) MTT assay of HUVECs treated with different concentrations of DHA for 24 h. n=6; * $P<0.05$ and ** $P<0.01$. (B) Representative images of HUVEC cell growth treated with 0 and 20 μ M DHA for 24 h. (C) Immunoblots of proliferating cell nuclear antigen (PCNA) from protein extracts of HUVECs treated with 0 and 20 μ M DHA for 24 h.

after 24 h incubation ($P<0.05$) (Fig. 1A). Treatment with 20 μ M DHA induced a marked reduction of cell density (Fig. 1B) and the expression of PCNA, an essential factor for DNA

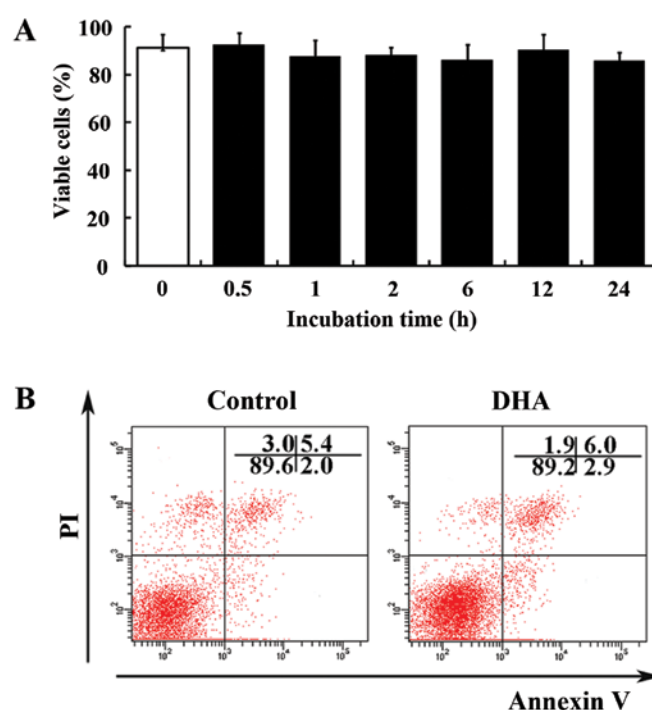


Figure 2. Effects of dihydroartemisinin (DHA) on apoptosis of human umbilical vein endothelial cells (HUVECs). (A) Trypan blue exclusion assay of HUVECs treated with 20 μ M DHA at different time points. (B) Representative image of flow cytometry detection with Annexin V/propidium iodide (PI) double-staining for HUVECs treated with 0 and 20 μ M DHA for 24 h.

replication and repair (Fig. 1C). The effects of DHA on cell viability were evaluated by the trypan blue exclusion assay. At 20 μ M, DHA did not significantly affect the percentage of viable cells <24 h (Fig. 2A). Subsequently, the apoptosis of

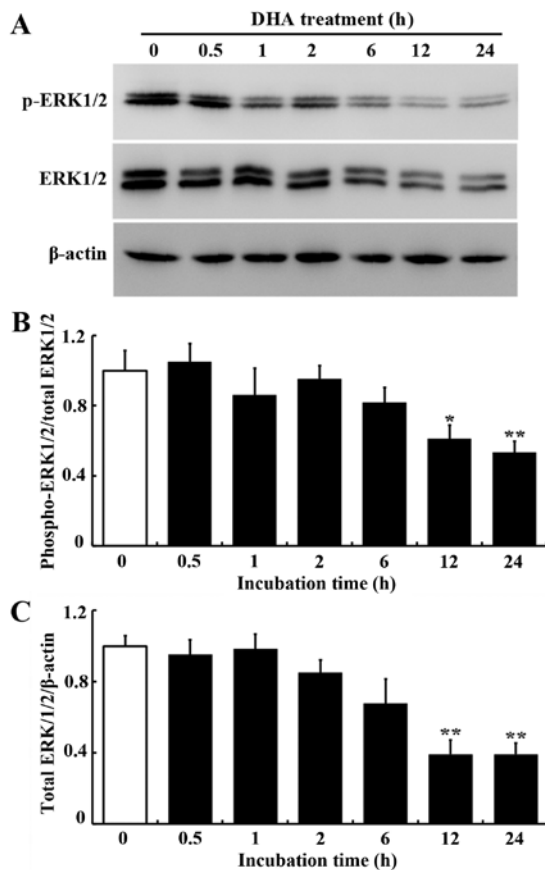


Figure 3. Effects of dihydroartemisinin (DHA) on the activation of extracellular signal-regulated kinase 1/2 (ERK1/2). (A) Representative immunoblots of phospho-ERK1/2, ERK1/2 and β -actin from protein samples of human umbilical vein endothelial cells (HUVECs) treated with DHA at different time points. (B) Densitometry analysis of the phospho-ERK1/2/total ERK1/2 blots. (C) Densitometry analysis of the total ERK1/2/ β -actin blots. $n=3$; * $P<0.05$ and ** $P<0.01$.

HUVECs treated with DHA was examined by double-staining of Annexin V and PI. Flow cytometry analyses showed that the percentage of Annexin V/PI positive cells and viable cells remain unchanged in the absence and presence of 20 μ M DHA (5.4 vs. 6.0%; 89.6 vs. 89.2%) (Fig. 2B). Therefore, at a concentration of 20 μ M, DHA inhibits proliferation without induction of apoptosis. For the following experiments, the concentration of 20 μ M was used to delineate the mechanisms of anti-proliferative effects of DHA.

DHA inhibits ERK1/2 expression and phosphorylation in HUVECs. The ERK pathway mediates cellular responses to growth factors and promotes endothelial cell proliferation (21). The effects of DHA on ERK signaling in endothelial cells were examined by western blot analysis. The protein levels of total and phospho-ERK1/2 were significantly decreased in HUVECs treated with 20 μ M DHA (Fig. 3A). Densitometry analysis showed that the ratios of total ERK1/2/ β -actin and phospho-ERK1/2/total-ERK1/2 were significantly reduced after 12 h incubation with DHA ($P<0.05$, Fig. 3B; $P<0.01$, Fig. 3C), suggesting that ERK1/2 protein expression and phosphorylation were inhibited by DHA. Subsequently, the mRNA expression of ERK1/2 was examined in HUVECs with DHA treatment. ERK1 (*MAPK3*) expression was downregulated at

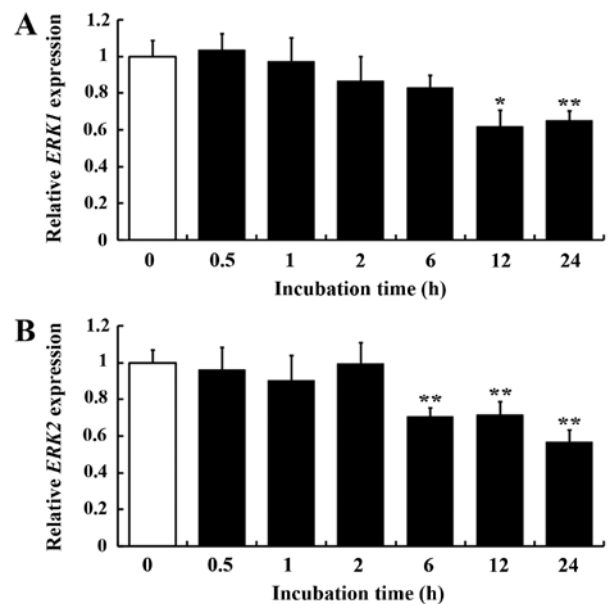


Figure 4. Dihydroartemisinin (DHA) suppresses the transcription of extracellular signal-regulated kinase 1/2 (ERK1/2). (A) Relative *ERK1* mRNA expression in human umbilical vein endothelial cells (HUVECs) treated with DHA by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). $n=4$; * $P<0.05$ and ** $P<0.01$. (B) Relative *ERK2* mRNA expression in HUVECs treated with DHA by RT-qPCR. $n=4$; ** $P<0.01$.

12 h incubation with DHA ($P<0.01$) (Fig. 4A), while ERK2 (*MAPK1*) expression was downregulated as early as 6 h incubation ($P<0.05$) (Fig. 4B). These data indicated that DHA inhibits the ERK signaling pathway in endothelial cells by suppression of ERK1/2 transcription and phosphorylation.

DHA downregulates downstream effectors of ERK1/2 in HUVECs. ERK signaling regulates transcription factors, such as c-Fos and c-Myc, which participate in the regulation of cell proliferation (22). In addition, *c-fos* and *c-myc* are immediate early genes that are expressed rapidly in response to extracellular stimuli (23). The effects of DHA were examined on *c-fos* and *c-myc* expression in HUVECs by RT-qPCR and western blot analysis. The mRNA levels of *c-fos* and *c-myc* were significantly decreased in HUVECs treated with 20 μ M DHA ($P<0.01$, Fig. 5A; $P<0.05$, Fig. 5B). The levels of c-Fos and c-Myc protein were also reduced by DHA treatment (Fig. 5C). Therefore, the downstream effectors of ERK1/2 were down-regulated by DHA.

Inhibitor of the ERK signaling compromises DHA-induced reduction of endothelial cell proliferation. PD98059 is a non-adenosine triphosphate competitive inhibitor that does not directly bind to ERK1 or ERK2, but specifically binds to the inactive forms of MEK1/2 and blocks the phosphorylation by upstream activators, such as Raf (24). As MEK1/2 are the only known direct activators of ERK1/2, PD98059 substantially inhibits ERK signaling. To validate the role of the ERK signaling in mediating the anti-proliferative effects of DHA, HUVECs were pretreated with 10 μ M PD98059 prior to the addition of DHA and after 24 h cell proliferation was examined by the MTT assay. PD98059 alone and the combination of PD98059 and DHA induced a similar reduction of the

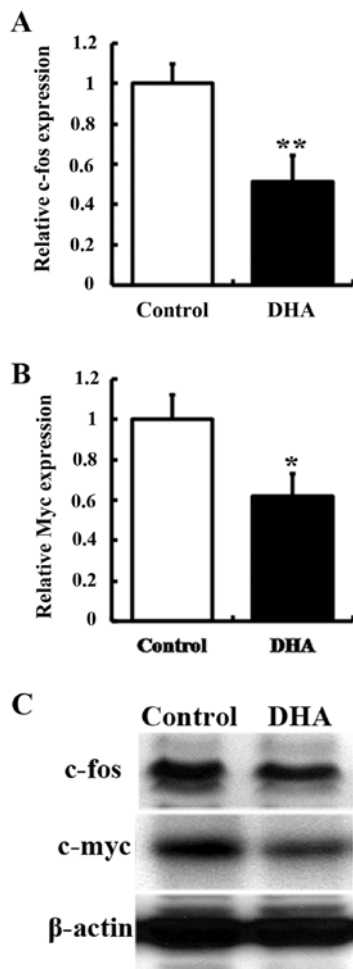


Figure 5. Dihydroartemisinin (DHA) suppresses the expression of c-Fos and c-Myc. (A) Relative *c-fos* mRNA expression in human umbilical vein endothelial cells (HUVECs) treated with 0 and 20 μ M DHA for 24 h. n=4; *P<0.05. (B) Relative *c-myc* mRNA expression in HUVECs treated with 0 and 20 μ M DHA for 24 h. n=4; **P<0.01. (C) Representative immunoblot of c-Fos and c-Myc in DHA treated HUVECs.

cell number (P=0.29) (Fig. 6A). In addition, real-time proliferation assays were performed using the ECIS system. In this setting, electrical resistance was measured over a monolayer of endothelial cells on electrodes, which is proportional to the cell density in the culture plates (25). Consistent with the results from the MTT assay, DHA or PD98059 alone induced a significant reduction in electrical resistance after 16 h of incubation (Fig. 6B). With the pretreatment of PD98059, DHA did not significantly induce additional reduction of electrical resistance <24 h (P=0.34 at 16 h; P=0.13 at 24 h; Fig. 6B and C). Therefore, the anti-proliferative effects of DHA were compromised by PD98059, suggesting that endothelial cell proliferation is suppressed by DHA through the inhibition of the ERK signaling pathway.

Discussion

Tumor angiogenesis is closely associated with tumor aggressiveness, metastasis and patient survival (1). Therefore, disrupting angiogenesis serves as an important strategy for cancer therapy (26). DHA, a more water-soluble metabolite of

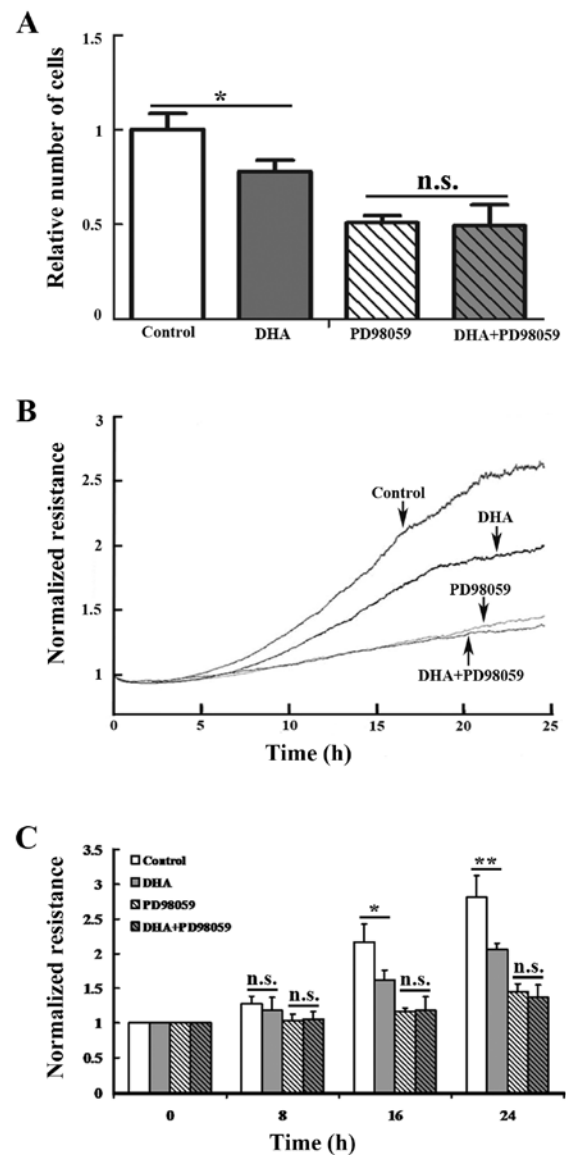


Figure 6. PD98059 compromises the anti-proliferative effects of dihydroartemisinin (DHA). (A) MTT assay of human umbilical vein endothelial cells (HUVECs) treated with DHA and PD98059. n=6; n.s., non-significant; *P<0.05. (B) Real-time resistance of electric cell-substrate impedance sensing (ECIS) measurement of HUVECs treated with DHA and/or PD98059. (C) Bar graph of normalized resistance measured at different time points. n=3; n.s., non-significant; *P<0.05 and **P<0.01.

artemisinin derivatives, has exhibited potent anti-angiogenic activities (11). Since DHA has been widely used as an anti-malarial drug and proved to be safe with minimal side effects, it could be used clinically as a component of cancer chemotherapy (11). In the present study, the molecular mechanisms underlying the anti-angiogenic activity of DHA were explored. The results suggested that the ERK signaling pathway mediates the anti-proliferative effects of DHA on endothelial cells.

A dose-responsive inhibition was demonstrated on HUVEC proliferation by DHA. This is similar to the results from previous studies using other artemisinin derivatives (14,27). A low concentration (20 μ M) of DHA was further defined to significantly inhibit cell proliferation, but does not induce cell death <24 h. Thus, the primary effect

of DHA at this concentration is restricted to inhibition of endothelial cell proliferation. In the time course study, 20 μ M DHA induced a significant reduction of ERK1/2 phosphorylation and expression. It has been reported that DHA prevented phosphorylation of ERK1/2 and other components of MAPK cascades in specific cell types (18,19). As the activation of the Ras-ERK cascade leading to endothelial cell proliferation and survival during angiogenesis (28), DHA may inhibit this cascade to reduce cell proliferation. In addition, DHA also decreases total ERK1/2 and suppresses the mRNA expression of *ERK1/2*. Although ERK1 and ERK2 proteins share a similar sequence and function, they are encoded by distinct genes located at Chr.22q11.2 and Chr.16p11.2, respectively (29-31). The gene expression of ERK1 (*MAPK3*) and ERK2 (*MAPK1*) are likely to be differentially regulated. DHA alters the expression of a variety of genes in the vascular system. In particular, DHA downregulates the transcription of key components of the VEGF/VEGFR2 axis, which promotes angiogenesis (32,33). In addition to inhibiting the activation of the ERK signaling, the present data suggest that downregulation of the ERK1/2 gene expression may also contribute to the anti-angiogenesis effects of DHA.

The proto-oncogenes *c-fos* and *c-myc* are downstream effectors of ERK signaling (22). The induction of the *c-fos* and *c-myc* gene expression is a common response of cells to stimulation of growth factors, including VEGF and bFGF (34,35). In the present study, DHA decreased the mRNA and protein expression of c-Fos and c-Myc. In tumor cells that express high levels of *c-myc*, DHA induces apoptosis and permanently reduces the protein level of c-Myc in these cells (36). In addition, DHA accelerates degradation of c-Myc protein in tumor cells, and forced expression of c-Myc sensitizes these cells to DHA-induced apoptosis (36). The effects of artemisinin and its derivatives on c-Fos have not been studied previously. The present data suggest that DHA suppresses responsive gene expression induced by growth factors, and this suppressive effect may result from the inhibition of ERK signaling.

The ECIS system monitors transendothelial cell electronic resistance and provided an accurate method to examine real-time cell proliferation (25). In this system, application of DHA to the MEK1/2 inhibitor PD98059 pre-treated HUVECs did not induce an additional decrease of cell proliferation. Thus, there was no synergistic or additive effect of DHA and PD98059 in the suppression of endothelial cell proliferation. Activation of the ERK signaling pathway increases cell growth, and numerous extracellular stimuli regulates endothelial cell proliferation through manipulation of this pathway (9). Pre-treatment with PD98059 reduces endothelial cell proliferation induced by adiponectin, ghrelin and adropin (37-39). The present results confirmed the role of ERK signaling in mediating DHA-induced inhibition of endothelial cell proliferation.

In conclusion, the present study demonstrated that DHA suppresses endothelial cell proliferation through inhibition of the ERK signaling pathway, and these findings provided important information for understanding the molecular mechanisms of the anti-angiogenic effects of the artemisinin family of drugs.

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