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Full Length Research Paper

Effect of ursolic acid extracted from Chinese herbs on the proliferation of vascular endothelial cells and its underlying mechanisms

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To investigate the role of ERK1, c-Jun, c-Myc and Cyclin D1 in the suppressive effect of ursolic acid (UA) on the proliferation of human umbilical vein endothelial cells (HUVECs), HUVECs were treated *in vitro* with UA at various concentrations (31.5, 62.5, 125, 250 and 500 µg/ml) for different durations (12, 24 and 48 h) and the proliferation inhibition rate of HUVECs was determined by MTT method. Then, HUVECs were treated with 125 µg/ml UA or 100 µmol/L PD98059 (an ERK inhibitor) for 48 h, or with various concentrations of UA for 24 h; or with 125 µg/ml UA for different durations and the protein and mRNA expressions of ERK1, c-Jun, c-Myc and Cyclin D1 were determined by RT-PCR and Western blot assay, respectively. UA exerted a suppressive effect on the proliferation of HUVECs in a time and concentration dependent manner. The protein and mRNA expressions of ERK1, c-Jun, c-Myc and Cyclin D1 in HUVECs were significantly decreased following treatment with UA and PD98059, which was also in a concentration and time dependent manner. UA can inhibit the proliferation of HUVECs by inhibiting the ERK signaling pathway.

Key words: Ursolic acid, endothelial cells, cell proliferation, extracellular-signal-regulated kinase.

INTRODUCTION

Angiogenesis plays an important role in the development of cancer. The sustainable growth, invasion and metastasis of cancers depend on the angiogenesis. Therefore, to suppress the angiogenesis is crucial for the inhibition of cancer growth (Hayden, 2009; Heath and Bicknell, 2009). Study had shown that the activation of extracellular-signal-regulated kinase (ERK) may promote the angiogenesis (Schäfer et al., 2003). Ursolic acid (UA) is a pentacyclic triterpene acid extracted from Chinese herbs.

UA is present in many plants, including apples, bilberries, cranberries, elder flower, peppermint, lavender, oregano, thyme, hawthorn, prunes. UA belongs to pentacyclic triterpenoids d-amyrin and its relative molecular weight is 456.68. The extensive biological activities of UA have been reported including the suppressive effect on the cancer growth. There is evidence showing that UA can inhibit the proliferation of endothelial cells, but the specific mechanism remains still unclear (Wang et al., 2001). In the present study, the role of ERK signaling pathway in the suppressive effect of UA on the proliferation of endothelial cells was investigated, in order to elucidate the mechanism of suppressive effect of UA on angiogenesis.

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MATERIALS AND METHODS

Cells

Human umbilical vein endothelial cells (HUVECs) were kindly provided by the Department of Pathology of Chongqing Medical University.

Main reagents

UA with purity >98% (Xuhuang Bio-tech Co., Ltd), RPMI1640 (Hyclone), Fetal bovine serum (FBS) (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd), PD98059 (ERK inhibitor) (Promaga), MTT (Amresco), DNA marker (TIANGEN), RNA Isolation Kit (BIOFLUX), RT-PCR Kit (TaKaRa), PRO-PREP™ (Beijing SBS Genetech Co., Ltd), Bradford Protein assay kit (Shanghai Sangon Bio-tech, Co., Ltd), Western Blotting Luminol Reagent, rabbit anti-human c-Jun, ERK1 or Cyclin D1 polyclonal antibodies, mouse anti-human β-actin monoclonal antibody (Santa

Cruz, USA), c-Myc antibody (Chemicon), HRP conjugated goat-anti rabbit IgG and goat anti mouse IgG (Beijing Zhongshan Golden Bridge Bio-tech, Co., Ltd) were used in the present study.

Cell culture

HUVECs were maintained in RPMI1640 containing 20% FBS at 37℃ in an atmosphere with 5% CO_2. Cells in logarithmic phase were used in the following experiments.

Effect of UA on the proliferation of HUVECs

The cell proliferation was determined by MTT assay. In brief, the HUVECs in logarithmic phase were seeded into 96-well plate at a density of 1×10^4/well followed by incubation at 37℃ for 24 h. Then, these cells were independently treated with UA at different concentrations (31.5, 62.5, 125, 250 and 500 μg/ml). In the solvent control group, cells were treated with only equal volume of 75% ethanol, and only medium was included in the blank control group. Five wells were included in each group and experiment was repeated three times. Incubation was performed for 12, 24 or 48 h and then 20 μl of MTT (5 g/L) was added to each well followed by additional incubation for 4 h. The medium was removed and 150 μl of DMSO added followed by gently shaking for 10 min. The absorbance (A) was determined at 570 nm in a microplate reader. The proliferation inhibition rate and half maximal inhibitory concentration (IC_{50}) were calculated.

Proliferation inhibition rate (%) = $(1-A_{UA}/A_{solvent}) \times 100\%$

Effect of UA on the mRNA expressions of ERK1, c-Myc, c-Jun and Cyclin D1

Grouping and treatment

HUVECs in logarithmic phase were collected and maintained for 24 h. The following 3 experiments were carried out: (1) Cells were treated with UA (125 μg/ml) or PD98059 (100 μmol/L) for 48 h. In the control group, cells were maintained in the medium without treatment; (2) Cells were treated with UA at different concentrations (0, 31.5, 62.5, 125, 250 and 500 μg/ml) for 24 h; (3) Cells were treated with 125 μg/ml for different durations (0, 12, 24 and 48 h). Five wells were included in each group.

Design and synthesis of primers

The primers for different target genes were designed according to the corresponding sequences in the Gene bank. The primers were as follows: ERK1: 5'-CTGACGGAGTATGTGGCTAC-3' (forward); 5'-GGGCAGAGACTGTAGGTAGTTTC-3' (reverse), anticipated size of 255 bp; c-Jun: 5'-TGGAAACGACCTTCTATGACGA-3' (forward), 5'-GTTGCTGGACTGGATTATCAGG-3'(reverse), anticipated size of 242 bp; c-Myc: 5'-AGGCGAACACACAACGTCTT-3' (forward), 5'-TTGGACGGACAGGATGTATGC-3' (reverse), anticipated size of 156 bp; Cyclin D1: 5'-GTGCTGCGAAGTGGAAACC-3' (forward), 5'-ATCCAGGTGGCGACGATCT-3' (reverse), anticipated size of 174 bp. The stated primers were synthesized in Beijing SBS Genetech Co., Ltd. The primers for β-actin were as follows: 5'-AACCGCGAGAAGATGACCCAG-3' (forward), 5'-CCACAGGACTCCATGCCCAG-3' (reverse), anticipated size of 467 bp, which were synthesized in Beijing Sunbiotech Co., Ltd. The primers for β-actin were as follows: 5'-TCCTGTGGCATCCACGAAACT-3' (forward), 5'-GTCAAGAAAA GGGTGTAACGCAAC-3' (reverse), anticipated size of 356 bp, which were synthesized in Beijing SBS Genetech Co., Ltd.

Extraction of total RNA

Cells in each group were collected and treated with BIOZOL. Total RNA was extracted by chloroform - isopropyl alcohol. The absorbance of extracted RNA was measured at 260 and 280 nm followed by the calculation of ratio of A_{260} to A_{280}.

RT-PCR

The extracted RNA was used as template and reverse transcribed into cDNA. The cDNA was then applied to amplification by PCR. β-actin served as an internal reference. The mixture (40 μl) for PCR included: 1 μl of cDNA, 0.5 μl of each primer, 10 μl of 5× buffer, 2 μl of $MgCl_2$, 1 μl of dNTP, 0.25 μl of DNA polymerase and sterile distilled water. The conditions for amplification of ERK1 gene were pre-denaturation at 94℃ for 5 min, 35 cycles of denaturation at 94℃ for 30 s, annealing at 62℃ for 30 s and extension at 72℃ for 30 s, followed by a final extension at 72℃ for 1 min. The conditions for amplification of c-Jun gene were pre-denaturation at 94℃ for 5 min, 35 cycles of denaturation at 94℃ for 30 s, annealing at 60℃ for 30 s and extension at 72℃ for 30 s, followed by a final extension at 72℃ for 1 min. The conditions for amplification of c-Myc and Cyclin D1 genes were pre-denaturation at 94℃ for 5 min, 35 cycles of denaturation at 94℃ for 30s, annealing at 59℃ for 30 s and extension at 72℃ for 30 s, followed by a final extension at 72℃ for 1 min. The products were subjected to 1% agarose gel electrophoresis for identification and the optical density of each target gene was determined and normalized by that of β-actin serving as the relative expression of corresponding target gene.

Effect of UA on the protein expressions of ERK1, c-Myc, c-Jun and Cyclin D1

Western blot assay was employed to detect the protein expression. Total protein was extracted and protein concentration was determined by Bradford method. Then, a proper amount of proteins was subjected to SDS-PAGE and transferred onto PVDF membrane followed by blocking in 5 g/ml non-fat milk at 37℃ for 1 h. The membranes were independently treated with rabbit anti-human ERK1 (1:100), c-Myc (1:200), c-Jun (1:100) and Cyclin D1 (1:100) polyconal antibody or mouse anti-human β-actinmonclonal antibody (1:100) in 5% non-fat milk at 4℃ overnight. After washing in TBST, these membranes were incubated with HRP conjugated goat anti-rabbit and goat anti-mouse secondary antibody (1:100) correspondingly at 37℃ for 2 h. Color development was done by enhanced chemiluminescence and representative photographs were captured using a chemiluminescence imaging system (ChemiDocXRS). The optical density and area of each band were determined with Quantity One 4.6.2 software. The optical density and area of each band were normalized by those of β-actin for semi-quantitation.

Statistical analysis

Data were expressed as mean ± standard deviation ($\bar{x} \pm s$). The normality test and homogeneity of variance test were performed firstly. Comparisons among different groups were performed one way or two way analysis of variance. Comparisons intra-group were

Table 1. Proliferation inhibition rate of HUVECs treated with UA at various concentrations (%, $\bar{x} \pm s$, n = 5).

Concentration (μg/ml)	Duration (h)		
	12	24	48
31.5	1.21±0.13	4.73±1.44	15.74±1.78
62.5	5.65±1.04	9.12±0.65	30.79±2.86
125	10.32± 2.01	20.88±0.50	55.90±2.31
250	15.00±2.36	30.79±1.98	78.10±2.60
500	20.31±3.01	40.18±0.96	91.48±0.26

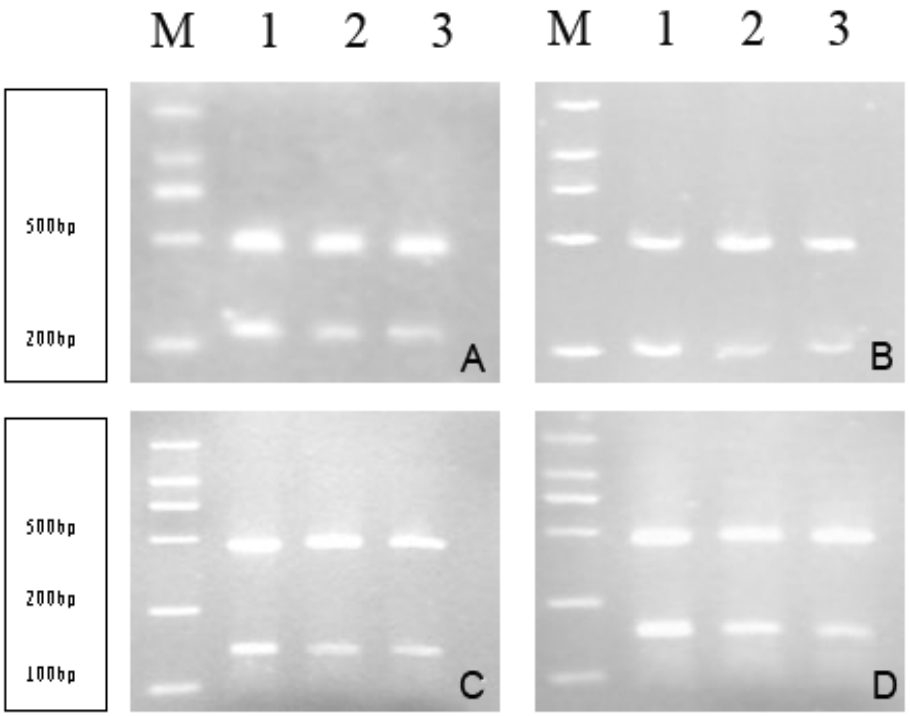

Figure 1. mRNA expressions of ERK1 (A), c-Jun (B), c-Myc (C) and Cyclin D1 (D) in HUVECs treated with 125 μg/ml UA and 100 μmol/L PD98059. M: DNA marker; 1: control group; 2: UA group; 3: PD98059 group.

done with SNK-q test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Effect of UA on the proliferation of HUVECs

Results showed UA at different concentration exerted suppressive effect on the proliferation of HUVECs, which was in a time and concentration dependent manner (Table 1). Significant differences were noted in the proliferation of HUVECs among treatments with different concentrations of UA and for different durations ($F = 6.25$, $P<0.05$; $F = 17.02$, $P<0.01$). The IC_{50} following UA treatment for 44 h was 166 μg/ml. Thus, the UA at 125 μg/ml was used in the following experiments.

Effect of UA on the mRNA expressions of ERK1, c-Myc, c-Jun and Cyclin D1 in HUVECs

In the experiment 1, the HUVECs treated with 125 μg/ml UA or 100 μmol/L PD98059 had significantly decreased mRNA expressions of ERK1, c-Myc, c-Jun and Cyclin D1 when compared with those in control group ($F = 362.92$, 18.19, 23.71 and 33.77, respectively, $P<0.05$ for all). There was no significant difference between cells treated with UA and PD98059 ($q = 2.4360$, 0.2467, 1.1300 and 2.3840, respectively, $P>0.05$ for all) (Figure 1 and Table 2). In the experiment 2, after treatment with UA at

Table 2. mRNA expressions of ERK1, c-Myc, c-Jun and Cyclin D1 in HUVECs treated with 125 μg/ml UA and 100 μmol/L PD98059 (target gene/β-actin, $\bar{x} \pm s$, n = 3).

Group	ERK1	c-Myc	c-Jun	Cyclin D1
Control	0.7482±0.013	0.8673±0.055	0.6629±0.018	0.9496±0.014
UA	0.4490±0.013	0.6838±0.026	0.4501±0.069	0.7335±0.053[a]
PD98059	0.4261±0.022	0.6888±0.040	0.4770±0.004	0.6739±0.046[a]

Note: [a] P<0.05 vs control group.

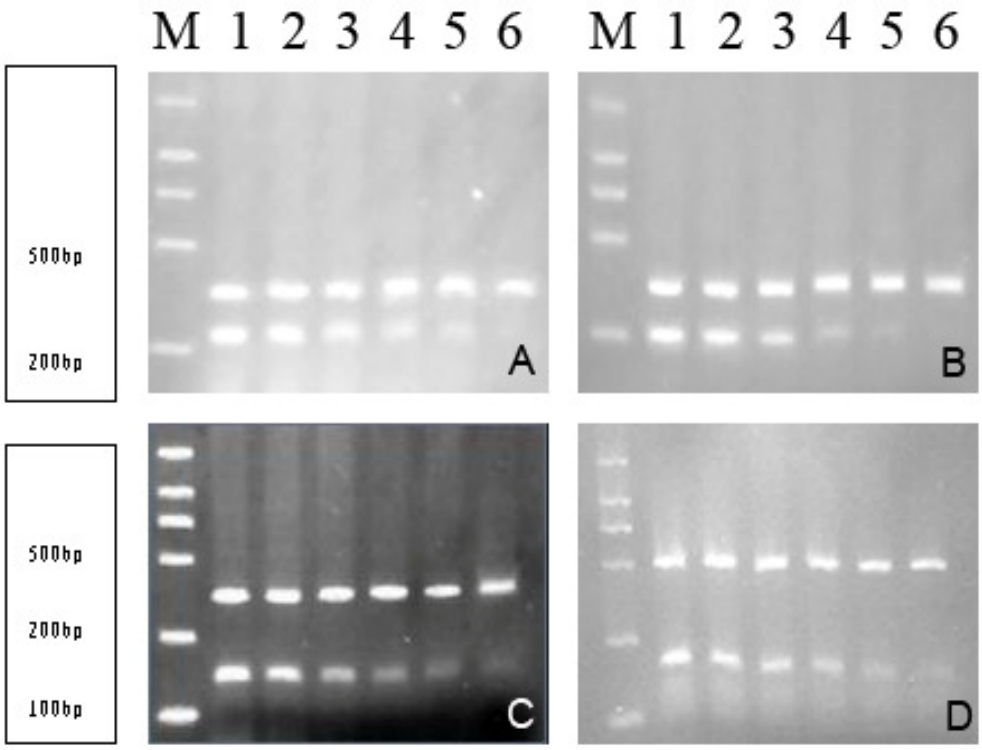

Figure 2. mRNA expressions of ERK1 (A), c-Jun (B), c-Myc (C) and Cyclin D1 (D) in HUVECs treated with UA at various concentrations. M:DNA marker; 1:0, 2:31.5, 3:62.5, 4:125, 5:250 and 6:500 μg/ml.

different concentrations, the mRNA expressions of ERK1, c-Myc, c-Jun and Cyclin D1 concentration-dependently decreased in the HUVECs. The higher the concentration, the lower the expression. UA at 125 μg/ml or higher concentration could significantly decreased the mRNA expressions of these target genes (F = 205.13, 152.00, 177.50 and 82.80, respectively, P<0.05 for all) (Figure 2 and Table 3). In the experiment 3, after treatment with 125 μg/ml UA for 12 h, the mRNA expressions of ERK1, c-Myc, c-Jun and Cyclin D1 decreased with the prolongation of treatment. When compared with control group (0 h), the mRNA expressions of these target genes were markedly decreased following treatment for different durations (F = 420.00, 207.38, 85.36 and 130.28, P<0.05 for all) (Figure 3 and Table 4).

Effect of UA on the protein expressions of ERK1, c-Myc, c-Jun and Cyclin D1 in HUVECs

In the experiment 1, after treatment with 125 μg/ml UA or 100 μmol/L PD98059, the protein expressions of ERK1, c-Myc, c-Jun and Cyclin D1 in HUVECs were marked decreased when compared with control group (F = 43.74, 304.88, 44.27 and 272.33, respectively, P<0.05 for all). However, there was no pronounced difference between UA group and PD98059 group (q = 0.2169, 0.2467, 0.1339 and 2.2940, respectively, P>0.05 for all) (Figure 4

Table 3. mRNA expressions of ERK1, C-Myc, C-Jun and Cyclin D1 in HUVECs treated with UA at various concentrations (target gene/β-actin, $\bar{x} \pm s$, n = 3).

UA (μg/ml)	ERK1	c-Myc	c-Jun	Cyclin D1
0	0.8886±0.029	0.9653±0.032	0.9647±0.000	0.8344±0.044
31.5	0.8281±0.031	0.9296±0.062	0.9188±0.003	0.7983±0.036
62.5	0.7819±0.049	0.8968±0.035	0.8833±0.022	0.7254±0.061
125	0.5436±0.030	0.6406±0.008	0.5699±0.045	0.5443±0.061[a]
250	0.4132±0.013	0.5563±0.006	0.5300±0.008	0.4178±0.079[a]
500	0.1492±0.008	0.4240±0.010	0.4890±0.008	0.3112±0.021[a]

Note: [a] $P<0.05$ vs 0 μg/ml UA.

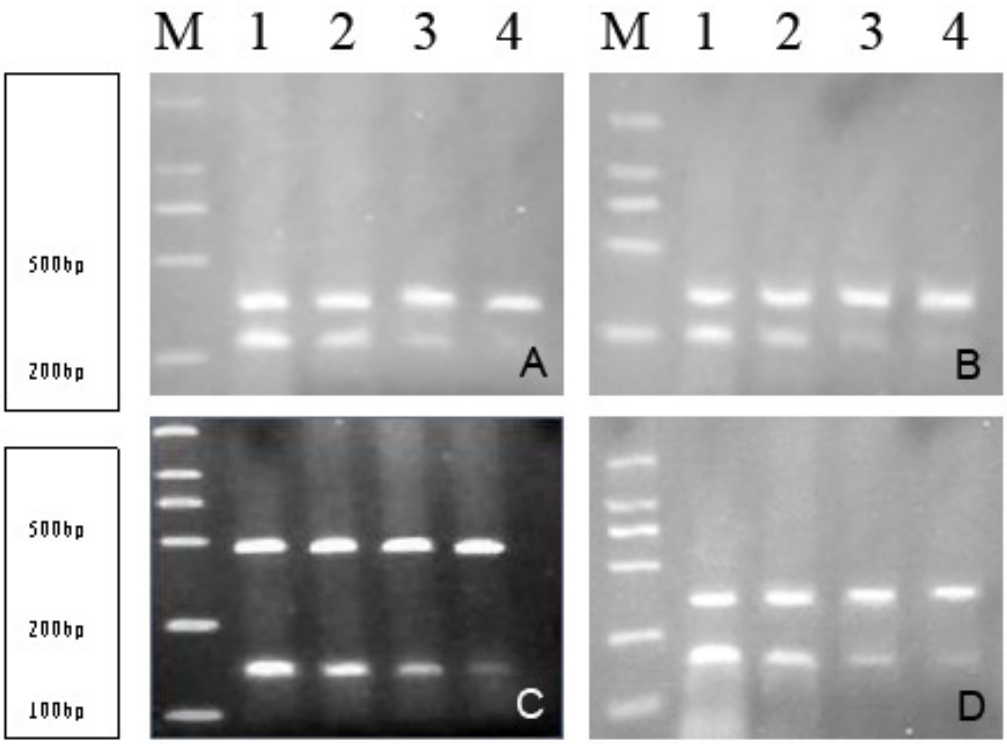

Figure 3. mRNA expressions of ERK1 (A), c-Jun (B), c-Myc (C) and Cyclin D1 (D) in HUVECs treated with 125 μg/ml UA for different durations. M: DNA marker; 1.0, 2.12, 3.24, 4.48 h.

and Table 5). In the experiment 2, after treatment with UA at different concentrations, the protein expressions of ERK1, c-Myc, c-Jun and Cyclin D1 in HUVECs were lower than those before treatment. The higher the UA concentration, the lower the protein expression. UA at 125 μg/ml or higher concentrations could significantly decrease the protein expressions of target genes (F = 70.89, 345.93, 201.78 and 510.45, $P<0.05$) (Figure 5 and Table 6). In the experiment 3, after 125 μg/ml UA treatment for 12 h, the protein expressions of ERK1, c-Myc, c-Jun and Cyclin D1 decreased with the prolongation of treatment. The protein expressions of target genes were dramatically decreased when compared with those in control group (0 h) (F = 34.11, 153.00, 235.60 and 444.37, respectively, $P<0.05$ for all) (Figure 6 and Table 7).

DISCUSSION

Angiogenesis is crucial for the growth and metastasis of cancer. To date, various inhibitors of angiogenesis including protamine, interferon and platelet factor 4 have been developed. However, these inhibitors are cytotoxic, which significantly limits the wide application of these inhibitors. UA is an efficient and low-toxic herb-derived

Table 4. mRNA expressions of ERK1, c-Myc, c-Jun and Cyclin D1 in HUVECs treated with 125 μg/ml UA for different durations (target gene/β-actin, $\bar{x} \pm s$, n = 3).

Duration (h)	ERK1	c-Myc	c-Jun	Cyclin D1
0	0.8966±0.051	0.8495±0.026	0.8838±0.003	1.0215±0.006
12	0.6530±0.017	0.7024±0.023	0.5609±0.032	0.7323±0.045[a]
24	0.4005±0.008	0.5027±0.014	0.4815±0.012	0.5391±0.027[a]
48	0.0191±0.006	0.4511±0.019	0.4815±0.013	0.4531±0.055[a]

Note: [a] $P < 0.05$ vs 0 h.

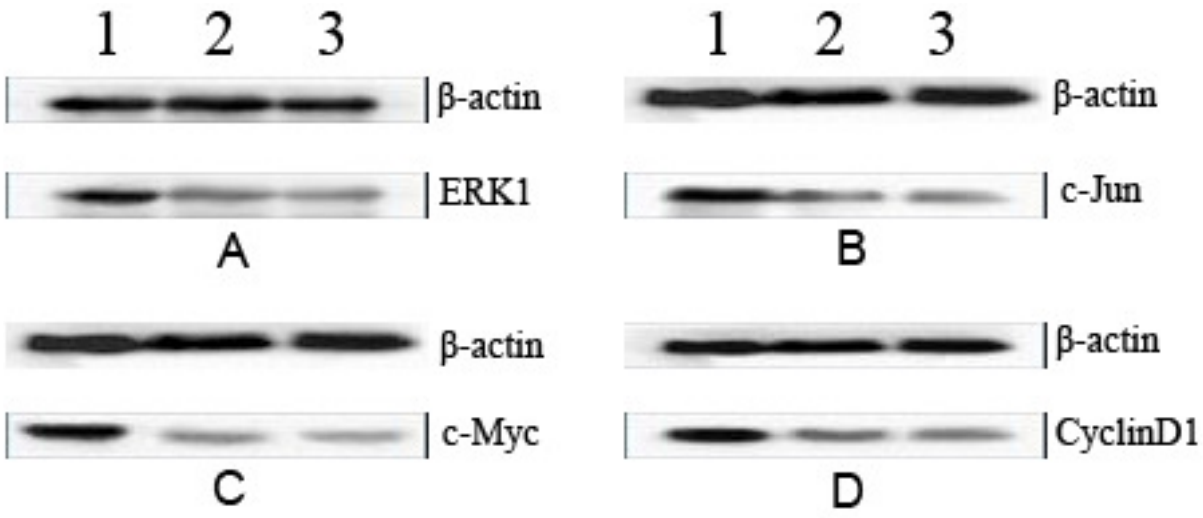

Figure 4. Protein expressions of ERK1 (A), c-Jun (B), c-Myc (C) and Cyclin D1 (D) in HUVECs treated with 125 μg/ml UA and 100 μmol/L PD98059. 1: control group; 2: UA group; 3: PD98059 group.

Table 5. Protein expressions of ERK1, c-Myc, c-Jun and Cyclin D1 in HUVECs treated with 125 μg/ml UA and 100 μmol/L PD98059 (target protein/β-actin, $\bar{x} \pm s$, n = 3).

Group	ERK1	C-Myc	C-Jun	Cyclin D1
control	0.9603±0.066	0.8027±0.018	0.8145±0.015	0.8421±0.007
UA	0.5768±0.062	0.4718±0.021	0.5369±0.053	0.5069±0.022[a]
PD98059	0.5840±0.040	0.4159±0.023	0.5401±0.020	0.4788±0.029[a]

drug and has a wide variety of applications and promising perspectives. The available evidence reveals that UA may become a novel anti-tumor drug. In recent years, treatment targeting the angiogenesis has been a hot topic in cancer research. To date, ERK, Notch and PI3K-AKT signaling pathways have been confirmed to involve in the regulation of angiogenesis in cancers. ERK signaling pathway is a predominant one and can stimulate the proliferation of vascular endothelial cells (VECs) in cancers (Lang et al., 2008; Kawasaki et al., 2008; Zhang et al., 2010). ERK belongs to MAPK family and ERK signaling pathway involves the signal transduction networks related to growth, development and division of numerous types of cells. The ERK signaling pathway consists of vascular endothelial growth factor (VEGF), VEGF receptor (KDR), Ras, Raf, MEK, ERK1/2 and nuclear transcription factors (c-myc, c-jun, Cyclin D1, etc). Wang et al (2001) found that UA could significantly inhibit the angiogenesis of VECs in logarithmic phase, which was positively related to the UA concentrations. UA can suppress the angiogenesis at different stages including VEC proliferation, migration and new blood vessel tube

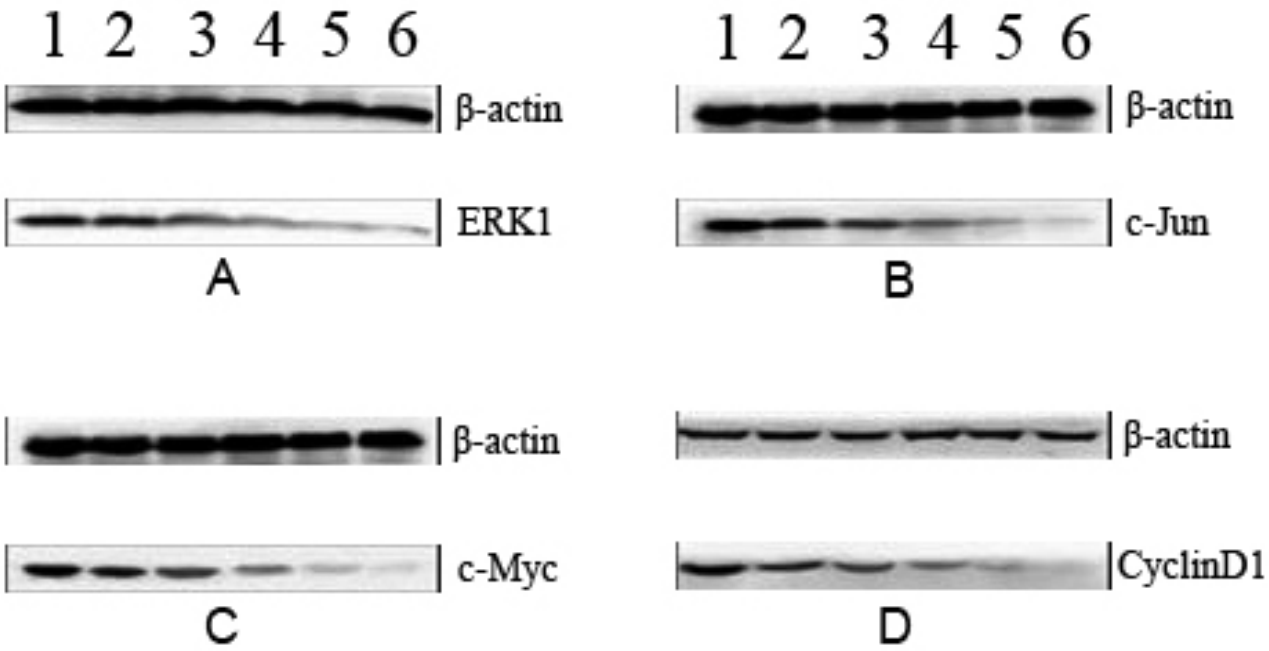

Figure 5. Protein expressions of ERK1 (A), c-Jun (B), c-Myc (C) and Cyclin D1 (D) in HUVECs treated with UA at various concentrations. 1:0, 2:31.5, 3:62.5, 4:125, 5: 250, 6: 500 μg/ml.

Table 6. Protein expressions of ERK1, c-Myc, c-Jun and Cyclin D1 in HUVECs treated with UA at various concentrations (target protein/β-actin, $\bar{x} \pm s$, n = 3).

UA (μg/ml)	ERK1	C-Myc	C-Jun	Cyclin D1
0	0.9116±0.023	0.8868±0.029	0.8168±0.052	0.8296±0.024
31.5	0.8373±0.015	0.7189±0.038	0.6533±0.041	0.6497±0.021
62.5	0.6683±0.090	0.6569±0.043	0.5619±0.043	0.5234±0.023
125	0.4610±0.109	0.4427±0.026	0.2874±0.024	0.3387±0.034[a]
250	0.2520±0.059	0.2144±0.008	0.1876±0.012	0.1845±0.014[a]
500	0.1494±0.024	0.1071±0.006	0.1018±0.007	0.0672±0.005[a]

Note: [a] $P < 0.05$ vs 0 μg/ml UA.

formation. Sohn et al. (1995) conducted chicken chorioallantoic membrane assay (CAM) and results also displayed potent suppressive effect of UA on the angiogenesis, which was in a concentration dependent manner. However, the potential mechanism underlying this suppressive effect of UA is still poorly understood.

The present study preliminarily investigated the mechanism underlying the suppressive effect of UA on the proliferation of VECs. The suppressed *in vivo* growth of glioma in nude mice and the potential mechanism were explored in our previous study (Liu et al., 2009). PD98059 is a specific ERK inhibitor and can selectively inhibit the ERK activity (Mijatovic et al., 2005). Our results showed that HUVECs treated with UA or PD98059 had markedly decreased mRNA and protein expressions of ERK1, c-Myc, c-Jun and Cyclin D1 when compared with control group ($P<0.05$). This suggests that both PD98059 and UA can inhibit the ERK signaling pathway in HUVECs, which is consistent with previously reports (Mijatovic et al., 2005; Huang et al., 2007). Huang et al (2007) investigated the effect of Tumstatin on the proliferation of microvascular endothelial cells of retina. Their results revealed that the inhibition of ERK1/2 signal pathway was accompanied by the suppressed proliferation of endothelial cells. Our results also demonstrated that the protein and mRNA expressions of ERK1, c-Myc, c-Jun and Cyclin D1 were remarkably decreased after treatment with UA at different concentrations or for different durations. The higher the UA concentration and the longer the treatment time, the

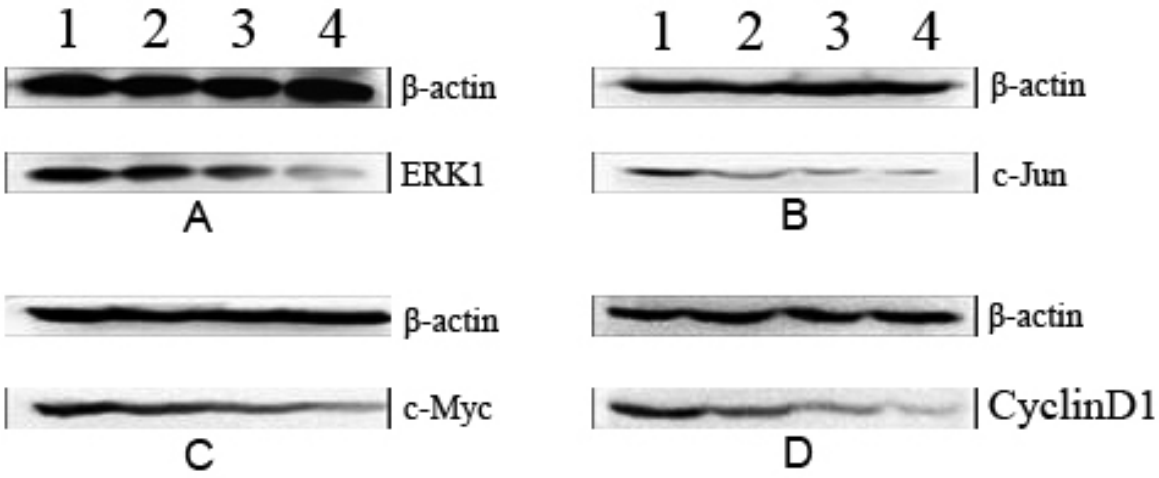

Figure 6. Protein expressions of ERK1 (A), c-Jun (B), c-Myc (C) and Cyclin D1 (D) in HUVECs treated with 125 μg/ml UA for different durations. 1:0, 2:12, 3:24, 4:48 h.

lower the expressions of these target genes. These findings suggest that UA can inhibit the ERK signaling pathway in HUVECs in a time and concentration dependent manner. Taken together, we speculate that inhibition of ERK signaling pathway in VECs is one of mechanisms underlying the suppressive effect of UA on angiogenesis and this effect is time and concentration dependent.

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