藥學碩士學位論文

PiB inhibits interaction between PIN1 and HIF-1α

PIN1과 HIF-1α의 상호작용에 대한 PiB의 저해효과 및 기전연구

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Abstract

PiB inhibits interaction between PIN1 and HIF-1α

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Hypoxia-inducible factor-1α (HIF-1α) is a key transcription factor that is induced by hypoxia. Specifically, the transcription of genes related to erythropoiesis, glycolysis, tissue invasion, metastasis and angiogenesis depends on the rate of HIF-1α binding to DNA at the hypoxia response elements of target genes. PIN1, the peptidyl-prolyl isomerase (PPIase), is an enzyme that changes the conformation of phosphoproteins. The conformational change induced by PIN1 alters the function and stability of the target proteins. PIN1 is overexpressed in many different types of malignancies, including breast, lung, cervical, brain and colorectal tumors. Thissuggests that PIN1 overexpression may function as a critical catalyst that amplifies multiple oncogenic signaling pathways during tumor

development. In this study, wefocused on the interaction of PIN1with HIF-1α by using PiB, a selective inhibitor of PIN1.PiB binds to the active site of PIN1 and thereby inhibits its catalytic activity in a competitive manner by masking thesubstrate binding site of PIN1. Under hypoxic conditions, PiB inhibits interaction of PIN1 with HIF-1α. In addition, it destabilizes HIF-1α protein, consequently reducing expression of vescular endothelial growth factor (VEGF) which is a major target protein of HIF-1α and also tube formation in HUVEC. Moreover, *in vivo* angiogenesis was decreased by PiB. In conclusion, PiB blocks interaction between PIN1 and HIF-1α, which leads to downregulation of VEGF expression and suppression of angiogenesis.

Keywords : PiB, HIF-1α,PIN1, VEGF,Colon Cancer HCT-116 cells

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Introduction

PIN1, the peptidyl-prolyl isomerase (PPIase), has been reported to beover-expressed in most types of human malignancies including breast, brain, lung and colorectal cancers[1].Thus, inappropriate overexpression of PIN1 is speculated to be implicated in pathogenesis of malignancy [2]. It controls functions of some essential proteins involved in various cellular processes such as immune response, cell development and cell growth[3, 4]. PIN1 has two domains; an N-terminal WW domain which is related to protein interaction and a C-terminal PPIase or a catalytic domain[5]. Both domains interact with the Ser/Thr-Pro moiety present in a variety of proteins, which causeschanges in the conformation and the functional properties of the phosphoproteins[6].

Hypoxia is a characteristic of most solid tumors. Cancer cells overcome a hypoxic condition by inducing the transcription of various genes involved in angiogenesis, metastasis, glycolysis and erythropoiesis[7]. The hypoxia-inducible factor-1α (HIF-1α) is a master regulator that mediates the upregulation of various hypoxia-regulated genes, such as vescular endothelial growth factor (VEGF)[8, 9]. Under normoxia, HIF-1α is hydroxylated by prolyl-hydroxylase domain (PHDs), resulting in the recognition of von Hippel-Lindau tumor suppressor protein (pVHL)followed byubiquitin proteasomal degradation of HIF-1α[10]. However, under hypoxic conditions, PHD activity decreases, resulting in accumulation of HIF-1α and subsequent to translocation to the nucleus. The stabilized HIF-1α dimerizes with HIF-1β and then binds to hypoxia response elements (HRE)present

in the VEGF promoter region[11].

Overexpression of both PIN1 and HIF-1α is prevalent in many different types of human cancers [12]. PIN1 binds to and stabilizes HIF-1α. In order to better investigate the interaction between PIN1 and HIF-1a, we utilized the PIN1 inhibitor PiB[13]. PiBbinds to the active site of PIN1, and thereby inhibits its catalytic activity [14]. In the present study, we investigated the effects of the PIN1 inhibitor PiB on angiogenesis related to HIF-1α signaling.Here, wereport thatPiB reduces stability of HIF-1α protein and expression of its downstream target gene, VEGF-Aunder hypoxic conditions.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin/fungizone mixtures, fatal bovine serum, TRIzol were purchased from Gibco BRL (Grand Island, NY, USA). Primary antibodies for PIN1, GFP, lamin B, α–tubulin and VEGF-A were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for phosphor-PIN1 and HA-tag were products of Cell Signaling Technology (Beverly, MA, USA).Antibodies against HIF-1α and HIF-2α were purchased from Novus Biologicals (Littleton, CO, USA). Secondary antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). The PIN1 inhibitor of PiB (diethyl-1,3,6,8-tetrahydro-1,3,6,8-tetraoxobenzol-phenanthroline-2,7-diacetate) and resuspended indimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St Louis, MO). Pico EPD Western blot detection kit was purchased from ELPIS (Republic of Korea). All other chemicals used were in the purest form available commercially.

Cell culture

HCT-116 cells were maintained in DMEM supplemented with 10% fetal bovine serum and 100 ng/ml penicillin/streptomycin/fungizone mixture at 37 °C in

humidified atmosphere of 5% CO_2 / 95% air. The cells were plated at appropriate density according to each experimental scale.

PIN1 activity assay

The specific activity of PIN1 was measured by the PPIase assay, using Suc-Ala-Ala-Pro-Phe-pNA (Bachem , Heidelberg, Germany) as a substrate for PIN1. After cells were treaetd with PiB, these were lysed with cell lysis buffer. Insoluble materials were removed by centrifugation at 13,000 x *g* for 15 min at 4°C and protein concentrations were determined by the Bradford method. Cell extracts (20μl, 10μg/μl) were placed in a cuvette, and 3μl of substrate and 3μl of α-chymotrypsin (Serva, Heidelberg, Germany) in 1 mM HCl were added. After incubation for 10 sec at room temperature, the cuvette absorbance was measured at 390nm.

Western blot analysis

HCT-116 cells (5 x 10^5 cells/ml) were plated in a 100 mm dish. After the cells were treated with PiB, these were grown in normoxia for 4h and then at 1% O_2 in a hypoxia chamber for additional 4h.The cells were exposed to the lysis buffer [5M NaCl, 0.5M EDTA, 0.5M Tris-HCl(pH8.0), 10% NP-40 and protease inhibitor] in the ice for 30 min. After centrifugation at 12,000 *g* for 15 min, the supernatant was

collected and stored at -70 °C until use. The protein concentration was determined by using the Bradford Assay Reagents kit (Bio-Rad, Hercules, CA).Protein samples were electrophoresed in a 8-12% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane at 200 mA for 1 h. Blots were incubated in fresh blocking buffer (0.1% Tween-20 in TBS containing 5% nonfat dry milk, pH 7.4) for 1h followed by incubation with appropriate primary antibodies in TBST with 3% BSA. After washing with TBST three times, blots were incubated with horseradish peroxidase-conjugated secondary antibody in TBST with 3% nonfat dry milk for 1 h at room temperature. Blots were washed again three times in TBST buffer. The immunoblots were visualized with a Pico EPD Western blot detection kit according to the manufacturer's instructions.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from HCT-116 cells using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. 10 μg of total RNA was reverse transcribed with murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) at 42 °C for 50 min and at 72 °C for 15 min. PCR was performed following standard procedures. The primers used for the reverse transcription-PCR are as follows (forward and reverse, respectively): HIF-1α,5'-CAAGACTTTCCTCAGTCAACA-3' and 5'-GGGAGAAAAATCAAGTCGTG-3', GAPDH, 5'-AAGGTCGGAGTCAACGGATTT-3' and 5'-

GCAGTGAGGGTCTCTCTCCT-3, VEGF, 5'-CCA TGA ACT TTC TGC TGT CTT G-3' and 5'-GGT GAG AGA TCT GGT TCC CGA-3' and HIF-2α, 5'-AAT GAC AGC TGA CAA GGA GAA GAA-3' and 5'-GAG TGA AGT CAA AGA TAC TAT GTC-3' Amplification products were analyzed on 1.2% agarose gel electrophoresis, stained with SYBR Green (Invitrogen) and photographed using fluorescence in LAS-4000 (GE Healthcare).

GST Pull-down Assays

HCT116 cells were seeded in a 100 mm dish. HA-HIF-1α was transfected into HCT-116 cells with FuGENE transfection reagents according to the manufacture's instruction (Promega, Madison, WI, USA). After 24h transfection, cells were grown at 1% O_2 in a hypoxia chamber for additional 4h. Cell were lysed in 120mM NaCl, 10mM EDTA, 40mM Tris-HCl(pH8.0), 0.1% NP-40 and a mixture of protease inhibitors.PiB was incubated with fresh GST-PIN1 beads for 2h at 4°C. The beads were pelleted by centrifugation, washed three times with PBS. Cell lysates were incubated with PiB-GST-PIN1 beads for 4h at 4°C. After centrifugation to pellet the beads, washed three times with PBS, denatured in 2X SDS-loading buffer, and analyzed by Western blot. To prepare the GST beads, *Escherichia coli* BL21 Lys cells (Promega) were transformed with the vectors pGEX-KG or pGEX-KG-PIN1 encoding for GST and GST-PIN1 proteins, respectively. The binding was detected by Western blot analysis.

Proximity Ligation Assay

To demonstrate the interaction of PIN1 and HIF-1α, *in situ* proximity assay was performed. HCT116 cells were plated on the chamber slide and then untreated or treated with PiB. These cells were grown in normoxia for 4h and then at 1% O_2 in a hypoxia chamber for additional 4h. After fixation with 4% paraformaldehyde solution for 10 min at room temperature, samples were incubated with Duolink Ⅱ blocking solution (Olink Bioscience, Uppsala Sweden) was performed as instructed in the manufacturer's protocol. Then the cells were immunostained and examined under a fluorescent microscope (NiKon).

Tube formation assay

Assay of the capillary tube-like structure formation of HUVEC was performed with commercial BD Matrigel. 96-well plates were coated with 100 μl Matrigel and incubated at 37 °C for 30 min to promote gelling. HUVEC cells were plated in confluent monolayers of 1×10^5cells per well in complete medium. PiB was added in given concentration. After 6h incubation, tube formation was evaluated bymicroscope.

Immunocytochemistry of PIN1 and HIF-1α

To demonstrate the nuclear translocation of PIN1 and HIF-1α, immunocytochemistry was performed. HCT-116 cells were plated on the chamber slide, were treated with PiB for 4h and then grown at 1% O_2 in a hypoxia chamberfor additional 4h. After fixation with 4% paraformaldehyde solution for 15 min at room temperature. After a rinse with PBS, cells were permeabilized with 0.1% Triton X-100(5min) and blocked with 5% bovine serum albumin(BSA) in PBST(30min). Anti-HIF 1α and Anti-PIN1 antibodies, diluted 1:100 in 3% BSA in PBS, were applied overnight at 4°C. After washing with PBS, samples were incubated with diluted (1:1,000) FITC-conjugated anti-rabbit and TRITC conjugated anti-mouse IgG secondary antibody for 1 h at room temperature. After washing (twice for 5min each), cells were treated with DAPI. The signals were detected using an inverted microscope Eclipse Ti-U (Nikon, Tokyo, Japan).

Aortic ring assay

Aortas excised from juvenile male Sprague Dawley rats were cleaned of periadventitial fat and connective tissues and cut into 1 to 1.5 mm long rings, placed into a 96 well coated with Matrigel, and then covered with another layer of Matrigel. Then, 150ul FBS-free DMEMcontaining VEGF (100ng/ml) and bFGF (100ng/ml) was added to each well. In some cases, PiB was added to the cultures in a dose dependent manner. The cultures were kept 37℃ in a humidified environment five-day cultures werephotographed using a Nikon microscope. For

quantitative assessment of sprouting, the sprouting of tissue was assessed using Image J software (National Institutes of Health)

In vivo Matrigel Angiogenesis Assay

To assess angiogenic effects *in vivo*, growth factor-reduced BD Matrigel (0.5ml) containing HCT-116 cells (4×10^6 cells/ml),heparin (10U/ml) andvarious concentrations of PiB were subcutaneously injected into BALB/c nude mice near the abdominal midline. 10 days after injection, mice were euthanized, and the Matrigel plugs were surgically removed. For macroscopic analysis of angiogenesis, hemoglobin content in Matrigel was quantifiedby using Drabkin's reagents (Sigma-Aldrich) by adding homogenated Matriglel. After thorough mixing, absorbance was measured by spectrometer at wave length 540nm to estimate hemoglobin.

Statistical analysis

When necessary, data were expressed as means ± SD of at least three independent experiments and statistical analysis for single comparison was performed using the Student's *t*-test.

Results

PPIase activity of PIN1 is decreased by PiB under hypoxic conditions

PiBhas a double-ring structure which isaknown PIN1 inhibitor **(Fig. 1A)**. To investigate whether PiB inhibits PIN1 activity, a PPIase assay was performed. When HCT-116 cells were treated with PiB under hypoxia, the activity of PIN1 was significantly reduced compared to the control **(Fig. 1B)**. According to the previous study, PiB selectively inhibitsthe catalytic activity of PIN1by competitively binding to the active site of PIN1 [13].

PiB inhibits interaction betweenPIN1 and HIF-1α

We performed a GST pull-down assay to determine whether PiB affects direct interaction of PIN1 with HIF-1α. After HCT-116 cells were transfected with HA-HIF-α, these cells were exposed to 1% O_2. The cell extract was then incubated with GST-PIN1 binding PiB. The interaction between PIN1 and HIF-1α was decreased by PiB **(Fig. 2A)**. We then determined whether endogenous HIF-1α interacts with PIN1 in HCT-116 cells using an *in situ* proximity assay. This assay measures nuclear localization of protein-protein interaction at single molecule resolution. We detected a number of strong fluorescence signals after 4h hypoxic conditions, indicative of interaction between HIF-1α and PIN1 in the nucleus,

whereas very weak signals were detected in PiB-treated HCT-116 cells **(Fig. 2B)**.

HIF-1α accumulation is decreased by PiB

When HCT-116 cells were treated with PiB under normoxic conditions for 4h and then maintained at 1% O_2 in a hypoxia chamber for additional 4h, the HIF-1α protein level was gradually decreased by PiB treatment. PIN1 expression was prolonged throughout PiB treatment. Also, the phsophorylated PIN1 (p-PIN1) level which is an inactive form of PIN1 was increased by PiB **(Fig. 3A)**. To determine whether PiB affects the HIF-2α protein level, HCT-116 cells were exposed to 1% O_2 for 24 h after these cells were treated with PiB under normoxic conditions for 4h. The HIF-2α protein level didn't change. However, HIF-1α, PIN1 and pPIN1 protein levels showed the same patterns under hypoxic conditions of 4h **(Fig. 3B)**. Next, we examined the changeat the mRNA level. These levels were not affected by PiB**(Fig. 3C)**. These results suggest that PiB affectsthe stability of HIF-1α protein.

Expression of HIF-1α and PIN1 under hypoxic conditions

To investigate the localization of HIF-1α and PIN1, we separated cytosol and nucleus of HCT -116 cells. PIN1 was localized not only in the nucleus but also in the cytoplasm. However,HIF-1α existed predominantly in the nucleus and its

expression was gradually decreased by PiB under hypoxic conditions **(Fig. 4A)**.Nuclear colocalization of HIF-1α and PIN1 was further confirmed by immunocytochemistry and this was inhibited by PiB **(Fig. 4B)**.

PIN1 and PiB were involved in the hypoxia inducible transcriptional activity of HIF-1

Next we investigated the role of PIN1 in HIF-1 transcriptional activity. HCT-116 cells were transfected with HRE-Luciferase construct (PGL3-Luc, HRE-Luc) or EPO-Luciferase construct (PGL3-Luc, EPO-Luc) and then treated with PiB in normoxic conditions for 4 h and additionally hypoxic conditions for 4 h. As shown in **Fig. 5A and B**, hypoxia increased the luciferase activity indicating that HIF-1α was indeed activated in these conditions. Compared to DMSO-treated cells, treatment of PiB strongly decreased the EPO and HRE luciferase activity. These results suggest that PIN1 plays a role in the regulation of hypoxia-induced activity of HIF-1.

HIF-1α protein stability was reduced by PiB

HIF-1α is stabilized under hypoxic conditions. Under normoxic conditions, however, it is degraded by E3-ubiquitin ligase proteasome. Therefore, we determined whether PIN1 inhibition affected the stability of HIF-1α. We measured

HIF-1α protein levels after inhibition of protein biosynthesis with cyclohexamide. In the absence of PiB, the half-life of HIF-1α protein was 25 min, whereas in the presence of PiB, its half-life was 45 min **(Fig. 6)**. These results suggest that PIN1 regulates HIF-1α stability by interfering with its proteasomal degradation.

PiB affects VEGF-A expression

To determine whether PiB affects VEGF-A, we performed Western blot analysis. When HCT-116 cells were treated with PiB under normoxic conditions for 4 h and then at 1% O_2 in a hypoxia chamber for additional 48 h. PIN1 expression was prolonged in the presence or absenceof PiB.However, these cells showed the reduced VEGF-A protein expression. PiBreduced the expression of VEGF-A proteinand its mRNA transcript under hypoxic conditions **(Fig. 7A and 7B)**.

PiB inhibits angiogenensis in* in vitro *and* in vivo *angiogenesis assays

To investigate whether PiB can inhibit hypoxia-induced angiogenesis, we performed a tube formation assay using HUVEC cells treated with PiB under hypoxic conditions. As shown in **Fig. 8**, tube structures were significantly inhibited in cells treated with a high concentration of PiB. To confirm the anti-angiogenic effect of PiB, we performed an*in vitro*rat aortic ring assasy. As shown in **Fig.**

9A,new blood vessel growth from rat aortic rings was strongly inhibited upon treatment with PiB. Also, we evaluated the anti-angiogenic effect of PiB using the matrigel plug-in assay that is widely used to detect newly formed blood vessel *in vivo*. Matrigel plugs containing HCT-116 cells and three different doses of PiB were injected subcutaneously in mice and the degree of vascularization into matrigel plugs was evaluated after 10 days. Macroscopic analysis of matrigel plugs in mice without PiB treatment showed intense vascularization, whereas the matrigel implant in mice treated with PiB showed weaker angiogenesis. The angiogenic response observed by macroscopic analysis was consistent with quantitative results obtained by measuring the Hb levels in the matrigel. Hb content in the matrigel plugs in mice treated with PiB was significantly lower **(Fig. 9B)**.

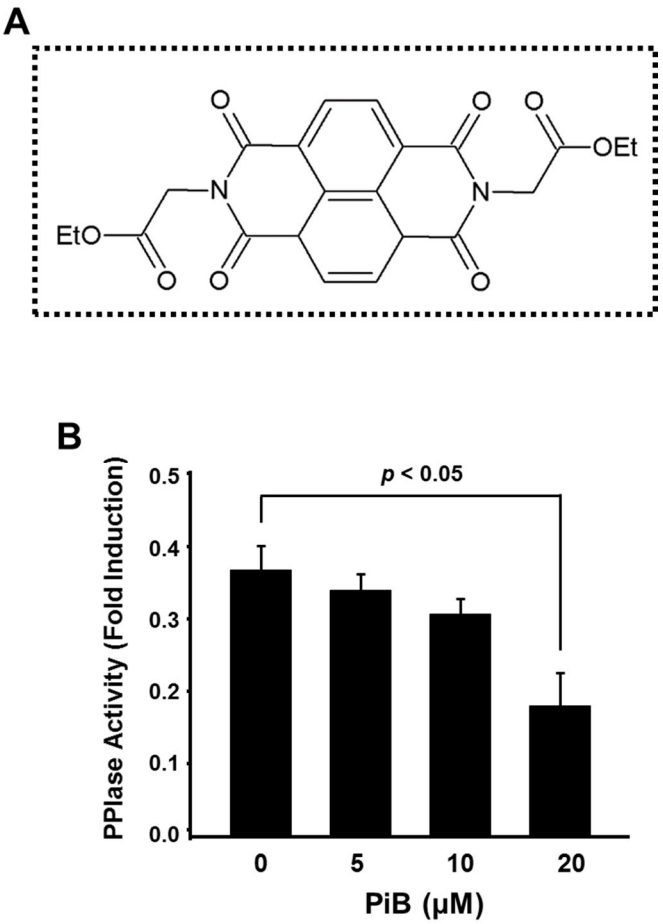

Fig. 1. The chemical stucture of PiB and its effects on PIN1 activity.(A) Chemical structure of PiB (B) HCT-116 cells were treated with PiB in normoxic conditions for 4h, and then maintained at 1% O_2 in a hypoxia chamber for additional 4h. Cell lysates were placed in a cuvette, and the substrate of PIN1 and α-chymotrypsin were added. After incubation at room temperature, the absorbance was measured at 390nm.

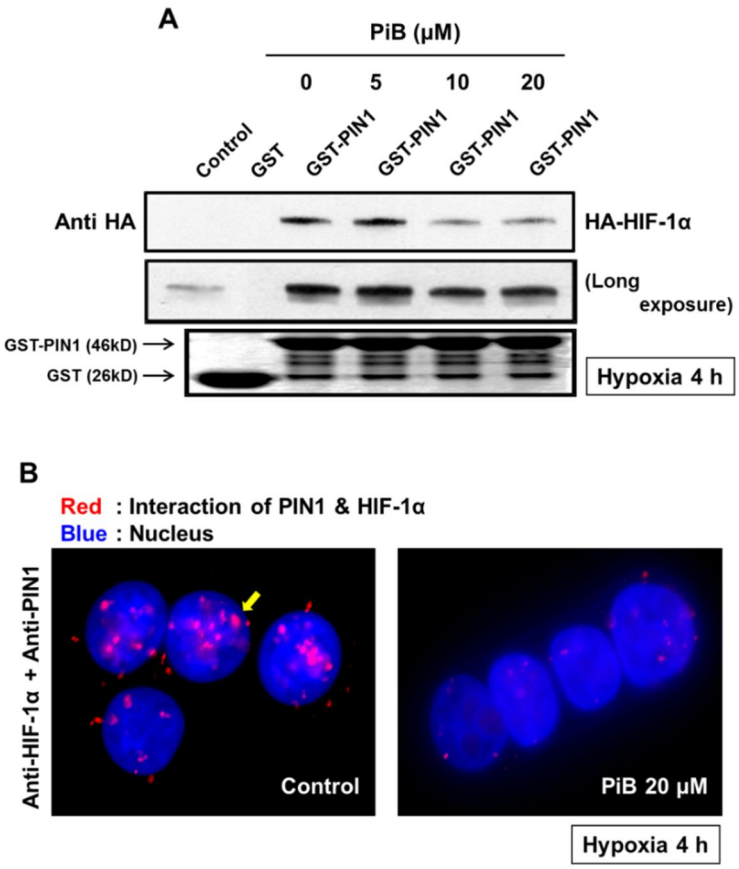

Fig. 2.Inhibition ofinteraction between PIN1 and HIF-1α and its inhibition by PiB.(A) *In vitro* interaction of PIN1 with HIF-1α under hypoxic conditions. HCT-116 cells were transfected with HA-HIF-1α and grown in hypoxic condition for 4h. The cell lysate was incubated with GST-PIN1 binding PiB. After incubation, interaction between PIN1 and HA-HIF-1α was determined by Western blot analysis. (B) *In situ* interaction of PIN1 and HIF-1α under hypoxic conditions. HCT-116 cellswere grown in normoxia in the absence or presence of PiB and then at 1% O_2

in a hypoxia chamber.Then these cells were determined by the proximity assay.

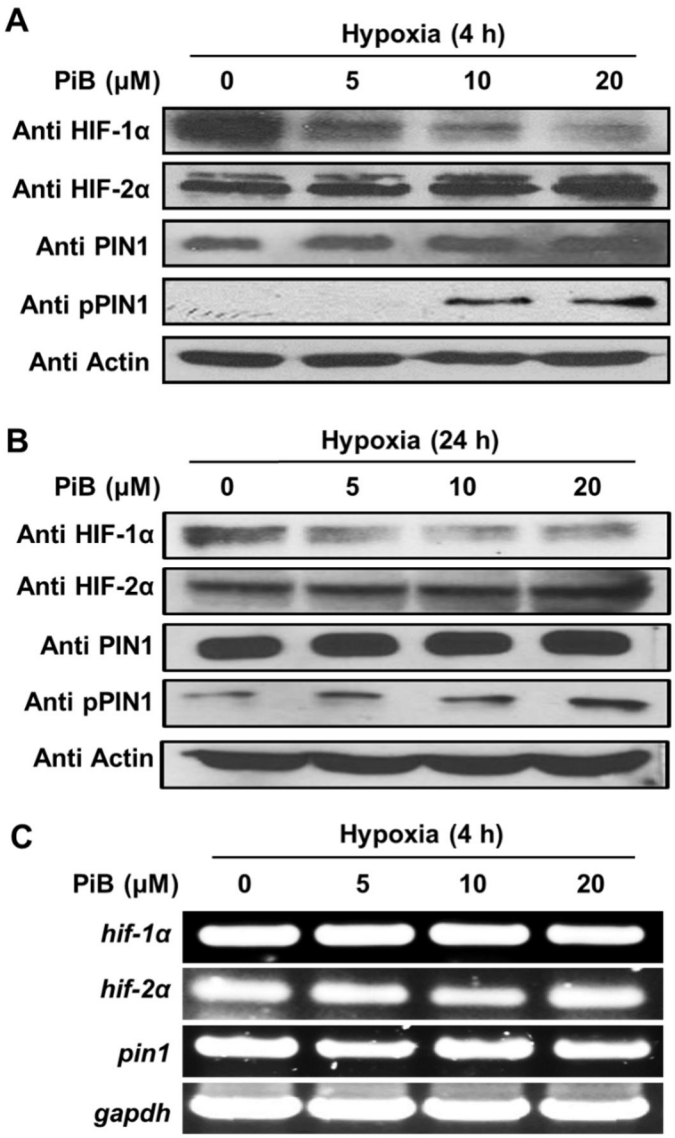

Fig. 3. Effects of PiB on the expression levels of HIF-1α and PIN1.(A),(B)Expression of HIF-1α, HIF-2α, PIN1 and pPIN1 were determined by Western blot analysis in the absence or presence ofthe indicated concentrations of PiB under hypoxic conditions for 4 h and 24 h. (C)Levels of HIF-1α, HIF-2α and

PIN1 were determined by RT-PCR analysis under hypoxia. HCT-116 cells were treated three different concentration of PiB and were grown in normoxia and then at 1% O_2 in a hypoxia chamber

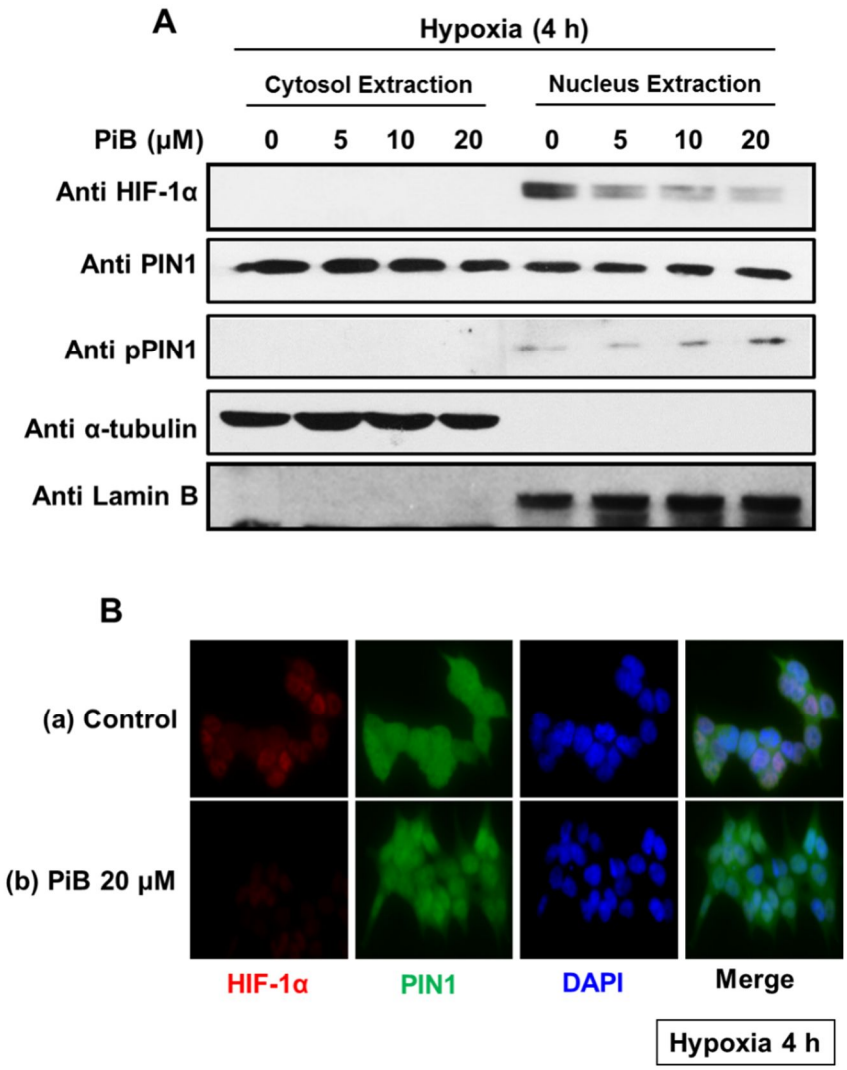

Fig. 4. Expression of HIF-1α and PIN1 under hypoxic conditions.(A) Cytosolic and nuclear extracts were immunoblotted for the measurement of HIF-1α and PIN1 localization under hypoxic conditions.Lamin B and α-tubulinwere measured to ensure accurate separation of nucleus and cytoplasm.(B) Nuclear colocalization of PIN1 and HIF-1α was determined by immunocytochemistry. HCT-116 cells plated

on the chamber slide were treated with PiB for 4 h and then grown at 1% O_2 in a hypoxia chamberfor additional 4 h. Anti-HIF 1α and anti-Pin1 antibodies were applied and then samples were incubated with secondary antibody. Lastly, cells were treated with DAPI. The signals were detected using an inverted microscope.

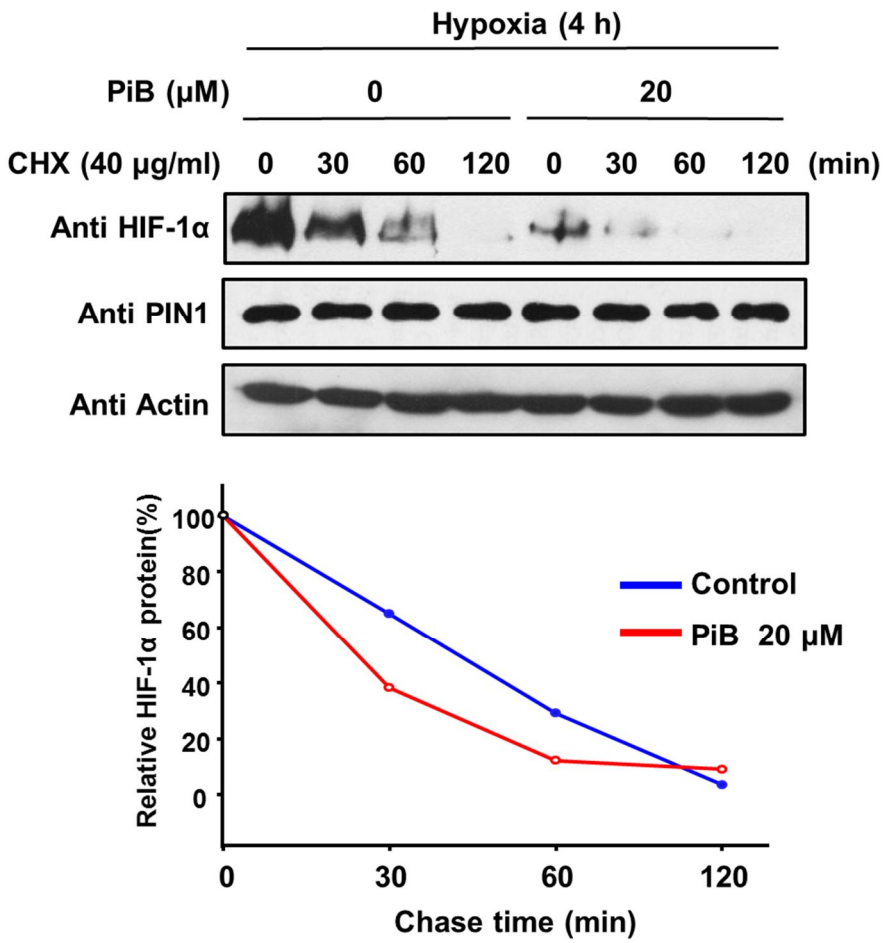

Fig. 5. Effect of PiB on the stability of HIF-1α in HCT-116 cells. Cells were treated with PiB under normoxic conditions for 4h and then incubated in 1% O_2 for 4h with cycloheximide (40 μg/ml), an inhibitor of protein biosynthesis for the indicated time periods. The expression of HIF-1α was assessed by Western blot analysis.Thegraph indicates half-lives of HIF-1αin the presence and absence of PiB.

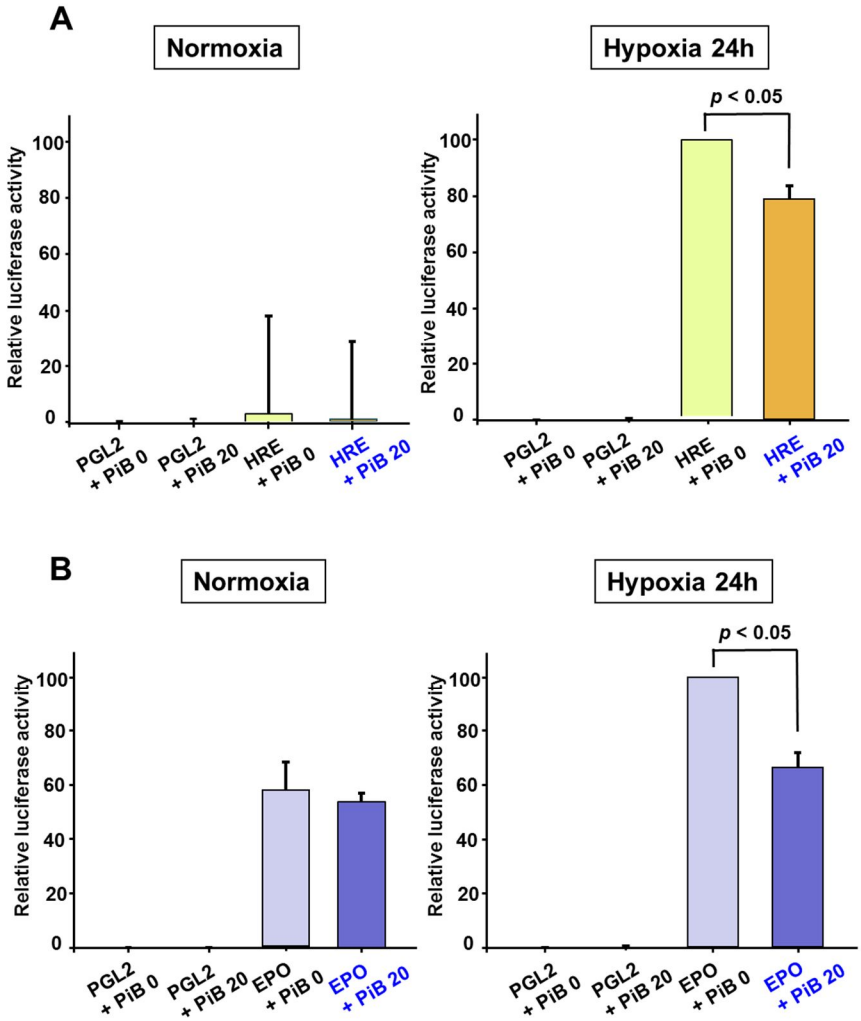

Fig. 6. Effectsof PiB in HIF-1α-mediated HRE and EPO activity.(A) Cells were transfected with HRE-luciferase construct (PGL3-Luc, HRE-Luc) after treatment of PiB. (B) Cells were transfected with EPO-luciferase construct (PGL3-Luc, EPO-Luc) after treatment of PiB. The luciferase activities were measured under normoxic or hypoxic conditions. PGL3-Luc plasmid was used as the mock vector. Luciferase activities were normalized by co-transfection with β-gal. Values represent means ± S.D. of three samples.

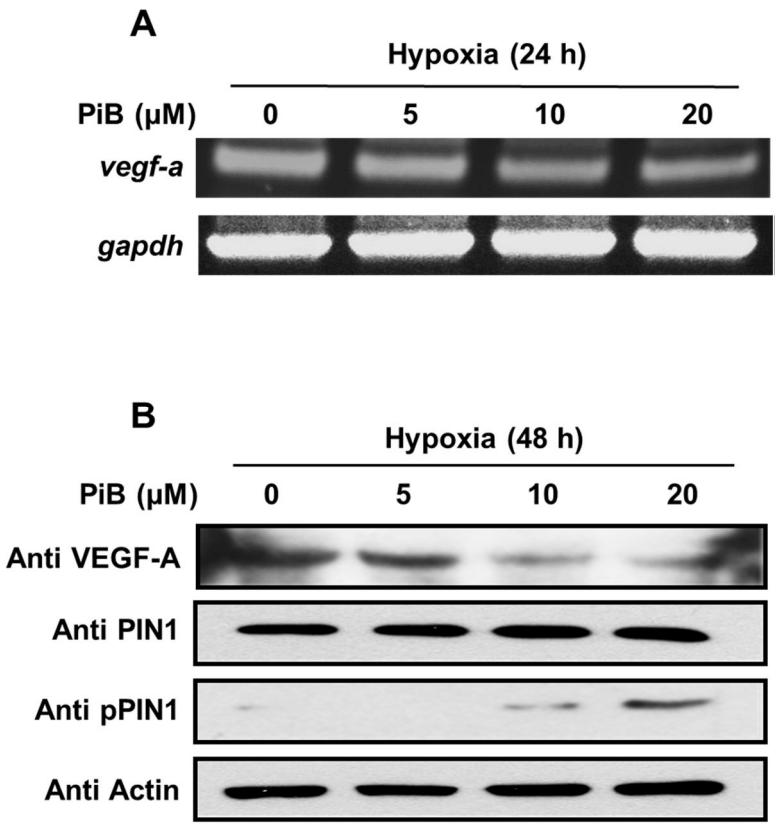

Fig. 7. Effects of PiB on expression of PIN1 and VEGF-A under hypoxic conditions. (A)The mRNA levels of VEGF-A and GAPDH were determined by quatitative RT-PCRat in the absence or presence of the indicated concentrations of PiB under hypoxic conditions for 24 h..(B) HCT-116 cells were treated with PiB under hypoxic conditions for 48 h. Lysates were immunoblotted with the indicated antibodies.Actin and GAPDH were used as internal controls.

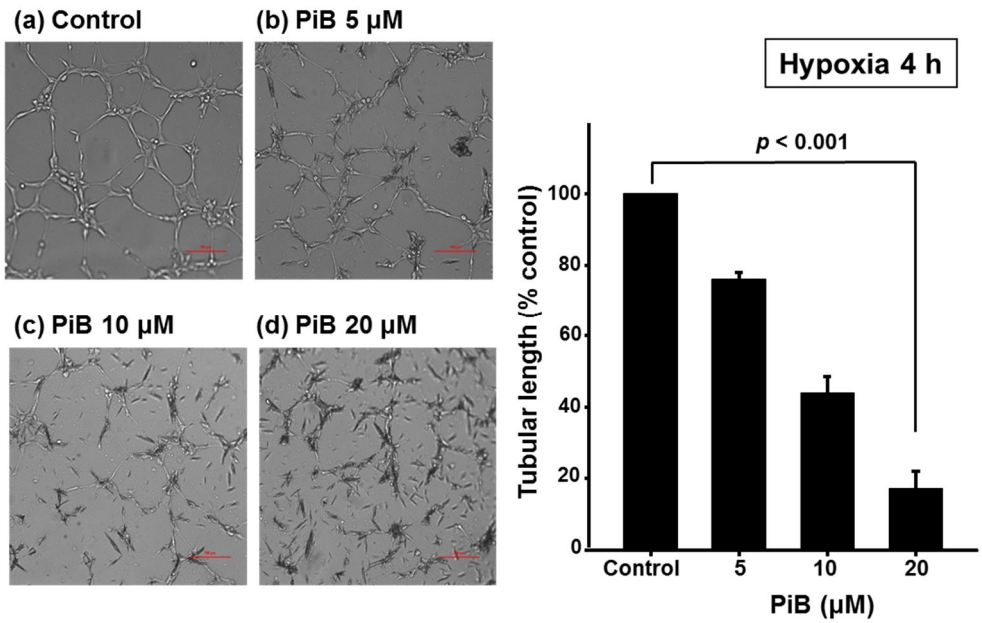

Fig. 8. Effect of PiB on tube formation in HUVEC cells under hypoxic conditions.HUVEC cells were plated in confluent monolayers of 1×10^5cells per well in complete medium. After 6 h incubation in the absence or presence of PiB, tube formation was evaluated by microscope.Tube formation was evaluated after 6h by under a microscope. Tube formations were quantified on the graph.

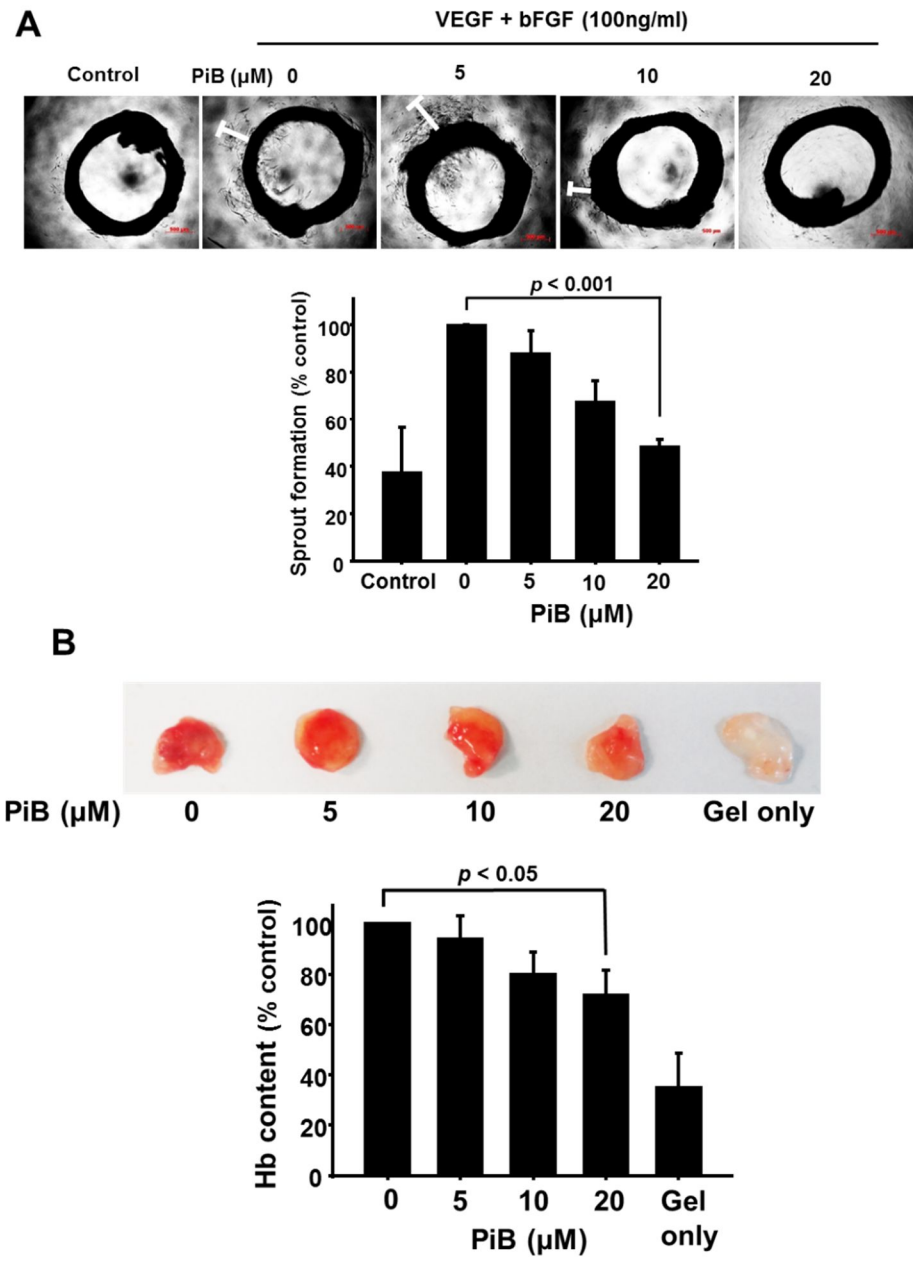
A
VEGF + bFGF (100ng/ml)
Control
PiB (μM) 0
5
10
20
p < 0.001
Sprout formation (% control)
100
80
60
40
20
0
Control
0
5
10
20
PiB (μM)
B
PiB (μM)
0
5
10
20
Gel only
p < 0.05
Hb content (% control)
100
80
60
40
20
0
0
5
10
20
Gel only
PiB (μM)

Fig. 9. Inhibition of angiogenesis by PiB.(A) The aortic rings from 6-week-old male Sprague-Dawley rats were ramdomly seeded into Matrigel-coated wells and sealed with an overlay of Matrigel. PiB, VEGF and bFGF in serum-free DMEM were added into the wells. After 5 days, microvessel sprouting was photographed using a microscope and vascular areas of each treatment were calculated (n=6). (B) HCT116 cells were suspended in Matrigel, DMEM and PiB in the indicated concentrations and subcutaneously injected into the flanks of mice photographed. The relative amount of angiogenesis was analyzed based on the RBC hemoglobin level, determined using the Drabkin method. The data are presented as the mean ± S.D. for each group.

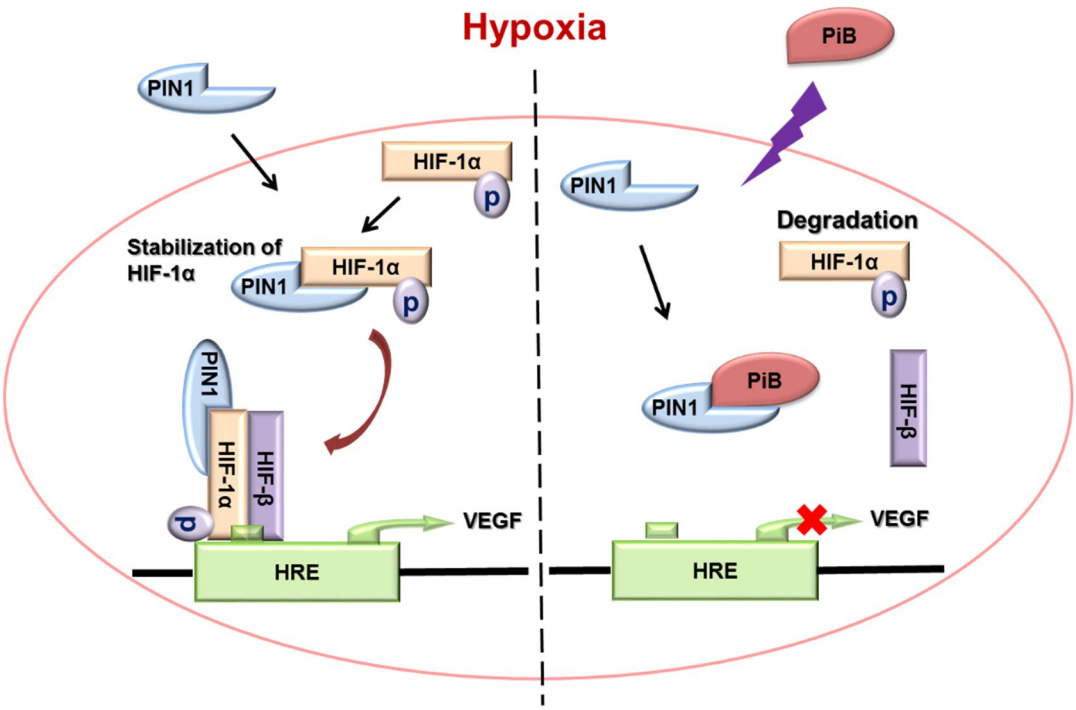

Fig. 10. Proposed mechanisms underlying the inhibitory effect of PiB on interaction between PIN1 and HIF-1α.

Discussion

PIN1 is an enzyme that specifically binds to phosphorylated Ser/Thr-Pro motifs and induces the conformational changes in phosphorylated proteins [15]. The PIN1-mediated isomerization has been considered to control many protein functions including the protein-protein interaction, catalytic activity and stability of proteins involved in cell cycle regulation and proliferation, thereby promoting carcinogenesis [16, 17]. PIN1 overexpression is observed in a number of tumors including colon cancer [18] and is considered a prognostic marker in cancer [16]. In solid tumors, the most common phenomenon is hypoxia. Under oxygen shortage, HIF-1α, an oxygen dependent transcriptional activator, is stabilized and activated in tumors as a means of adaptation and survival[19, 20]. Protein levels of HIF-1α are very low by the proteasomal degradation under normoxic conditions. However, HIF-1α is stabilized and accumulated in hypoxia [21]. The stabilized HIF-1α subunit forms a dimer with HIF-1β and binds to HRE of target genes. These genes encode proteins involved in angiogenesis, glucose metabolism, erythropoietin production, cell proliferation, invasion and metastasis [22, 23]. It has been reported that HIF-1α is overexpressed in human cancer cells, and HIF-1α expression correlates with tumor development [24]. HIF-1α is well known as the master regulator of VEGF[25]. The HIF-1α-VEGF axis plays a critical role during tumor angiogenesis [26].Recently, stability of HIF-1αhas been reported to be associated with PIN1 activity in Alzheimer's disease [27]. So, we examined angiogenesis induced by the interaction of PIN1 and HIF-1α in the presence and absence of the

PIN1 inhibitor, PiB. PiB binds PIN1 at the active site and inhibits its activity by blocking substrate binding sites [14]. The present study focused on the possible blockade of angiogenesis by using PiB in human mammary cancer cells.

PiB inhibits the PPIase activity of PIN1 under hypoxic conditions in HCT-116 cells. Our present study demonstrates that PIN1 co-localizes with HIF-1α in nucleus under hypoxic conditions and PiB inhibits direct binding of PIN1 and HIF-1α. In addition, we found that the protein level of HIF-1α was decreasedby PiB whereas the protein level of phosphorylated PIN1 (pPIN1) which is an inactive form of PIN1 was increased. PIN1 also interacts with some cell cycle regulators such as Cdc25 and Plx1 [28] and with transcription factors, such as p53[29]. PIN1 has been reported to differentially regulate activities of several transcription factors through direct physical binding [30]. The data from the present study reveals the direct interaction between PIN1 and HIF-1α under hypoxic conditionsis inhibited by PiB. In the presence of PiB, the stability and expression of HIF-1α were decreased. We found that both HRE and EPO reporter luciferase activities were decreased in the presence of PiB. The half-life of HIF-1α was decreased when in the presence of PiB, indicating PIN1 may play an important role in stabilizing HIF-1α.

Among various hallmarks of cancer, angiogenesis represents an important indicator of cancer progression [31]. VEGF plays a crucial role as a principal mediator of angiogenesis. There are several transcription factor binding sites within thepromoter region of VEGF gene, among which HIF-1α plays a key role in

regulation of VEGF transcription[32]. It has been reported that PIN1 is an inducer of VEGF [33]. However, there has been no study reported on the effect of PiB on the angiogenesis through inhibition of the interaction between PIN1 and HIF-1α. Here, we found that the mRNA and protein levels of VEGF-A were decreased in PiB treated cells. These findings suggest that PiB reduces both the stability and the expression of HIF-1α by blocking the binding of PIN1 to HIF-1α, and subsequently HIF-1α medicated VEGF-A expression. We found that tube formation formed in HUVEC cells was completely suppressed in the presence of PiB.In addition, an *in vivo* matrigel assay results reveal that PiB is capable of suppressing vessel formation.

In summary, our study demonstrates that PiB inhibits VEGF-mediatedangiogenesis by disrupting stabilization of HIF-1α through disruption of its interaction with PIN1, suggesting that PiB can be a potent therapeutic agent for angiogenic disorders, such as cancer.

References

1. Ryo, A., et al., *Stable suppression of tumorigenicity by Pin1-targeted RNA interference in prostate cancer.* Clin Cancer Res, 2005. **11**(20): p. 7523-31.

2. Lu, K.P. and X.Z. Zhou, *The prolyl isomerase PIN1: a pivotal new twist in phosphorylation signalling and disease.* Nat Rev Mol Cell Biol, 2007. **8**(11): p. 904-16.

3. Liou, Y.C., X.Z. Zhou, and K.P. Lu, *Prolyl isomerase Pin1 as a molecular switch to determine the fate of phosphoproteins.* Trends Biochem Sci, 2011. **36**(10): p. 501-14.

4. Innes, B.T., et al., *Non-catalytic participation of the Pin1 peptidyl-prolyl isomerase domain in target binding.* Front Physiol, 2013. **4**: p. 18.

5. Nicole Tsang, Y.H., et al., *Prolyl isomerase Pin1 downregulates tumor suppressor RUNX3 in breast cancer.* Oncogene, 2013. **32**(12): p. 1488-96.

6. Boussetta, T., et al., *The prolyl isomerase Pin1 acts as a novel molecular switch for TNF-alpha-induced priming of the NADPH oxidase in human neutrophils.* Blood, 2010. **116**(26): p. 5795-802.

7. Harris, A.L., *Hypoxia--a key regulatory factor in tumour growth.* Nat Rev Cancer, 2002. **2**(1): p. 38-47.

8. Koshiji, M., et al., *HIF-1alpha induces cell cycle arrest by functionally counteracting Myc.* EMBO J, 2004. **23**(9): p. 1949-56.

9. Fang, J., et al., *Apigenin inhibits tumor angiogenesis through decreasing HIF-1alpha and VEGF expression.* Carcinogenesis, 2007. **28**(4): p. 858-64.

10. Greer, S.N., et al., *The updated biology of hypoxia-inducible factor.* EMBO J, 2012. **31**(11): p. 2448-60.

11. Toffoli, S., et al., *Intermittent hypoxia changes HIF-1alpha phosphorylation pattern in endothelial cells: unravelling of a new PKA-dependent regulation of HIF-1alpha.* Biochim Biophys Acta, 2007. **1773**(10): p. 1558-71.

12. Dales, J.P., et al., *Overexpression of hypoxia-inducible factor HIF-1alpha predicts early relapse in breast cancer: retrospective study in a series of 745 patients.* Int J Cancer, 2005. **116**(5): p. 734-9.

13. Uchida, T., et al., *Pin1 and Par14 peptidyl prolyl isomerase inhibitors block cell proliferation.* Chem Biol, 2003. **10**(1): p. 15-24.

14. Moretto-Zita, M., et al., *Phosphorylation stabilizes Nanog by promoting its interaction with Pin1.* Proc Natl Acad Sci U S A, 2010. **107**(30): p. 13312-7.

15. Kim, J.A., et al., *Amurensin G inhibits angiogenesis and tumor growth of tamoxifen-resistant breast cancer via Pin1 inhibition.* Food Chem Toxicol, 2012. **50**(10): p. 3625-34.

16. Bao, L., et al., *Prevalent overexpression of prolyl isomerase Pin1 in human cancers.* Am J Pathol, 2004. **164**(5): p. 1727-37.

17. Lee, T.H., et al., *Death-associated protein kinase 1 phosphorylates Pin1 and inhibits its prolyl isomerase activity and cellular function.* Mol Cell, 2011. **42**(2): p. 147-59.

18. Duncan, K.E., et al., *Discovery and characterization of a nonphosphorylated cyclic peptide inhibitor of the peptidylprolyl isomerase, Pin1.* J Med Chem, 2011. **54**(11): p. 3854-65.

19. Lee, J.W., et al., *Hypoxia-inducible factor (HIF-1)alpha: its protein stability and biological functions.* Exp Mol Med, 2004. **36**(1): p. 1-12.

20. Semenza, G.L., *Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy.* Trends Pharmacol Sci, 2012. **33**(4): p. 207-14.

21. Mylonis, I., et al., *Identification of MAPK phosphorylation sites and their role in the localization and activity of hypoxia-inducible factor-1alpha.* J Biol Chem, 2006. **281**(44): p. 33095-106.

22. Uchida, T., et al., *Prolonged hypoxia differentially regulates hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression in lung epithelial cells: implication of natural antisense HIF-1alpha.* J Biol Chem, 2004. **279**(15): p. 14871-8.

23. Kim, K.H., H.J. Jung, and H.J. Kwon, *A new anti-angiogenic small molecule, G0811, inhibits angiogenesis via targeting hypoxia inducible factor (HIF)-1alpha signal transduction.* Biochem Biophys Res Commun, 2013. **441**(2): p. 399-404.

24. Isaacs, J.S., et al., *HIF overexpression correlates with biallelic loss of*

fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability. Cancer Cell, 2005. **8**(2): p. 143-53.

25. Eubank, T.D., et al., *Opposing roles for HIF-1alpha and HIF-2alpha in the regulation of angiogenesis by mononuclear phagocytes.* Blood, 2011. **117**(1): p. 323-32.

26. Nordgren, I.K. and A. Tavassoli, *Targeting tumour angiogenesis with small molecule inhibitors of hypoxia inducible factor.* Chem Soc Rev, 2011. **40**(8): p. 4307-17.

27. Lonati, E., et al., *Pin1, a new player in the fate of HIF-1alpha degradation: an hypothetical mechanism inside vascular damage as Alzheimer's disease risk factor.* Front Cell Neurosci, 2014. **8**: p. 1.

28. Crenshaw, D.G., et al., *The mitotic peptidyl-prolyl isomerase, Pin1, interacts with Cdc25 and Plx1.* EMBO J, 1998. **17**(5): p. 1315-27.

29. Wulf, G.M., et al., *Role of Pin1 in the regulation of p53 stability and p21 transactivation, and cell cycle checkpoints in response to DNA damage.* J Biol Chem, 2002. **277**(50): p. 47976-9.

30. Wulf, G., et al., *Phosphorylation-specific prolyl isomerization: is there an underlying theme?* Nat Cell Biol, 2005. **7**(5): p. 435-41.

31. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.

32. Wang, F.S., et al., *Ras induction of superoxide activates ERK-dependent angiogenic transcription factor HIF-1alpha and VEGF-A expression in shock wave-stimulated osteoblasts.* J Biol Chem, 2004. **279**(11): p. 10331-7.

33. Kim, M.R., et al., *Induction of vascular endothelial growth factor by peptidyl-prolyl isomerase Pin1 in breast cancer cells.* Biochem Biophys Res Commun, 2008. **369**(2): p. 547-53.

국문초록

PIN1은 peptidyl-prolyl isomerase로 인산화 단백질의 입체 형태를 바꿀 수 있어 단백질 기능과 안정도에 영향을 주며 세포주기 발달에서 중요한 역할을 하는 많은 단백질들을 조절한다. 또한 본 실험의 선행연구를 통해 대부분의 암 조직에서의 PIN1의 축적농도가 정상조직에 비해 높은 수준으로 존재한다는 것을 밝혔다. 따라서 PIN1의 과발현은 과대 증식 질병인 암의 성장에 영향을 줄 수 있다고 사료된다. Mammalian cell은 저산소 상태인 Hypoxia 상태를 극복하기 위하면 Hypoxia inducible factor (HIF)라는 전사인자를 활성화시킨다. 그 중 HIF-1도 종양 조직에서 통상 그 발현이 증가되어 있는 전사인자로서 VEGF와 같이 혈관 신생이나 암화 과정에 관여하는 단백질의 합성 유도에 관여한다. 선행연구로 알츠하이머에서 HIF-1α와 PIN1이 연관되어 있다고 밝혀졌다. 그래서 본 연구에서는 PIN1의 inhibitor인 PiB를 이용하여 HIF-1α와 연관되어 혈관신생을 유발하는 VEGF를 발현시키는 기전에 대해 연구하였다. PiB는 PIN1의 active site에 결합하는 specific inhibitor이다. Colorectal cancer cell인 HCT-116 cell에서 Hypoxia 상태일 때 PiB의 활성을 나타내는지 보기 위해 PIN1 activity 실험을 실행하여 PiB가 PIN1의 활성을 저해한다는 것을 확인하였다. PiB로 인해 PIN1과 HIF-1α의 direct binding이 저해되었다. HCT-116 세포에 PiB를 농도별로 처리하였을 때, HIF-1α와 VEGF-A의 축적이 억제됨을 확인하였다. PiB를 전처리하고 단백질 합성을 억제하는 cyclohexamide를 처리한 경우에도 HIF-1α의 발현이 더 빠르게 감소하였다. 이는 PIN1이 HIF-1α의 안정도 조절에 관여하고 이를 PiB가 저해한다는 것

을 시사한다. HIF-1α가 최종적으로 결합하여 target gene을 발현시키는 HRE와 EPO luciferase activity를 측정하였을 때도 PiB를 처리한 경우에 그 활성도가 현저히 감소함을 확인하였다. 또한 HUVEC 세포를 이용한 tube 형성을 관찰하였을 때 역시 PiB를 처리하여 PIN1 activity를 저해시킨 세포에서 tube의 형성이 잘 이루어지지 않았고, Aortic ring assay에서 PiB를 처리한 rat 혈관에서 혈관신생작용이 확연히 억제됨을 확인하였다. 또한, *in vivo* Matrigel assay를 통해 PiB를 처리한 Matrigel에서 혈관신생 작용이 줄어들었다. 결론적으로 PIN1이 HIF-1α에 결합하여 그 안정도를 높여 혈관신생을 유발하는 VEGF의 발현을 증가시키는데 PiB가 PIN1과 HIF-1α의 결합을 방해하여 VEGF의 발현을 저해시키며, 그로 인해 암화 과정이 감소될 것으로 사료된다.