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## 抗血管增生藥物的研發與其分子機轉的探討

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# Anti-proliferation effect of 5,5-diphenyl-2-thiohydantoin (DPTH) in human vascular endothelial cells

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**Abstract**

The aim of this study was to examine the anti-proliferation effect of 5,5-diphenyl-2-thiohydantoin (DPTH), an analogue of antiepileptic drug phenytoin (5,5-diphenylhydantoin), on human umbilical vein endothelial cells (HUVEC) and its possible molecular mechanism underlying. Here we demonstrated that DPTH at a range of concentrations (12.5–50 μM) dose- and time-dependently inhibited DNA synthesis and decreased cell number in cultured HUVEC, but not human fibroblasts. DPTH was not cytotoxic at these concentrations. [$^3$H]Thymidine incorporation and flow cytometry analyses demonstrated that treatment of HUVEC with DPTH arrested the cell at the G0/G1 phase of the cell cycle. Western blot analysis revealed that the protein level of p21 increased after DPTH treated. In contrast, the protein levels of p27, p53, cyclins A, D1, D3 and E, cyclin-dependent kinase (CDK)2, and CDK4 in HUVEC were not changed significantly after DPTH treatment. Immunoprecipitation showed that the formations of the CDK2–p21 and CDK4–p21 complex, but not the CDK2–p27 and CDK4–p27 complex, were increased in the DPTH-treated HUVEC. Kinase assay further demonstrated that both CDK2 and CDK4 kinase activities were decreased in the DPTH-treated HUVEC. Pretreatment of HUVEC with a p21 antisense oligonucleotide reversed the DPTH-induced inhibition of [$^3$H]thymidine incorporation into HUVEC. In conclusion, these data suggest that DPTH inhibits HUVEC proliferation by increasing the level of p21 protein, which in turn inhibits CDK2 and CDK4 kinase activities, and finally interrupts the cell cycle. The findings from the present study suggest that DPTH might have the potential to inhibit the occurrence of angiogenesis.


*Keywords:* DPTH; Angiogenesis; p21; p53; Cyclin-dependent kinase; Endothelial cells

## 1. Introduction

Angiogenesis, the formation of new capillary blood vessels as extensions of existing vessels, is a complex process regulated by multiple stimulatory and inhibitory factors [1]. In the physiological condition, the activity of stimulators and inhibitors of angiogenesis maintains it in balance. However, persistent and unregulated angiogenesis is often found to be a critical causal factor in many pathological conditions [2].

The events that are thought to be essential for angiogenesis include: local degradation of the basement membrane of the parent vessel, allowing protrusion of endothelial cells; outward migration of endothelial cells in tandem to form a capillary sprout; proliferation of endothelial cells within the sprout; and the formation of a lumen with subsequent branching. Angiogenesis is essential for many physiological processes and important in the pathogenesis of many disorders [1]. Normally, vascular proliferation occurs only during embryonic development, the female reproductive cycle and wound healing. In contrast, many

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*Abbreviations:* DPTH, 5,5-diphenyl-2-thiohydantoin; DPH, 5,5-diphenylhydantoin; HUVEC, human umbilical vein endothelial cells; HDMVEC, human dermal microvascular endothelial cells; CDK, cyclin-dependent kinase; ECGS, endothelial cell growth supplement; M199, medium 199; FBS, fetal bovine serum; CKI, CDK-inhibitory protein; FACS, fluorescence-activated cell sorter.

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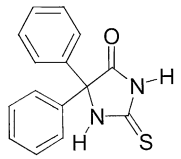


5,5-diphenyl-2-thiohydantoin (DPTH)

Fig. 1. Chemical structure of DPTH.

pathological conditions (e.g. atherosclerosis, cancer, and diabetic retinopathy) are characterized by persistent, unregulated angiogenesis [2]. Control of vascular development could permit new therapeutic approaches to these disorders. During the past few years, experimental and clinical investigators continue to search for new therapeutic strategies for preventing the occurrence of angiogenesis. One approach, as pursued in this study, seeks to identify medicinal agents capable of retarding the cell cycle in the vascular endothelial cells.

DPTH (Fig. 1), an analogue of antiepileptic drug phenytoin (5,5-diphenylhydantoin, DPH), has been suggested to be a potential hypolipidemic agent [3] and a potent goitrogenic compound [4]. Structural difference is that 2-oxo functionality of DPH is replaced as 2-thio of DPTH, but their biological activity is very different. DPTH is inactive as an anti-convulsant, and its prominent activities were reported as being inhibitory against thyroxine-stimulated response in mitochondria and being hypolipidemic [5]. As far as anti-angiogenic activity is concerned, DPTH has not been assessed yet.

This study was designed to investigate the inhibitory effect of DPTH in HUVEC proliferation and its molecular mechanism underlying. Here, we demonstrate that DPTH dose-dependently inhibited the growth of HUVEC by interrupting the transition of cell cycle from the G1 into S phase. The DPTH-induced cell cycle arrest in HUVEC occurred when the CDK2 and CDK4 activities were inhibited just as the level of p21 protein increased.

## 2. Materials and methods

### 2.1. Materials

DPTH was synthesized as previously described [6]. Briefly, a mixture of benzil (2.1 g, 10 mmol) and thiourea (0.8 g, 10.5 mmol) in 95% ethanol (15 mL) was added 8 M aqueous KOH (3 mL) and the whole was heated for 2.5 hr and then cooled. Thin layer chromatography ($SiO_2$, $CH_2Cl_2$) showed the disappearance of benzil. Concentrated HCl was added to acidify the mixture at 0° and the resulted precipitate was collected, washed with water and then dried. Recrystallization with alcohol gave 2.53 g (94%) yield, mp 237–239° (Lit. 239–240°). HEPES, glycerol, phenylmethylsulphonyl fluoride (PMSF), SDS, Nonident P-40 (NP-40), and endothelial cell growth supplement (ECGS) were purchased from Sigma Chem. Medium 199 (M199), trypsin-EDTA, and kanamycin were purchased from Life Technologies. Fetal bovine serum (FBS) was purchased from HyClone. Antibodies specific for cyclins, CDKs, and CKIs were purchased from Transduction Laboratories. An antibody specific for G3PDH was purchased from Biogenesis. Anti-mouse IgG conjugated alkaline phosphatase was purchased from Jackson Immuno Research Laboratories. 4-Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were purchased from Kirkegaard & Perry Laboratories. Protein assay agents were purchased from Bio-Rad.

### 2.2. Cell culture

HUVEC or human dermal microvascular endothelial cells (HDMVEC) were grown in M199 containing 10% FBS, ECGS (0.03 mg/mL) and kanamycin (50 U/mL) in a humidified 37° incubator. After the cells had grown to confluence, they were disaggregated in trypsin solution, washed with M199 containing 10% FBS, centrifuged at 125 *g* for 5 min, resuspended, and then subcultured according to standard protocols. Cells from passages 5 to 9 were used.

### 2.3. [$^3$H]Thymidine incorporation

The [$^3$H]thymidine incorporation was performed as previously described [7,8]. Briefly, HUVEC were applied to 24-well plates in growth medium (M199 plus 10% FBS and ECGS). After the cells had grown to 70–80% confluence, they were rendered quiescent by incubation for 24 hr in M199 containing 2% FBS. M199 supplemented with 10% FBS and 0.05% DMSO (control) or various concentrations of DPTH was added to the cells and the cultures were allowed to incubate for 21 hr. During the last 2 hr of the incubation with or without DPTH, [$^3$H]thymidine was added at 1 μCi/mL (1 μCi = 37 kBq). Incorporated [$^3$H]thymi- N NaOH and measured in a liquid scintillation counter.

### 2.4. Cell counting

As a measurement of cell proliferation, the cells were seeded onto 6-well 1% gelatin-coated plates and grown in M199 supplemented with 10% FBS and ECGS. Media without (control) and with DPTH were changed daily until cell counting. At various times of incubation, cultures were treated with trypsin-EDTA and the released cells were counted in a Coulter apparatus.

### 2.5. Viability assay

Cell viability was estimated by a modified MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay as previously described [8]. Four samples were analyzed in each experiment.

### 2.6. Protein preparation and Western blotting

To determine the expression levels of cyclins, CDKs, CKIs, and G3PDH in HUVEC, the total proteins were extracted and Western blot analyses were performed as described previously [8,9]. Briefly, HUVEC were cultured in 10 cm petri dishes. After reaching subconfluence, the cells were rendered quiescent and then treated with various concentrations of DPTH for 21 hr, and then incubated in a humidified incubator at 37°. After incubation, the cells were washed with PBS (pH 7.4), incubated with extraction buffer (Tris 50 mM, pH 7.5, NaCl 150 mM, PMSF 1 mM, NP-40 1%, 0.1% SDS, 10 μg/mL Aprotinin and EDTA 10 mM) on ice, and then centrifuged at 12,000 *g* for 30 min. The cell extract was then boiled in a ratio of 3:1 with sample buffer (Tris–HCl 250 mM, pH 6.8, glycerol 40%, β-mercaptoethanol 20%, SDS 8% and bromophenol blue 0.04%). Electrophoresis was performed using 12% SDS-polyacrylamide gel (2 hr, 110 V, 40 mA, 50 μg protein per lane). Separated proteins were transferred to PVDF membranes (1 hr, 400 mA), treated with 5% fat-free milk powder to block the nonspecific IgGs, and incubated for 1 hr with specific antibody for cyclins, CDKs, CKIs, or G3PDH. The blot was then incubated with anti-mouse or anti-rabbit IgG linked to alkaline phosphatase (1:1000) for 1 hr. Subsequently, the membrane was developed with NBT/BCIP as a substrate.

### 2.7. RNA extraction and RT–PCR

Total RNA was isolated from cultured HUVEC and prepared by guanidinium isothiocyanate extraction and centrifugation through cesium chloride. The mRNA levels were detected by reverse transcription–polymerase chain reaction (RT–PCR) technique as previously described [9]. The p21 cDNA amplication was performed by incubating 20 ng equivalents of cDNA in 100 mM Tris–HCl buffer, pH 8.3, containing 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin, 200 μM concentration of each dNTP, and 50 U/mL Super Taq DNA polymerase with specific oligonucleotide primers: 5′-AGGAGGCCCGTGAGCGAGCGATGGAAC-3′ and 5′-ACAAGTGGGGAGGAAGTAGC-3′. The cDNA sequence of GAPDH was also amplified as a control in the same method using the following primers: 5′-CCACCCATGGCAAATTCCATGGCA-3′ and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. PCR products were analyzed on 1.0% agarose gels. Thermal cycle conditions were as follows: 1 cycle at 94° for 5 min, 30 cycles at 94° for 1 min (for p21) or 45 s (for GAPDH), 55° for 1 min (for $p21^{Cip1}$) or 59° (for GAPDH) for 45 s, 72° for 2 min (for p21) or 1 min (for GAPDH), and 1 cycle at 72° for 10 min. PCR products were analyzed on 1.8% agarose gels.

### 2.8. Immunoprecipitation

As previously described [8], CDK2 or CDK4 was immunoprecipitated from 200 μg of protein by using anti-CDK2 or anti-CDK4 antibody (2 μg/mL) and protein A agarose beads (1/10 V). The precipitates were washed five times with washing buffer, and then resuspended in sample buffer (250 mM Tris–HCl, pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, and 0.04% bromophenol blue) and incubated at 95° for 10 min before electrophoresis to release the proteins from the beads.

### 2.9. CDK kinase assay

As previously described [8], CDK2 or CDK4 immunoprecipitates from DPTH-treated and control HUVEC were washed three times with lysis buffer and twice with kinase assay buffer [50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, and 1 mM dithiothreitol (DTT)]. Phosphorylation of histone H1 (for CDK2) and glutathione-*S*-transferase/retinoblastoma (Gst-Rb) fusion protein (for CDK4) were measured by incubating the beads with 40 μL of "hot" kinase solution [0.25 μL (2.5 μg) histone H1, 0.5 μL [$\gamma$-$^{32}$P]ATP (10 mCi/mL), 0.5 μL 0.1 mM ATP, and 38.75 μL kinase buffer] at 37° for 30 min. The reaction was stopped by boiling the sample in SDS sample buffer for 5 min. The products were analyzed by 10% SDS–PAGE. The gel was dried and visualized by autoradiography.

### 2.10. Flow cytometry

As previously described [10], the cells were seeded onto 10-cm petri dishes and grown in M199 supplemented with 10% FBS and ECGS. After the cells had grown to subconfluence, they were rendered quiescent and challenged with 10% FBS. Then, after release using trypsin-EDTA, they were washed twice with PBS and fixed in 70% ethanol at 4°. Nuclear DNA was stained with a reagent containing propidium iodine (8 μg/mL) and DNase-free RNase (100 μg/mL) and measured using a fluorescence-activated cell sorter (FACS).

### 2.11. Statistical analysis

Values represent the means ± SEM. Three to four samples were analyzed in each experiment. Comparisons were subjected to one-way ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$.

## 3. Results

### 3.1. Inhibition of [$^3$H]thymidine incorporation and cell proliferation in HUVEC by DPTH

To study the anti-proliferative effect of DPTH on the vascular endothelial cells, we examined changes in [$^3$H]thymidine incorporation (a measurement of DNA synthesis) in response to DPTH treatment in subcultured

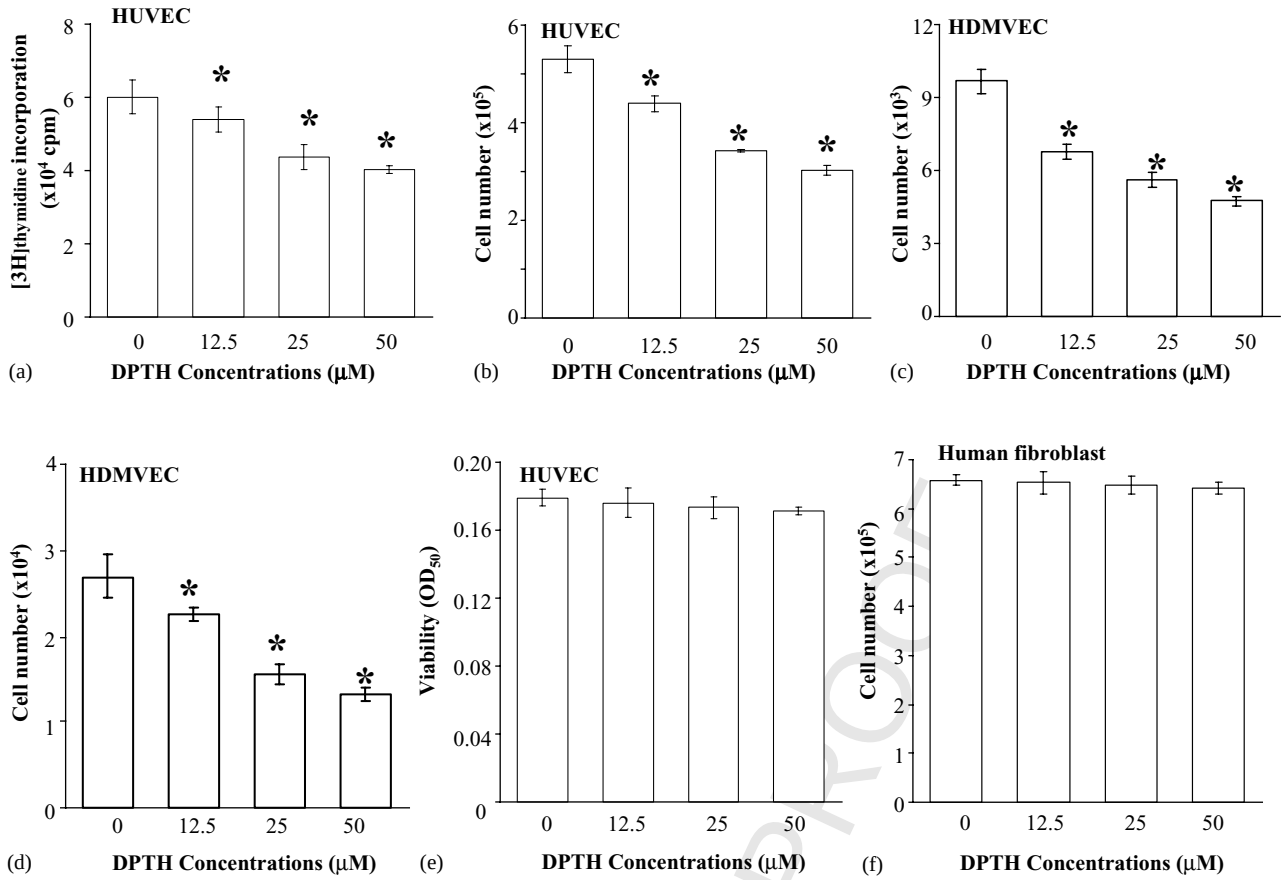


Fig. 2. Effects of DPTH on [$^3$H]thymidine incorporation and cell growth rate in subcultured HUVEC. (a) Dose-dependent inhibition of [$^3$H]thymidine incorporation in HUVEC by DPTH. (b and c) Dose-dependent inhibition of vascular endothelial cell growth by DPTH treatment. The HUVEC (b) or HDMVEC (c) were treated with DMSO without or with various doses of DPTH for 3 days, and then the cells were harvested and counted. (d) Continuous presence of DPTH is not necessary for its anti-proliferation effect in vascular endothelial cells. The HDMVEC were treated with DMSO without or with various doses of DPTH for 1 day, and then counted the number of cells after 6 days. A dose-dependent inhibition of cell number was still observed. (e) There was no significant difference in viability between control and DPTH-treated HUVEC. (f) Treatment with DPTH (12.5–50 μM) for 3 days did not affect cell growth of human fibroblasts. Three to four samples were analyzed in each group, and values represent the means ± SEM. Significance was accepted at $P < 0.05$. (∗) DPTH-treated group different from control group.

HUVEC. As illustrated in Fig. 2a, treatment of HUVEC with DPTH (12.5–50 μM) for 21 hr induced a decrease in [$^3$H]thymidine incorporation in a dose-dependent manner. We further examined the effect of DPTH on cell number of HUVEC. In the experiment of Fig. 2b, HUVEC were cultured for 3 days with or without DPTH (12.5–50 μM), and then the cells were harvested and counted. These data showed that a reduced cell count was observed in the DPTH-treated HUVEC as compared with DMSO-treated cells. This DPTH-induced decrease in the cell growth rate was dose-dependent, consistent with the inhibitory effect of DPTH on [$^3$H]thymidine incorporation. The DPTH-induced decrease in the cell growth rate was also observed in the other endothelial cells, HDMVEC (Fig. 2c). In contrast, treatment of human fibroblasts with DPTH (12.5–50 μM) for 3 days did not cause any significant growth inhibition (Fig. 2f), suggesting the specific effect of DPTH on HUVEC growth inhibition. However, the continuous presence of DPTH is not necessary for its anti-proliferation effect in vascular endothelial cells. As illustrated in Fig. 2d, the HDMVEC were treated with DPTH for 1 day and then counted the number of cells after 6 days. A dose-dependent inhibition of cell number was still observed. The DPTH-induced reduction in [$^3$H]thymidine incorporation may be due to retardation of cell cycle or cell death. To confirm that the results of our studies of DNA synthesis and cellular proliferation in HUVEC were not due to cell death caused by DPTH treatment, we conducted viability assay by treating the cells with DPTH for 21 hr at the doses (12.5–50 μM) used in the studies of cell growth inhibition. MTT assays indicated that there was no significant difference in cell viability between control and DPTH-treated HUVEC (Fig. 2e), suggesting that there was an inhibitory effect of DPTH on the mechanisms for cell division in the subcultured HUVEC.

### *3.2. Arrest of cell cycle in G0/G1phase by DPTH treatment*

To further investigate the cellular mechanism of the DPTH-induced growth inhibition, FACS analyses of DNA content in both DMSO- and DPTH-treated HUVEC

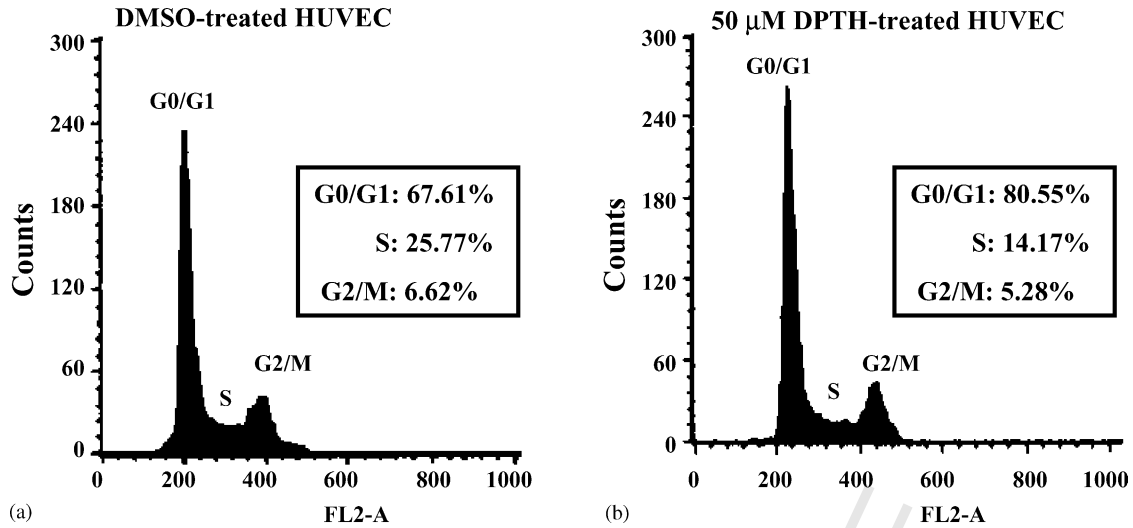


Fig. 3. Retardation of cell cycle in HUVEC by DPTH. FACS analysis of DNA content was performed after 24 hr release from quiescence by incubation in culture media supplemented with 10% FBS and 0.05% DMSO without (a) or with 50 μM DPTH (b). Results from a representative experiment are shown. Percentage of cells at the G0/G1, S, or G2/M phase of the cell cycle was determined using established CellFIT DNA analysis software.

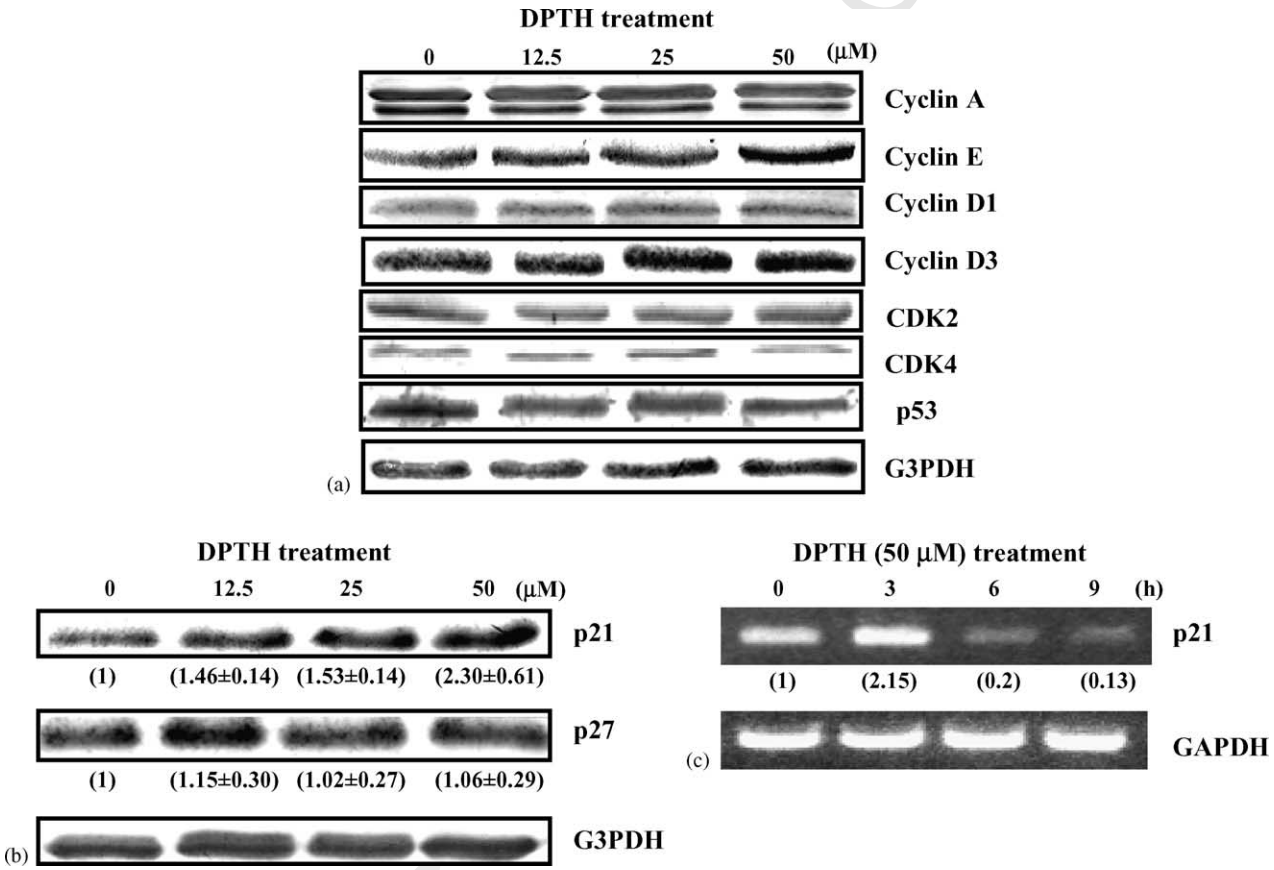


Fig. 4. Effect of DPTH on the protein levels of cyclins, CDKs and CKIs. Western blot analysis was performed to examine the changes of protein levels of cyclins, CDKs, and CKIs in the DPTH-treated HUVEC. Proteins were extracted from the cultured HUVEC at 21 hr after DPTH treatment and probed with proper dilutions of specific antibodies. (a) DPTH (12.5–50 μM) did not induce any significant change of the protein levels of cyclin A, D1, D3, and E, CDK2, and CDK4. Results from a representative experiment are shown. (b) Treatment of HUVEC with DPTH (12.5–50 μM) for 21 hr dose-dependently increased the protein levels of p21, but not p27 and p53. Results from a representative experiment are shown. Three samples were analyzed in each group, and values shown in parentheses represent the means ± SEM. Membrane was probed with anti-G3PHD antibody to verify equivalent loading. (c) The time course of p21 mRNA changes in HUVEC in response to 50 μM DPTH treatment. Values shown in parentheses represent the relative intensities (ratios of p21/GAPDH mRNA in the HUVEC treated with DPTH vs. DMSO). RT–PCR products of GAPDH were used as an internal control. CDK, cyclin-dependent kinase.

were conducted. Initially, the cells were switched to media with 2% FBS for 24 hr to render them quiescent and to synchronize their cell cycle activities. Then they were returned to media with 10% FBS without or with DPTH (50 μM). Figure 3 showed that after 24 hr treatment of HUVEC with DPTH induced a significant accumulation of cells at the G0/G1 phase of the cell cycle as compared with the cell treated with vehicle, suggesting that the observed growth inhibition effect of DPTH was due to an arrest of DNA replication thereby inhibiting further progress in the cell cycle.

### 3.3. Alterations in cell cycle activity by DPTH treatment

To delineate the molecular mechanism of DPTH-induced inhibition of HUVEC cell proliferation, we further examined the levels of cell cycle regulatory proteins in the DPTH-treated HUVEC. It has been generally believed that progression of cell cycle activity is regulated by coordinated successive activation of certain CDKs. This CDK activation is in turn modulated by association with a number of regulatory subunits called cyclins, and with a group of CDK-inhibitory proteins designated CKIs. Accordingly, we examined the changes in cyclin and CDK protein level in the DPTH-treated HUVEC. As shown in Fig. 4a, treatment of HUVEC with DPTH for 21 hr at a range of concentrations (12.5–50 μM), which caused the inhibition of [$^3$H]thymidine incorporation and cell growth, did not induce any significant changes of the protein levels of cyclin A, cyclin D1, cyclin D3, cyclin E, CDK2 and CDK4, suggesting that DPTH-induced cell cycle arrest in HUVEC is not through alternations of the protein levels of cyclins and CDKs. Since the CDK activity can be also controlled by a group of CKIs, we further examined the changes of protein levels of p21 and p27, two known CKIs, in the DPTH-treated HUVEC. Figure 4b showed that the protein levels of p21, but not p27, were dose-dependently increased in the DPTH-treated HUVEC as compared with the DMSO-treated cells (control). Using

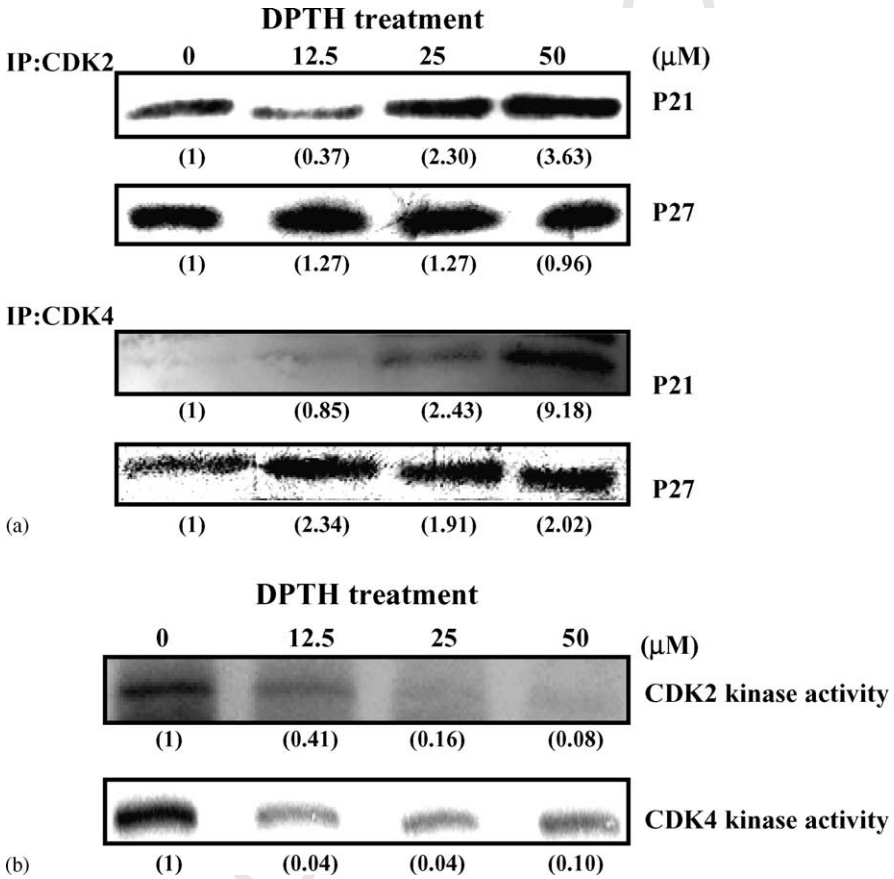


Fig. 5. Effect of DPTH on the formations of CKI–CDK complex and CDK kinase activity. (a) DPTH-induced upregulation of the formations of CDK2–p21 and CDK4–p21 complex in a dose-dependent manner. The formations of CDK2–p27 and CDK4–p27 complex were not affected significantly by DPTH treatment. CDK2 was immunoprecipitated by anti-CDK2 antibody, and CDK2–p21 association was detected by anti-p21 antibody, whereas CDK2–p27 association was detected by anti-p27 antibody. CDK4 was immunoprecipitated by anti-CDK4 antibody, and CDK4–p21 association was detected by anti-p21 antibody, whereas CDK4–p27 association was detected by anti-p27 antibody. Results from a representative experiment are shown. Values shown in parentheses represent the relative intensities (ratios of CDK–p21 or CDK–p27 complex in the HUVEC treated with DPTH vs. DMSO). (b) Treatment of the HUVEC with DPTH dose-dependently decreased the CDK2 and CDK4 kinase activities. Results from a representative experiment are shown. The CDK2 and CDK4 kinase activities were determined as described in Section 2. Values shown in parentheses represent the relative intensities (ratios of CDK2 or CDK4 kinase activity in the HUVEC treated with DPTH vs. DMSO). CDK, cyclin-dependent kinase.

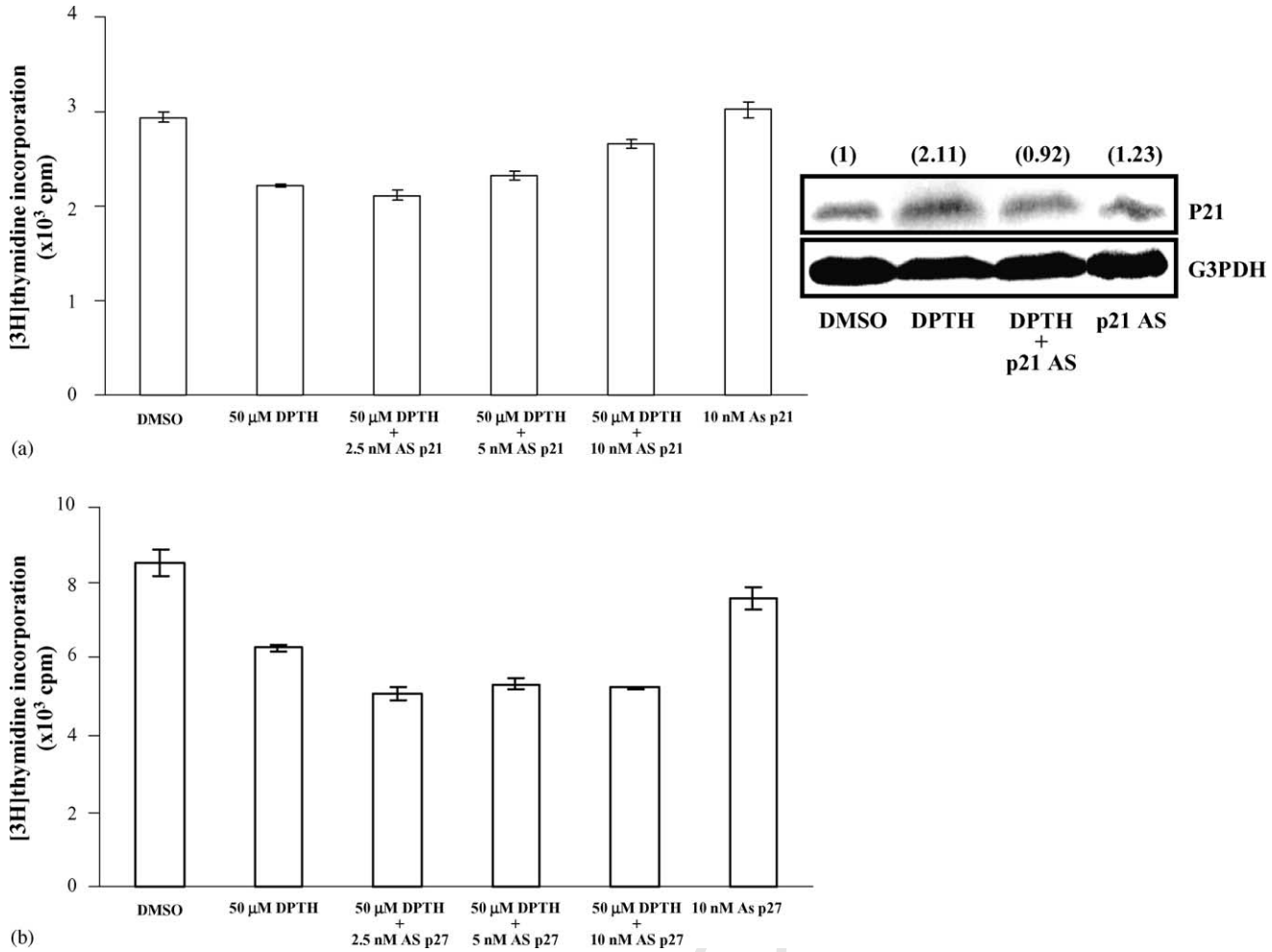


Fig. 6. Involvement of p21 in the DPTH-induced decrease of [$^3$H]thymidine incorporation in HUVEC. Antisense p21 or p27 oligonucleotide was added to HUVEC at a final concentration up to 10 nM at 16 hr before the cell was challenged with 10% FBS and 50 μM DPTH for additional 21 hr. The DPTH-induced up-regulation of p21 protein was reduced by pre-treatment of the cells with p21 antisense oligonucleotide (a, right panel). Membrane was probed with anti-G3PHD antibody to verify equivalent loading. The levels of p21 protein in each treatment after normalized with the levels of G3PDH protein were shown in parentheses. Pretreatment of HUVEC with AS p21 (a, left panel), but not AS p27 (b), dose-dependently reversed the DPTH-induced decrease of [$^3$H]thymidine incorporation. Values represent the means ± SEM. AS p21, antisense p21 oligonucleotide; AS p27, antisense p27 oligonucleotide.

RT–PCR technique, we further demonstrated that treatment of HUVEC with 50 μM DPTH for 3 hr induced an up-regulation of p21 mRNA (Fig. 4c), suggesting that transcriptional regulation was involved in the DPTH-induced increase in p21 protein levels. The CKI exerts its inhibitory effect on the kinase activity through binding to cyclin–CDK complex. Accordingly, we further conducted immunoprecipitation assay to examine the effect of DPTH on the formation of CDK–CKI complex. In the DPTH-treated cells, the formations of the CDK2–p21 and CDK4–p21 complex, but not CDK2–p27 and CDK4–p27 complex, were increased (Fig. 5a). To demonstrate that the increased p21 protein is associated with inhibition of CDK activation, we examined the CDK kinase activity. Figure 5b showed that the assayable CDK2 and CDK4, kinase activities were significantly decreased in the HUVEC treated with DPTH. To further demonstrate that the increased p21 expression observed in the DPTH-treated HUVEC correlated with G0/G1 arrest, the experiment illustrated in Fig. 6 was conducted. Thus, in the sample labeled DPTH (for 50 μM DPTH-treated alone), the [$^3$H]thymidine incorporation was decreased. Sample DPTH + AS p21 was pretreated with a p21 antisense oligonucleotide (AS), which blocked the expression of p21 protein (Fig. 6a, right panel). Treatment of HUVEC with AS p21 or AS p27 alone did not cause any significant change in [$^3$H]thymidine incorporation into HUVEC. Consequently, pretreatment of the HUVEC with AS p21 dose-dependently reversed the DPTH-induced decrease in [$^3$H]thymidine incorporation (Fig. 6a, left panel). In contrast, pretreatment of HUVEC with AS p27 failed in preventing the DPTH-induced decrease in [$^3$H]thymidine incorporation (Fig. 6b).

## 4. Discussion

Control of vascular development has been suggested to be new therapeutic approaches to many angiogenesis-

related disorders. In the present study, we demonstrated that DPTH at a range of concentrations (12.5–50 μM) inhibited DNA synthesis and decreased cell number in cultured HUVEC in a dose- and time-dependent manner (Fig. 2). These results were not due to cell death, indicating that there was an inhibitory effect of DPTH on the mechanisms for cell division in the subcultured HUVEC. To our knowledge, this is the first demonstration that DPTH inhibits the growth of human vascular endothelial cells.

By flow cytometry analyses, we demonstrated that DPTH treatment decreased DNA synthesis and arrested the cells at the G0/G1 phase of the cell cycle (Fig. 3). It has been suggested that the cell cycle is regulated by the activation of specific CDKs and coordinated successive activation of certain CDKs occurs late in the G1 phase and is instrumental in the transition from the G1 to the S phase [11,12]. This CDK activation is in turn modulated positively by their assembly with a series of regulatory subunits called cyclins, and negatively by association with a group of CDK-inhibitory proteins designated CKIs [13]. Cyclins have been identified as cyclins A, D1, D3 and E, whereas the most common CDKs are designated CDK2 and CDK4. The formations of cyclin A–CDK2 and cyclin E–CDK2 complex occur late in the G1 phase as cells prepare to synthesize DNA [14], and formation of the cyclin E complex is a rate-limiting step in the G1/S transition [15]. The basic mechanisms for cell cycle regulation appear to be universal. In the present study, we demonstrated that DPTH at a concentration of 50 μM, which inhibited cell cycle arrest, did not induce any significant changes of the protein levels of cyclins A, D1, D3 and E, CDK2, and CDK4 in the HUVEC, indicating that the action of DPTH on the growth of endothelial cells is not through a mechanism to reduce the cyclins or CDKs. Examination of the expression levels of CDK inhibitory proteins, we found that treatment of HUVEC with DPTH resulted in an increase in the protein level of p21, but not p27. In accord with the established notion that p21 is one known CDK inhibitor, we found that the formations of the CDK2–p21 and CDK4–p21 complex, but not CDK2–p27 and CDK4–p27complex, were increased and the assayable CDK2 and CDK4 kinase activities were decreased in the DPTH-treated HUVEC. These findings suggest that DPTH inhibits the CDK2 and CDK4 kinase activities through an increase in p21 expression. The important role of p21 in the DPTH-induced anti-proliferation in the HUVEC is confirmed by the antisense p21 oligonucleotide experiment showing that pretreatment with a p21 antisense oligonucleotide, but not p27 antisense oligonucleotide, reversed the DPTH-induced inhibition in [$^3$H]thymidine incorporation. Accordingly, we concluded that DPTH induced an increase in p21 expression, which in turn inhibited the CDK2 and CDK4 enzyme activities and led to the impairment of HUVEC in the transition from the G1 to S phase.

How does DPTH act to regulate the level of p21 protein remains unsolved in the present study? In response to a 50 μM DPTH treatment for 18 hr, we observed that the level of p21 protein increased significantly (Fig. 4b). Interestingly, the level of p21 mRNA in HUVEC increased after a 3 hr exposure and then declined after 6 hr (Fig. 4c). Similarly, our previous study done in the 12-*o*-tetradecanoylphorbol-13-acetate-treated COLO-205 cell line showed that the p21 mRNA level increased after 1 hr treatment with 12-*o*-tetradecanoylphorbol-13-acetate, peaked at 3 hr and then declined at 6 hr, whereas the level of p21 protein increased significantly after 6 hr exposure and peaked after 24 hr [9]. These data indicated that the half-life of p21 protein is much longer than its mRNA, or else a post-translational regulation might also be involved in the DPTH-induced increase in the p21 protein. Although we did not perform the time course experiment of DPTH-induced increases in the protein levels of p21, our data suggested that transcriptional regulation might be involved in the DPTH-induced increase in p21 protein levels. p21 is a transcriptional target of the tumor suppressor gene p53 [16,17]. Expression of p53 in the cells can induce cell growth arrest through transcriptional activation of p21 [18]. However, treatment of HUVEC with DPTH did not affect the expression level of p53 protein, suggesting that p53 protein is not involved in this process. To identify the primary target molecules of DPTH in regulating the p21 up-regulation, more experiments need to be done. In conclusion, the results from the present studies indicate that DPTH-induced cell cycle arrest in HUVEC occurred when the cyclin-CDK system was inhibited just as p21 protein levels increased. The findings from the present studies suggest the potential applications of DPTH in the treatment of angiogenesis-related disorders.

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