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YC-1 inhibits proliferation of human vascular endothelial cells through a cyclic GMP-independent pathway

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Abstract

This study was designed to investigate the effect of YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole, in human umbilical vein endothelial cells (HUVECs) proliferation and its underlying mechanism. YC-1 at a range of concentrations (5–50 μM) inhibited DNA synthesis and decreased cell number in cultured HUVEC in a dose- and time-dependent manner. YC-1 was not cytotoxic at these concentrations. [^{3}H]thymidine incorporation and flow cytometry analyses revealed that YC-1 treatment decreased DNA synthesis and arrested the cells at the G0/G1 phase of the cell cycle. Western blot analysis demonstrated that YC-1 (5–50 μM) increased the levels of cyclin-dependent kinase (CDK)-inhibitory proteins (CKIs), p21 and p27, but did not induce any significant changes of cyclins and CDKs. In the YC-1-treated HUVEC, the formation of CDK2–p21 complex, but not CDK2–p27 complex, was increased and the assayable CDK2 kinase activity was decreased. These changes were in a dose-dependent manner. In contrast, the formations of CDK4–p21 and CDK4–p27 complex were slightly increased and the assayable CDK4 kinase activity was slightly decreased (if there were any changes). Pretreatment with guanylyl cyclase inhibitors, 1*H*-(1,2,4)oxadiazolo[4,3-*a*]quinozalin-1-one (ODQ) and methylene blue, inhibited the YC-1-induced increase of cyclic GMP level, but did not change significantly the magnitude of the YC-1-induced inhibition of thymidine incorporation and cell number in HUVEC. These results indicate that YC-1-induced cell cycle arrest in HUVEC occurred when the cyclin–CDK system was inhibited just as p21 and p27 protein levels were augmented. This YC-1-induced antiproliferation effect in HUVEC is via a cyclic GMP-independent pathway.

Keywords: YC-1; Cyclic GMP; p21; p27; Cyclin; Endothelial cells

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Abbreviations: YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole; sGC, soluble guanylate cyclase; HUVEC, human umbilical vein endothelial cells; NO, nitric oxide; VEGF, vascular endothelial growth factor; CKI, cyclin-dependent kinase inhibitor; cyclic GMP, 3′,5′-cyclic guanosine monophosphate; CDK, cyclin-dependent kinase; PKG, cyclic GMP-dependent protein kinase; HEPES, 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulfate; NP-40, Nonident P-40; ODQ, 1*H*-(1,2,4)oxadiazolo[4,3-*a*]quinozalin-1-one; ECGS, cell growth supplement; M199, medium 199; FBS, fetal bovine serum; NBT, 4-Nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PCNA, proliferation cell nuclear antigen; Gst-Rb, glutathione s-transferase retinoblastoma; PBS, phosphate buffer saline; FACS, fluorescence-activated cell sorter.

1. Introduction

Angiogensis, the formation of new blood vessels as extensions of existing vessels, 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 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. The events that are thought to be essential for angiogenesis include: local degradation of the basement membrane of the parent vessel, allowing protrusion of

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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. 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.

YC-1, a unique NO-independent activator of sGC, has been shown to increase the intracellular cyclic GMP concentration in platelets [3], vascular smooth muscle cells [4], and HUVEC [5]. The cyclic GMP-increasing effect of YC-1 has been reported to result in an inhibition of platelet aggregation [3,6] as well as to mediate vasorelaxation [4,7]. Furthermore, it has been demonstrated that YC-1 not only stimulated sGC but also inhibited cyclic GMP-hydrolyzing phosphodiesterase in human platelets [8]. Exposure of HUVEC to VEGF, a regulator of angiogenesis, led to a dose-dependent increase in cyclic GMP levels, an indicator of NO production [9]. NO mediates aspects of VEGF signaling required for endothelial cell proliferation. Accordingly, this study was designed to investigate the effect of YC-1 in HUVEC proliferation and its underlying mechanism.

2. Methods and materials

2.1. Materials

YC-1 was provided by Yung-Shin Pharma Ind. Co. DTT, HEPES, EDTA, glycerol, PMSF, pepstatin A, leupeptin, SDS, NP-40, ODQ, ECGS, and methylene blue were purchased from Sigma. KT-5823 was purchased from Biomol. M199, trypsin-EDTA, and kanamycin were purchased from Life Technologies. 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. NBT and BCIP were purchased from Kirkegaard & Perry Laboratories. Protein assay agents were purchased from Bio-Rad.

2.2. Cell culture

HUVEC were grown in M199 containing 10% FBS, ECGS (0.03 mg mL^{-1}) and kanamycin (50 U mL^{-1}) 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 5–9 were used.

2.3. [^{3}H]thymidine incorporation

As previously described [10,11], HUVEC at a density of 1×10^4 cells cm^{-3} 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 18 hr in M199 containing 0.5% FBS. M199 supplemented with 10% FBS and 0.05% DMSO (control) or various concentrations of YC-1 in 0.05% DMSO were added to the cells and the cultures were allowed to incubate for 24 hr. During the last 2 hr of the incubation with or without YC-1, [^{3}H]thymidine was added at 1 μCi mL^{-1} (1 mCi = 37 kBq). Incorporated [^{3}H]thymidine was extracted in 0.2 N NaOH and measured in a liquid scintillation counter.

2.4. Cell counting

As a measurement of cell proliferation, the cells at a density of 1×10^4 cells/cm^{-3} were seeded onto 6-well 1% gelatin-coated plates and grown in M199 supplemented with 10% FBS and ECGS. Media with and without YC-1 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 assay as previously described [10]. Briefly, HUVEC were applied to 24-well plates in growth medium (M199 plus 10% FBS and ECGS). After the HUVEC had grown to 70–80% confluence, they were rendered quiescent by incubation 24 hr with M199 containing 0.5% FBS. A fresh medium (M199 plus 10% FBS and ECGS) was applied and YC-1 was added at a concentration of 50 μM and the mixture was allowed to incubate for 24 hr. MTT assay is based on the conversion of the tetrazolium salt 3-(4,5-dimethyl thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfopheyl)2-*H*-tetrazolium by mitochondrial dehydrogenase to a formazan product, as measured at an absorbance of 490 nM. Four samples were analyzed in each experiment.

2.6. Protein preparation and Western blotting

To determine the expression levels of cyclins, CDKs, CKIs, PCNA and G3PDH in HUVEC, the total proteins were extracted and Western blot analyses were performed as described previously [12]. Briefly, HUVEC were cultured in 15-cm petri dishes. After reaching subconfluence, the cells were rendered quiescent and then treated with various concentrations of YC-1 for 18 hr, or 50 μM YC-1 for indicated time intervals, and incubated in a humidified incubator at 37°. In some experiments, cells were pretreated with specific inhibitors as indicated followed by YC-1 (50 μM) and incubated in a humidified incubator at 37°.

After incubation, the cells were washed with PBS (pH 7.4), incubated with extraction buffer (Tris 10 mM, pH 7.0, NaCl 140 mM, PMSF 2 mM, DTT 5 mM, NP-40 0.5%, pepstatin A 0.05 mM and leupeptin 0.2 mM) with gentle shaking, and then centrifuged at 12,500 *g* for 30 min. The cell extract was then boiled in a ratio of 1:1 with sample buffer (Tris 100 mM, pH 6.8, glycerol 20%, SDS 4% and bromophenol blue 0.2%). 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 (3 hr, 40 V), treated with 5% fat-free milk powder to block the nonspecific IgGs, and incubated for 1 hr with specific antibody for cyclins, CDKs, CKIs, PCNA, or G3PDH. The blot was then incubated with anti-mouse or -rabbit IgG linked to alkaline phosphatase (1:1000) for 1 hr. Subsequently, the membrane was developed with NBT/BCIP as a substrate.

2.7. Immunoprecipitation

As previously described [13], CDK2 or CDK4 was immunoprecipitated from 200 μg of protein by using anti-CDK2 or anti-CDK4 antibody (2 μg) and protein A agarose beads (20 μL). The precipitates were washed five times with washing buffer and once with PBS. The pellet was then resuspended in sample buffer (50 mM Tris, pH 6.8, 100 mM bromophenol blue and 10% glycerol) and incubated at 90° for 10 min before electrophoresis to release the proteins from the beads.

2.8. CDK kinase assay

As previously described [12,14], CDK2 or CDK4 immunoprecipitates from YC-1-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 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 [γ-^{32}P] ATP, 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 and the products were analyzed by 10% SDS–PAGE. The gel was dried and visualized by autoradiography.

2.9. Flow cytometry

As previously described [15,16], the cells were seeded onto 100-mm 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. After release using trypsin-EDTA, they were harvested at various times, washed twice with PBS/0.1% dextrose, and fixed in 70% ethanol at 4°. Nuclear DNA was stained with a reagent containing propidium iodine (50 mg mL^{-1}) and DNase-free RNase (2 U mL^{-1}) and measured using a FACS. The proportion of nuclei in each phase of the cell cycle was determined using established CellFIT (Becton Dickinson) DNA analysis software.

2.10. Cyclic GMP assay

The levels of cyclic GMP were examined using cyclic GMP enzyme immunoassay kit according to manufacture's instruction (Cayman Chemical). Briefly, HUVEC grown at the 10-cm^2 dishes were pretreated with cyclic GMP inhibitors, 0.1 μM ODQ or 1 μM methylene blue, or with PKG inhibitor, 0.5 μM KT-5283, for 30 min and then followed by 2 hr treatment with 10 μM YC-1. After treatment, the cells were collected for protein extraction using 10% TCA in phosphate buffer and followed by sonication, and the protein concentrations were measured. Samples (400 μg protein), cyclic GMP acetylcholinesterase tracer and rabbit antiserum-cyclic GMP were subsequently added to each well, which was pre-coated with mouse anti-rabbit monoclonal antibody, incubated for 18 hr at room temperature, and then washed with buffer. Ellman's reagent, substrate of acetylcholinesterase, was added to each well for color development and measured at OD_{412}.

2.11. Statistical analysis

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

3. Results

3.1. YC-1 inhibits [^{3}H]thymidine incorporation into HUVEC

To study the effect of YC-1 on the DNA synthesis of HUVEC, we examined changes in [^{3}H]thymidine incorporation (a measurement of DNA synthesis) in response to YC-1 in subcultured HUVEC. As illustrated in Fig. 1a, YC-1 inhibited [^{3}H]thymidine incorporation into HUVEC. This inhibition was dose-dependent, and it occurred at YC-1 dose as low as 5 μM.

3.2. YC-1 inhibits HUVEC proliferation

In the experiment of Fig. 1b, HUVEC were cultured for 6 days with or without YC-l (5–50 μM), and then the cells were harvested and counted. These data show that YC-1 reduced cell counts in HUVEC. This YC-1-induced reduction in the cell growth rate was dose-dependent, consistent

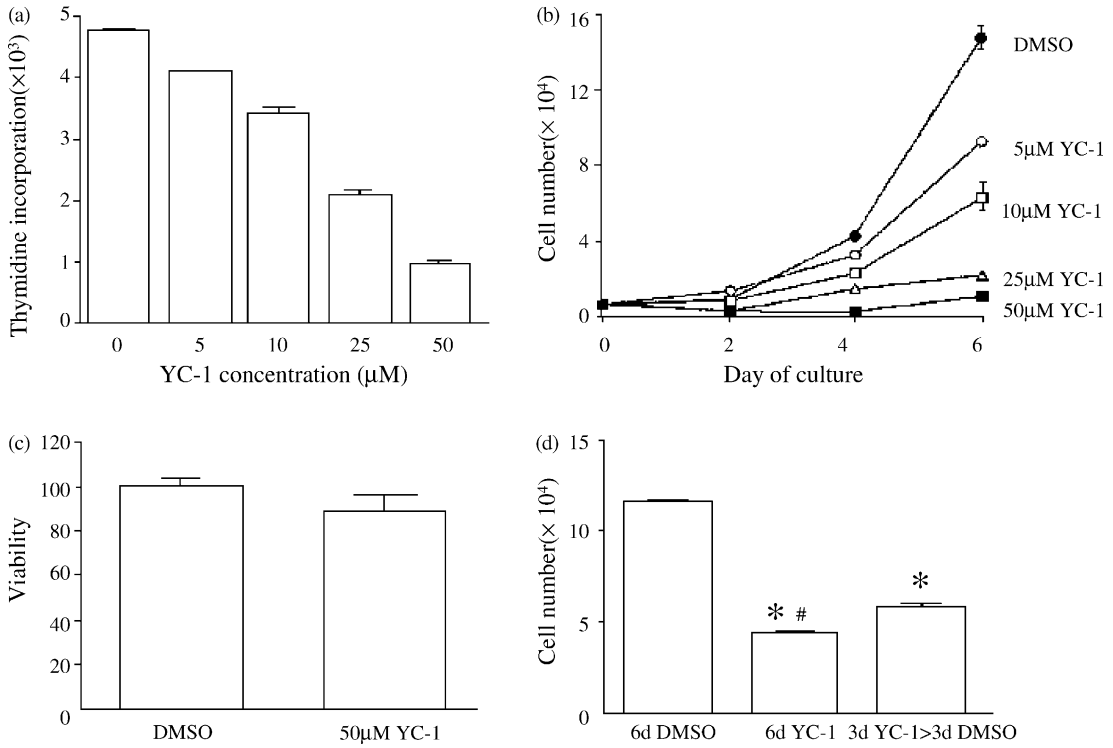

Fig. 1. Effects of YC-1 on [^{3}H]thymidine incorporation and cell growth rate in subcultured HUVEC. (a) Dose-dependent inhibition of [^{3}H]thymidine incorporation in HUVEC by YC-1. (b) Dose-dependent inhibition of HUVEC growth by YC-1. (c) There was no significant difference in viability between vehicle-treated and 50 μM YC-1-treated HUVEC. (d) YC-1-induced inhibition of HUVEC proliferation was reversed by removal of YC-1. Treatment of HUVEC with 25 μM YC-1 for 6 days induced a significant inhibition of cell number as compared with the cells treated with 0.05% DMSO. However, treatment of the cells with 25 μM YC-1 for 3 days followed by 0.05% DMSO without YC-1 for an additional 3 days reversed the inhibition. Three to four samples were analyzed in each group, and values represent the means ± SEM. Comparisons were subjected to ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$. *6 d YC-1-treated group and 3 d YC-1 + 3 d DMSO group different from control (6 d DMSO) group. #6 d YC-1-treated group different from 3 d YC-1 + 3 d DMSO group.

with the inhibitory effect of YC-1 on [^{3}H]thymidine incorporation. To confirm that the results of our studies of DNA synthesis and cellular proliferation in HUVEC were not due to cell death caused by YC-1 treatment, we conducted a viability assay by treating the cells with YC-1 for 6 days at the maximal dose (50 μM) used in the studies of cell growth inhibition. MTT assays indicated that there was no significant difference in cell viability between vehicle- and YC-1-treated HUVEC (Fig. 1c).

3.3. YC-1-induced inhibition of HUVEC proliferation is reversible

We also examined the reversibility of the YC-1-induced inhibition of cell proliferation in HUVEC. As illustrated in Fig. 1d, treatment of HUVEC with 25 μM YC-1 for 6 days induced a 63% reduction of cell number as compared with the cells treated with 0.05% DMSO for 6 days. However, treatment of the cells with 25 μM YC-1 for 3 days followed by 0.05% DMSO without YC-1 for an additional 3 days induced only 50% inhibition as compared with the cells treated with 0.05% DMSO for 6 days. These results suggest that the YC-1-induced inhibition of cell growth is reversible.

3.4. Arrest of cell cycle in G0/G1

In order to examine further the actions of YC-1 on the cell cycle, the cells were switched to media with 0.5% FBS for 18 hr to render them quiescent and to synchronize their cell cycle activities. Then they were returned to media with 10% FBS and, at various times thereafter, they were treated with [^{3}H]thymidine. Figure 2a shows a reduction of the thymidine incorporation into HUVEC during the S phase of the cell cycle. Figure 2b shows the FACS analyses of DNA content at various times after release from quiescence by incubation in culture media supplemented with 10% FBS and 0.05% DMSO or 20 μM YC-1 in 0.05% DMSO. The data reveal that YC-1 induced a significant accumulation of cells at the G0/G1 phase of the cell cycle, suggesting that the observed growth inhibition effect of YC-1 was due to an arrest of DNA replication thereby inhibiting further progress in the cell cycle.

3.5. Alterations in cell cycle activity

It has been generally believed that coordinated successive activation of certain CDKs occurs late in the G1 phase and is

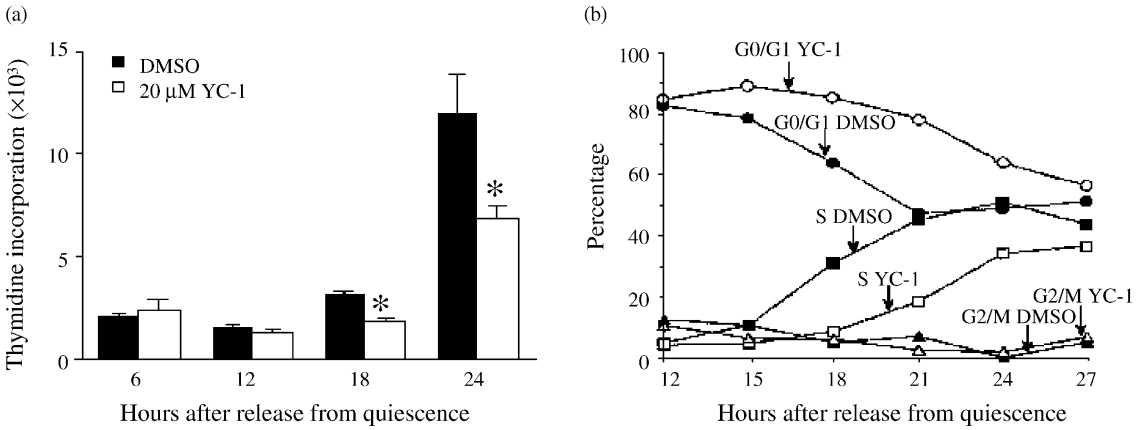

Fig. 2. Time-dependent inhibition of cell cycle in HUVEC by YC-1. To study the time-dependent of YC-1 on the cell cycle, [^{3}H]thymidine incorporation was conducted after HUVEC release from quiescence by incubation in culture media supplemented with 10% FBS and 0.05% DMSO (control) or 20 μM YC-1 in 0.05% DMSO (a). Comparisons were subjected to ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$. *YC-1-treated group different from DMSO-treated group. FACS analysis of DNA content was performed after 12 hr release from quiescence by incubation in culture media supplemented with 10% FBS and 0.05% DMSO without (control) or with 20 μM YC-1 (b). Percentage of cells at the G0/G1, S, or G2/M phase of the cell cycle was determined using established CellFIT DNA analysis software. Four samples were analyzed in each group, and values represent the mean ± SEM.

instrumental in the transition from the G1 to the S phase [17,18]. 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 [19]. Cyclins have been identified as cyclins A, D1, D3, and E. As illustrated in Fig. 3, YC-1 at a concentration of 20 μM, which caused the inhibition of thymidine incorporation and growth arrest, did not induce any significant changes of the levels of cyclins A, D1, D3 and E, CDK2, CDK4 and PCNA. Since the CDK activity can be controlled by a group of CKIs, we examined the protein levels of p21 and p27, two known CKIs, in the YC-1-treated HUVEC. Figure 4A showed that the protein levels of p21 and p27 were increased in the YC-1-treated HUVEC as compared with the DMSO-treated cells (control). These upregulations occur at 18 hr after YC-1 treatment and then decline to the control levels. We further conducted immunoprecipitation assay to examine the effect of YC-1 on the formation of CDK–CKI complex. In YC-1-treated cells, the formation of the CDK2–p21 complex, but not CDK2–p27 complex, was increased and the assayable CDK2 kinase activity was decreased (Fig. 4B). These changes are in a dose-dependent manner. In contrast, the formations of the CDK4–p21 and CDK4–p27 complex were increased slightly and the assayable CDK4 kinase activity was decreased slightly (if there was any changed) in the YC-1-treated HUVEC (Fig. 4C). These findings suggest that YC-1 induced an inhibition of CDK2 activity and led to the impairment of HUVEC in the transition from G1 to S phase.

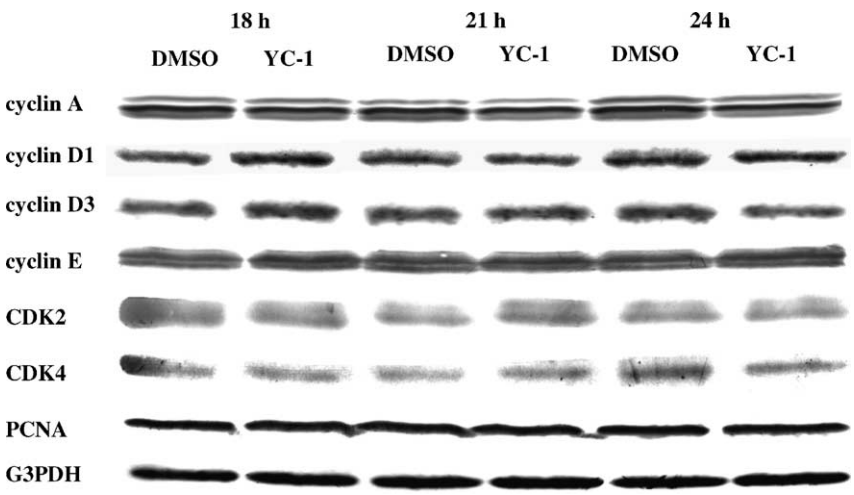

Fig. 3. Effect of YC-1 on cyclin and CDK protein levels. Proteins were extracted from the cultured HUVEC at 18, 21 and 24 hr after YC-1 treatment and probed with proper dilutions of specific antibodies. YC-1 at a concentration of 20 μM did not induce any significant changes of the levels of cyclins A, D1, D3, and E, CDK2, CDK4, and PCNA. Membrane was probed with anti-G3PHD antibody to verify equivalent loading.

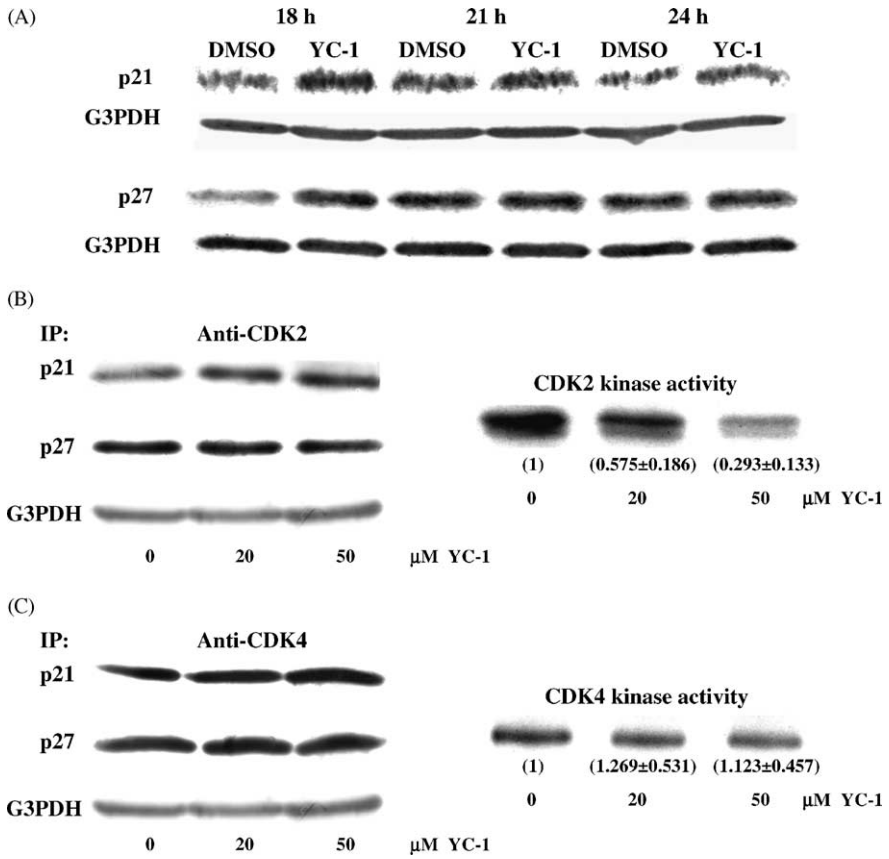

Fig. 4. Effect of YC-1 on CKI protein levels, CKI–CDK association and CDK kinase activity. (A) Treatment of HUVEC with YC-1 at a concentration of 50 μM increased the levels of p21 and p27 protein at 18 hr after YC-1 treatment. Membrane was probed with anti-G3PHD antibody to verify equivalent loading. (B) YC-1 induced upregulation of CDK2–p21 association (left panel) and downregulation of CDK2 kinase activity (right panel) in a dose-dependent manner. The CDK2–p27 association was not affected by YC-1 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. (C) The CDK4–p21 and CDK4–p27 associations and CDK4 kinase activity were only slightly affected by YC-1 treatment. 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.

3.6. Cyclic GMP-pathway is not involved in the YC-1-induced inhibition of HUVEC proliferation

To determine whether cyclic GMP pathway was involved in the YC-1-induced growth inhibition in HUVEC, the sGC inhibitors, ODQ and methylene blue, or PKG inhibitor, KT-5823, were used in this study. Pretreatment of the HUVEC for 30 min with ODQ (0.1 μM) or methylene blue (1 μM), which did not significantly affect the basal cyclic GMP levels, blocked the YC-1-induced increase in cyclic GMP level (Fig. 5). In contrast, pretreatment the cells with the PKG inhibitor, KT-5823 (0.5 μM), did not have significant effect on the YC-1-induced increase in the cyclic GMP level. However, pretreatments of the HUVEC for 30 min with ODQ or methylene blue at these concentrations, which blocked the YC-induced increases in cyclic GMP levels, did not affect the basal levels and the YC-1-induced inhibition in [^{3}H]thymidine incorporation (Fig. 6a) and cell number (Fig. 6b). These results indicate that cyclic GMP pathway might not be involved in the YC-1-induced inhibition of HUVEC proliferation.

4. Discussion

In the present study, we demonstrated that YC-1 at a range of concentrations (5–50 μM) inhibited DNA synthesis and decreased cell number in cultured HUVEC in a dose- and time-dependent manner. These results were not due to cell death and indicated that there was an inhibitory effect of YC-1 on the mechanisms for cell division in the subcultured HUVEC. The findings of the present study suggest that the cyclic GMP pathway might not be involved in the signal transduction leading to the inhibition of DNA synthesis and proliferation caused by YC-1 in cultured HUVEC. To our knowledge, this is the first demonstration that YC-1 inhibits the growth of HUVEC through a cyclic GMP-independent pathway.

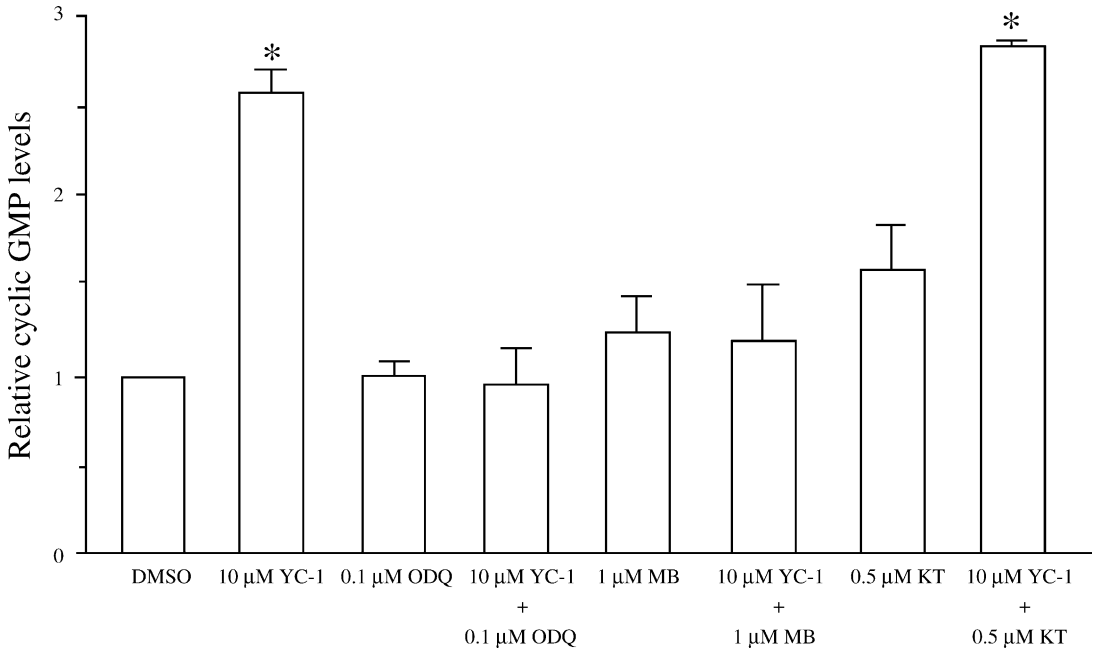

Fig. 5. Effect of YC-1 on the levels of cyclic GMP in HUVEC. YC-1-induced increases of cyclic GMP levels in HUVEC were blocked by 30 min pretreatment of the cells with cyclic GMP antagonists, 0.1 μM ODQ and 1 μM MB, but not with PKG inhibitor, 0.5 μM KT-5823. PKG, cyclic GMP-dependent protein kinase; ODQ, 1*H*-(1,2,4)oxadiazolo[4,3-*a*]quinozalin-1-one; MB, methylene blue; KT, KT-5823. Three samples were analyzed in each group, and values represent the means ± SEM. Comparisons were subjected to ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$. *10 μM YC-1-treated group or 10 μM YC-1 plus 0.5 μM KT-treated group different from DMSO-treated group.

By [^{3}H]thymidine incorporation and flow cytometry analyses, we demonstrated that YC-1 treatment decreased DNA synthesis and arrested the cells at the G0/G1 phase of the cell cycle (Fig. 2). Observation of intracellular events associated with the progression of cell cycle activity have suggested that 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 [17,18]. This CDK activation is in turn modulated by association with a series of regulatory subunits called cyclins, and with a group of CDK-inhibitory proteins designated CKIs [19]. Cyclins have been identified as cyclins A, D1, D3 and E, whereas the most common CDKs are designated CDK2 and CDK4. Cyclin A–CDK2 and cyclin E–CDK2 complexes form late in the G1 phase as cells prepare to synthesize DNA [20], and formation of the cyclin E complex is a rate-limiting step in the G1/S transition [21]. YC-1 at a concentration of 50 μM did not induce any significant changes of the levels of cyclins A, D1, D3 and E, CDK2, CDK4, and PCNA in the HUVEC. However, treatment of HUVEC with YC-1 resulted in increases in the levels of p21 and p27 protein at 18 hr after treatment. In accord with the established notion that p21 and p27 are two known CDK inhibitors, we found in YC-1-treated cells that the formation of the CDK2–p21 complex, but not CDK2–p27 complex, was increased and the assayable CDK2 kinase activity was decreased. In contrast, the formations of the CDK4–p21 and CDK4–p27 complex and the assayable CDK4 kinase activity were changed only slightly. Accordingly, we concluded that such a decreased CDK2 kinase activity could account (in part at least) for the impairment in the transition from G1 to S phase.

YC-1 has been shown to increase the intracellular cyclic GMP concentration in various cell types including HUVEC [5]. YC-1 inhibits vascular smooth muscle cell proliferation through an activation of cyclic GMP pathway [22]. Moreover, cyclic GMP has been indicated to be involved in the signaling of VEGF-induced proliferation of vascular endothelial cells [9]. As shown in the present study, pretreatment of the HUVEC for 30 min with sGC inhibitors, ODQ and methylene blue, or PKG inhibitor, KT-5823, at the doses 10 times higher than the IC_{50}, did not affect the YC-1-induced inhibition in [^{3}H]thymidine incorporation (Fig. 6a) and cell number (Fig. 6b). These results indicate that cyclic GMP pathway might not be involved in the proliferation inhibition of HUVEC caused by YC-1 at a range of concentrations (5–50 μM). How YC-1 acts to regulate the level of p21 protein, which in turn modulates the CDK kinase activity, and finally causes growth inhibition of HUVEC, needs further investigation.

In conclusion, the results from the present studies indicate that YC-1-induced cell cycle arrest in HUVEC occurred when the cyclin–CDK system was inhibited just as p21 and p27 protein levels were augmented. This YC-1-induced antiproliferation effect in HUVEC is via a

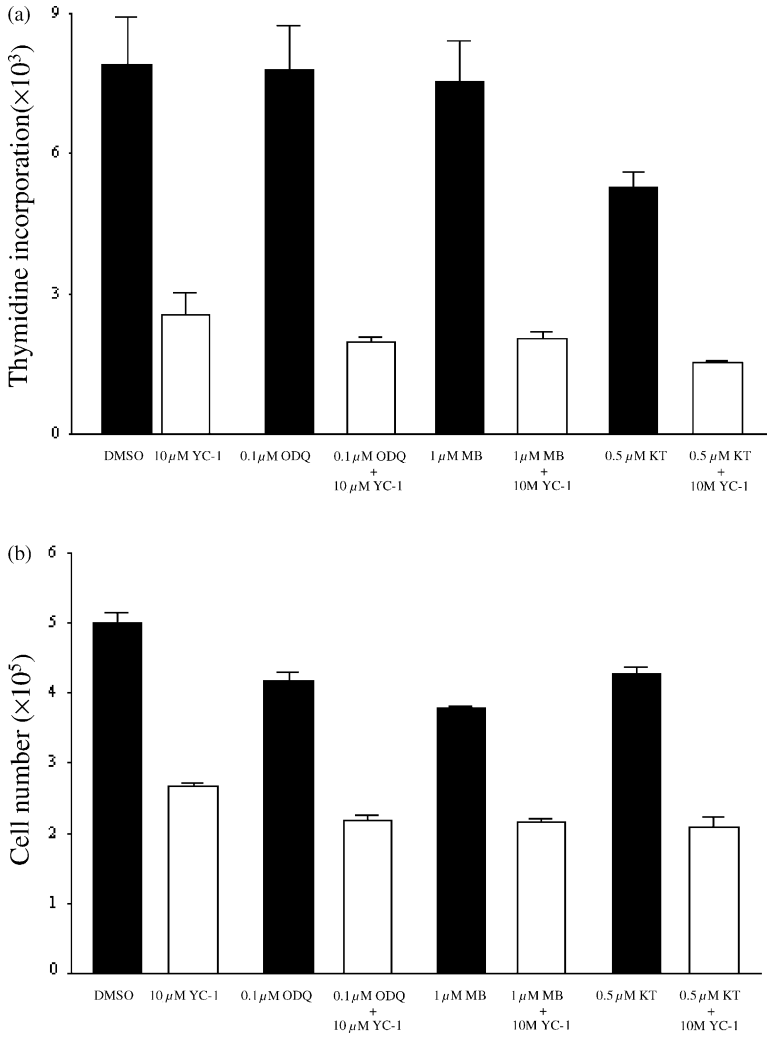

Fig. 6. Involvement of cyclic GMP-pathway on the YC-1-induced inhibition of HUVEC proliferation. Pretreatment of HUVEC with cyclic GMP antagonists, 0.1 μM ODQ and 1 μM MB, or PKG inhibitor, 0.5 μM KT-5823, did not affect YC-1-induced inhibition of [^{3}H]thymidine incorporation (a), and cell growth (b). PKG, cyclic GMP-dependent protein kinase; ODQ, 1*H*-(1,2,4)oxadiazolo[4,3-*a*]quinozalin-1-one; MB, methylene blue; KT, KT-5823. Six samples were analyzed in each group, and values represent the means ± SEM.

cyclic GMP-independent pathway. Although animal studies of YC-1-mediated anti-angiogenesis are still ongoing, the findings from the present studies suggest the potential applications of YC-1 in the treatment of angiogenesis-related disorders.

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