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Molecules and Cells

Vitexin, an HIF-1α Inhibitor, Has Anti-metastatic Potential in PC12 Cells

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Vitexin, a natural flavonoid compound identified as apigenin-8-C-β-D-glucopyranoside, has been reported to exhibit antioxidative and anti-inflammatory properties. In this study, we investigated its effect on hypoxia-inducible factor-1α (HIF-1α) in rat pheochromacytoma (PC12), human osteosarcoma (HOS) and human hepatoma (HepG2) cells. Vitexin inhibited HIF-1α in PC12 cells, but not in HOS or HepG2 cells. In addition, it diminished the mRNA levels of hypoxia-inducible genes such as vascular endothelial growth factor (VEGF), smad3, aldolase A, enolase 1, and collagen type III in the PC12 cells. We found that vitexin inhibited the migration of PC12 cells as well as their invasion rates, and it also inhibited tube formation by human umbilical vein endothelium cells (HUVECs). Interestingly, vitexin inhibited the hypoxia-induced activation of c-jun N-terminal kinase (JNK), but not of extracellular-signal regulated protein kinase (ERK), implying that it acts in part via the JNK pathway. Overall, these results suggest the potential use of vitexin as a treatment for diseases such as cancer.

Keywords: c-jun N-terminal Kinase; Hypoxia-inducible Factor-1α; Vascular Endothelial Growth Factor; Vitexin.

Introduction

Hypoxia-inducible factors (HIF) are transcription factors that turn up the activity of a variety of genes when oxygen becomes scarce, which can happen in a variety of both normal and pathological conditions. The genes controlled by the HIFs include those coding for proteins that stimulate red blood cell production and angiogenesis, as well as for glycolytic enzymes that can produce energy from glucose without the aid of oxygen (Zagorska and Dulak, 2004). HIFs are heterodimers composed of one of the three α subunits (HIF-1α, HIF-2α or HIF-3α) and the β subunit (HIF-1β) or ARNT [aryl hydrocarbon receptor nuclear translocator]). HIF-1α is ubiquitously expressed while two other members of this family (HIF-2α or HIF-3α) are tissue specific (Wenger, 2002). Although HIF-1β is readily detectible in all cells, HIF-1α is virtually undetectable in normoxic conditions. When cells are subjected to hypoxic conditions, the expression of HIF-1α protein rapidly increases. Instead of acting on the transcription or translation of HIF-1α, hypoxia increases HIF-1α protein levels by inhibiting the rapid ubiquitination and proteasomal degradation of HIF-1α protein (Page *et al.*, 2002). HIF-1α is involved in various human diseases such as cancer (Vaupel, 2004), heart disease (Semenza, 2004), preeclampsia (Rajakumar *et al*., 2003), liver cirrhosis (Cejudo-Martin *et al.*, 2002), and rheumatoid arthritis (Hollander *et al.*, 2001). Vascular endothelial growth factor (VEGF), the major mediator of angiogenesis and vascular permeability, is induced by HIF-1α (Tsuzuki *et al.*, 2000), and hypoxia is an important selective force in the clonal evolution of tumors (Graeber *et al.*, 1996). HIF-1α is overexpressed in common human cancers (Zhong *et al.*, 1999) and thereby up-regulates the expression of various genes favoring tumor growth, metastasis, and resistance to anticancer therapies (Park *et al.*, 2004). Since HIF-1α regulates the expression of essential genes associated with hypoxia, modulation of HIF-1α

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Abbreviations: HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor.

activity may be a good strategy for treating a wide rage of hypoxia- or ischemia-related pathologies. HIF-1α inhibitors may be useful for treating various diseases associated with the over-activation of HIF-1α, such as tumors, cardiovascular remodeling, preeclampsia, and other angiogenesis-related diseases.

Vitexin, identified as apigenin-8-C-β-D-glucopyranoside, is a flavonoid compound found in *Anthurium versicolor* (Aquino *et al.*, 2001), *Ficaria verna Huds.* (Ranunculaceae) (Tomczyk *et al.*, 2002), *Cucumis sativus L.* (Cucurbitaceae) (McNally *et al.*, 2003), and *Acer palmatum* (Aceraceae) (Kim *et al.*, 2005). It has antioxidative (Kim *et al.*, 2005) and antiviral activity (Li *et al.*, 2002), and other potent actions (Gaitan *et al.*, 1995; Hien *et al.*, 2002). However nothing is known of its inhibitory effect on HIF-1α. Here, we report that it inhibits HIF-1α in PC12 cells and that this leads to a decrease in the level of hypoxia-induced genes such as those encoding VEGF, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), collagen III, aldolase A, enolase 1, transforming growth factor-β1 (TGF-β1), and platelet-derived growth factor α (PDGFα). We also performed *in vitro* experiments to examine the capacity of vitexin to attenuate the metastatic potential of PC12 cells and neovascularization by HUVECs.

Materials and Methods

Materials Cell culture media, fetal bovine serum (FBS) and horse serum were obtained from Invitrogen (USA). All of the other chemicals were purchased from Sigma (USA) unless otherwise indicated.

Cell culture and treatment Rat pheochromocytoma PC12 cells, HepG2 (human hepatocellular carcinoma) and HOS (human osteosarcoma) cells were purchased from the American Type Culture Collection (ATCC). PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). HepG2 and HOS cells were grown in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Human umbilical vein endothelium cells (HUVECs) were purchased from Clonetics (USA) and grown in EGM-2 supplemented with growth factors (Clonetics). All cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C. For incubation of cells in a hypoxic state, they were incubated in serum-free media for 18 h in an airtight chamber (Thermo Forma Co., Marietta, OH, USA) which was flushed with a mixture of 1% O_2, 5% CO_2 and 94% N_2 for the indicated times at 37°C.

Cell viability Cells (5×10^3 cells) were seeded in a 96-well plate in medium supplemented with 10% FBS and incubated for 24 h. They were then serum-starved for 16 h and incubated for 24 h with various concentrations of vitexin. Thereafter they were washed with phosphate-buffered saline (PBS) and treated with 100 μg/ml of MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] for 2 h in a 37°C incubator, washed with PBS, and solubilized with 200 μl of DMSO. The resulting intracellular purple formazan was quantified by absorbance at 540 nm.

Reverse transcription-polymerase chain reaction (RT-PCR) Total RNAs were isolated from cultured cells using TRIzol (Invitrogen, USA) and cDNA synthesis was performed with MMLV reverse transcriptase (Invitrogen). Hypoxia time, PCR condition and primer sequences are listed at Table 1. After initial denaturation at 95°C for 5 min, PCR was performed for various cycles (30 s at 94°C, 1 min at annealing temperature and 2 min at 72°C) using *Taq* polymerase (Promega, USA). Reaction products (10 μl per lane) were electrophoresed in 1% agarose, stained with ethidium bromide and analyzed densitometrically. Band intensities were analyzed by densitometry using a Phosphoimager and Quantity One software (Version 4.3.1) (Bio-Rad).

Western blot analysis Cells were harvested and resuspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 10% glycerol, 1% Trixon X-100, 1 mM Na_3VO_4, and 1 mM PMSF). After centrifugation at 13,000 × *g* for 30 min, supernatants were used as whole cell lysates. For the detection of HIF-1α, cells were resuspended in 500 μl of hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF) for 10 min on ice and centrifuged 13,000 × g for 1 min. The pellets were resuspended in 100 μl of high salt buffer (10 mM HEPES, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF) for 20 min on ice. After centrifugation at 13,000 × *g* for 30 min, the supernatants were used as nuclear extracts. Whole cell lysates or nuclear extracts were separated on 10% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, USA). These were blocked with 5% nonfat skim milk in Tris-buffered saline (TBS) containing 0.5% Tween-20 (TTBS) at room temperature for 1 h and then incubated for 16 h at 4°C with rabbit anti-HIF-1α, anti-VEGF (Santa Cruz Biotechnology, USA), anti-phospho ERK, anti-ERK (Cell Signaling Technology Inc., USA) antibody diluted 1:1000 in 5% nonfat milk in TTBS. Horseradish peroxidase-conjugated anti-rabbit or mouse antibody (Santa Cruz Biotechnology Inc., USA) was used as a secondary antibody (1:5000–1:10000 dilution in 5% nonfat skim milk in TTBS, for 1 h at room temperature) and antigen-antibody complexes were visualized with an ECL Plus kit (Amersham Biosciences, USA). Western blot experiments were repeated at least three times with independent cell preparations.

***In vitro* kinase assay** Immunocomplex JNK activity was assayed as described, with modifications (Soh *et al*., 2000). Cells treated with vitexin for the indicated times under hypoxic conditions were harvested and resuspended in ice-cold lysis buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 0.5 mM dithiothreitol, 12.5 mM β-glycerophosphate,

Table 1. Primer sequences and conditions for RT-PCR.

Target genes	Incubation time in hypoxia (h)	Primers (Forward, reverse)	Annealing Tm (°C)	PCR cycles
HIF-1α	24	5′-ggctttgttatggtgctaac-3′ 5′-acttgatgttcatcgtcctc-3′	49	27
VEGF	24	5′-ctggctttactgctctacct-3′ 5′-gtctgcattcacatctgcta-3′	49	26
PDGF	4	5′-cttggagacaaacctgagag-3′ 5′-tggcttcttcctgacatact-3′	49	25
TGF-β1	4	5′-ccagatcctctccaaactaa-3′ 5′-gctccacagttgacttgaat-3′	49	25
Smad3	24	5′-cctactacgagctgaaccag-3′ 5′-ggttgacagactgagctagg-3′	49	27
TGF-β receptor	24	5′-ccctttttgattact-3′ 5′-aatttcccagaatac-3′	31.5	32
Aldolase A	24	5′-cccaagttatcaagtccaag-3′ 5′-ggtcactcagagccttgtag-3′	48.5	21
Enolase 1	48	5′-gacggcacagagaataaatc-3′ 5′-cttctttgttctccaggatg-3′	48.5	21
GAPDH	48	5′-gaacatcatccctgcatcca-3′ 5′-ccagtgagcttcccgttca-3′	55.5	21
Collagen III	24	5′-cctgaagatgtccttgatgt-3′ 5′-agggtgcgatatctatgatg-3′	48.5	25
β-actin	24	5′-tctgtgtggattggtggctcta-3′ 5′-ctgcttgctgatccacatctg-3′	55.5	22

1 mM Na_3VO_4, and 1 μg/ml leupeptin. Cell debris and particulate fractions were removed by centrifugation at 14,000 × *g* for 20 min at 4°C. To assay JNK1, GST-c-Jun beads (Cell Signaling Technology) were added to cell extracts (300 μg/reaction) with shaking for 18 h at 4°C. The kinase reaction buffer contained 20 mM HEPES (pH 7.4), 1 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na_3VO_4, 20 μM ATP, and 2 μCi [γ-^{32}P] ATP. Reactions were initiated by the addition of radiolabeled ATP. After incubation for 30 min at 30°C, the reactions were stopped with 4× SDS sample buffer and the reaction mixtures were subjected to electrophoresis on 12% SDS-polyacrylamide gels followed by staining with 0.25% Coomassie blue R250, drying and autoradiography with an intensifying screen.

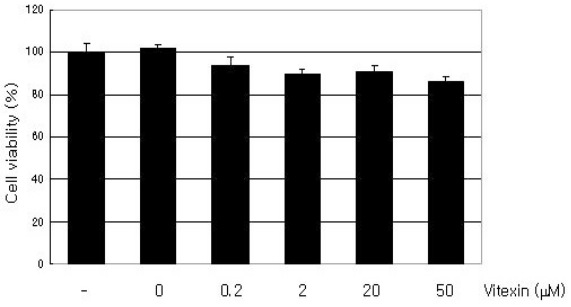

Fig. 1. The effect of vitexin on the viability of PC12 cells. Cells were treated with 0.2, 2, 20, and 50 μM vitexin for 24 h and cell viability was measured by MTT assay. Cell viability was compared to vehicle control. *Bars*: ± SE.

Transient expression of transfected cDNAs cDNA constructs for HA-JNK, SEK1 wt or the dominant negative mutant, SEK1 KR, were transfected into PC12 cells (70% confluence) with LipofectAMINE reagent (Life Technologies). In each 90 mm dish, 2.5 μg of pcDNA3-HA-JNK cDNA was cotransfected with 2.5 μg of the cDNAs of pEBG-SEK1 wt or pEBG-SEK1 KR as indicated in the Figure legend.

Immunocytochemistry For Immunocytochemical staining we used the DakoCytomation LSAB 2 system-HRP (DakoCytomation, USA) according to the manufacturer's protocol. Cells grown on poly-L-lysine-coated plates were washed several times with PBS and fixed with 3% paraformaldehyde in PBS for 15 min at room temperature. They were permeabilized by incubation in 0.2% Triton X-100 for 2 min at room temperature and then quenched in 3% H_2O_2 in the dark to block endogenous peroxidase activity. Plates were incubated with 3% BSA in PBS for 1 h to block non-specific binding and incubated with rabbit polyclonal anti-HIF-1α diluted in antibody diluent solution (Zymed Labora-

tories Inc., USA). Cells were washed 3 times with PBS and then incubated with biotinylated anti-rabbit immunoglobulins in PBS containing carrier protein and 15 mM sodium azide. After further rinsing, cells were incubated with streptavidin conjugated to horseradish peroxidase-containing carrier protein, and then exposed to a diaminobenzidine/urea/H_2O_2 solution (DakoCytomation) in distilled water. They were then counterstained with Mayer's hematoxylin (DakoCytomation). Finally, they were washed, and the coverslips were mounted in Faramount Aqueous Mounting medium (DakoCytomation) to inhibit photobleaching.

***In vitro* migration and invasion assays** Migration was analyzed in a modified Boyden chamber assay using cell culture inserts with a polycarbonate-filter (PVP-free, pore size 8 μm, Corning Incorporated, USA). Analysis of invasive properties was achieved by using cell culture inserts covered with Matrigel (BD Biosciences, USA). For both assays, 100 μl of cell suspension (1×10^5 cells) in DMEM supplemented with 1% FBS was added to the upper wells. The lower compartment was filled with DMEM supplemented with 1% FBS, 5 μg/ml of fibronectin and the indicated concentrations of vitexin. Chambers were incubated for 24 h at 37°C in a 5% CO_2 atmosphere. Cells on the lower side of the filter were stained with 0.1% crystal violet, photographed and quantitated by dissolving the cell-bound crystal violet in 10% acetic acid followed by spectrophotometric analysis at 540 nm. Data are expressed as percentages compared to control.

***In vitro* angiogenesis assay (tube formation assay)** To reconstitute a basement membrane, Matrigel was added to 48-well tissue culture plates (150 μg/well) on ice. The 48-well plates were incubated for 1 h at 37°C to allow the Matrigel to solidify. HUVECs were trypsinized, resuspended in medium containing the indicated concentrations of vitexin and plated on the reconstructed basement membrane (3×10^4 cells/well). The cells were incubated in a hypoxic chamber for 24 h to induce various factors such as VEGF and to form capillary-like endotube structures.

Statistical analysis All the experimental data shown are expressed as means ± SE and were obtained from experiments repeated at least three times, unless otherwise indicated. Statistical analysis was by one-way analysis of variance (ANOVA) followed by Student's *t*-test, and P-values less than 0.05 were considered significant.

Results

Cytotoxic effect of vitexin To assess the cytotoxic effect of vitexin, PC12 cells were treated with various concentrations of vitexin for 24 h and cell viability was measured by MTT assay. As shown in Fig. 1, there was some cell death after treatment with 50 μM of vitexin (> 86%). Therefore, less than 20 μM of vitexin was used in all subsequent experiments.

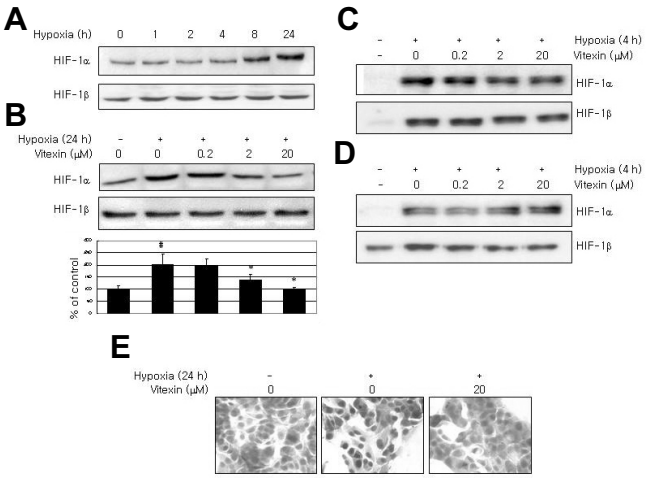

Fig. 2. Inhibition of HIF-1α by vitexin in PC12 cells under hypoxia. PC12 cells were incubated for the indicated times under hypoxia to measure the time-dependent increase of HIF-1α (A). PC12 cells were treated with 0.2, 2, and 20 μM vitexin and incubated under hypoxia for 24 h. The nuclear protein levels of HIF-1α were analyzed by Western blot analysis using an antibody against HIF-1α and compared to the levels of HIF-1β (B). The graph represents the level of HIF-1α protein (%) compared to the normoxic control, and the asterisk (*) indicates a significant difference ($P < 0.05$) compared with the positive control ($^{\#}$). *Bars*: ± SE. HepG2 cells (C) and HOS cells (D) were also treated with 0.2, 2, and 20 μM vitexin and incubated under hypoxia for 4 h. Their protein levels of HIF-1α were analyzed in nuclear extracts by Western blot analysis and compared to the levels of HIF-1β. Immunocytochemical staining was performed in PC12 cells with 20 μM vitexin under normoxia or hypoxia for 24 h (× 400) (E). Negative controls were performed with normal IgG (data not shown).

Inhibition of HIF-1α protein by vitexin To see whether vitexin affected the level of HIF-1α protein in HepG2, HOS and PC12 cells, we determined its level in nuclei by Western blot analysis after treatment with vitexin under hypoxia. In PC12 cells, the level of HIF-1α protein increased up to 2-fold within 24 h in response to hypoxia, compared with HIF-1β as a control (Fig. 2A). In HepG2 and HOS cells the level of HIF-1α protein markedly increased within 4 h. However, vitexin significantly reduced the HIF-1α level only in PC12 cells (Fig. 2B), not in HepG2 or in HOS cells (Figs. 2C and 2D). Treatment with 20 μM of vitexin under hypoxia significantly reduced nuclear HIF-1α, as shown by immunocytochemical staining (Fig. 2E).

Transcriptional inhibition of hypoxia-inducible genes To investigate the effect of vitexin on HIF-1α mRNA, we carried out RT-PCR with total PC12 RNA isolated after treatment with various concentrations of vitexin. As shown in Fig. 3, vitexin did not affect the level of HIF-1α mRNA;

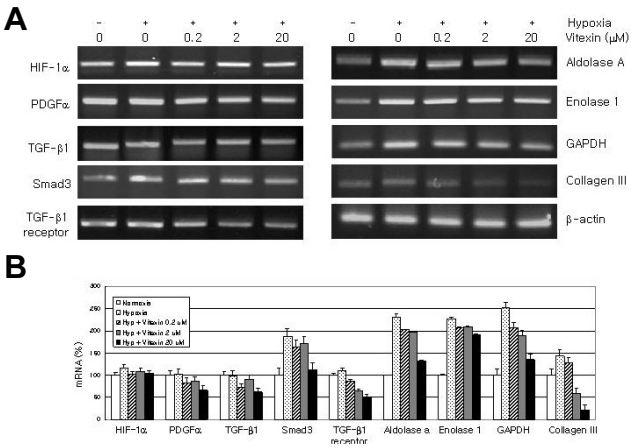

Fig. 3. RT-PCR analysis of hypoxia-inducible genes in PC12 cells. RT-PCR analysis was performed to examine the expression pattern of various hypoxia-inducible genes (A). Total RNA (1 μg) was isolated from PC12 cells which were cultured under hypoxia for various times in the presence of vitexin. Incubation times and PCR conditions are listed in Table 1. β-actin was used as a control. The graph represents the level of mRNA (%) compared to control (B). *bars*: ± SE.

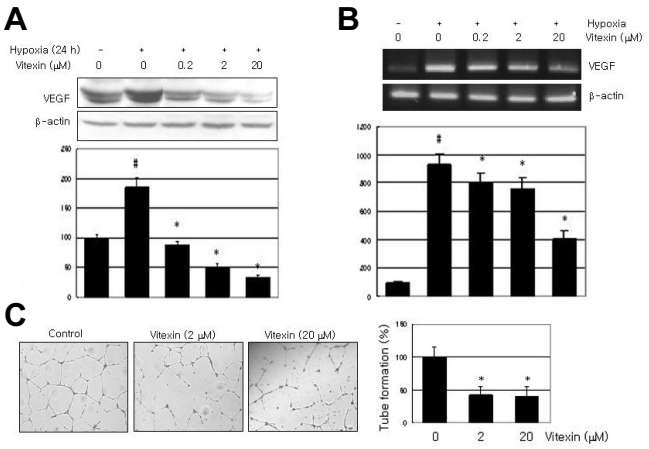

Fig. 4. Vitexin inhibits VEGF expression in PC12 cells, and tube formation by HUVECs. The protein levels of VEGF were analyzed in whole cell lysates by Western blot analysis using an antibody against VEGF and the protein concentration of each lane was normalized by β-actin. The graph represents the levels of VEGF protein (%) compared to control, and the asterisk (*) indicates a significant difference ($P < 0.05$) compared with the positive control ($^{\#}$) (A). RT-PCR analysis was performed to examine the expression pattern of VEGF (B). β-actin was used as a control. Culture plates were coated with Matrigel and HUVECs (3×10^4 cells) were added together with 2 and 20 μM vitexin and incubated under hypoxia for 40 h. Photographs of tube formation (× 40) were taken (C). The graph represents the number of tubes per microscopic field (× 100) in three separate experiments. Data were normalized to the control group and an asterisk (*) indicates significant difference ($P < 0.05$) compared with the control. *Bars*: ± SE.

on the other hand treatment with cycloheximide, a protein synthesis inhibitor, did reduce HIF-1α protein (data not shown). The results suggested that vitexin affected HIF-1α at the protein level. The effect of vitexin on HIF-1α inducible genes was examined using RT-PCR. As shown in Fig. 3, vitexin reduced the mRNA levels of hypoxia-inducible genes involved in glucose metabolism (aldolase A, enolase 1, GAPDH), skeletal protein synthesis (smad3, TGF-β1, TGF-β1 receptor, collagen type III) and cell proliferation (PDGFα).

Inhibition of angiogenesis by vitexin VEGF, the major angiogenic factor, is markedly induced by HIF-1α (Osada *et al.*, 2004). To investigate the effect of vitexin on angiogenesis, we measured VEGF protein and mRNA level in PC12 cells after treatment with vitexin under hypoxia. As shown in Figs. 4A and 4B, vitexin reduced the levels of VEGF protein and mRNA in a dose-dependent manner. We also assessed the effect of vitexin on angiogenesis by examining its effect on tube formation by HUVECs. Although serum, VEGF, or FGF are usually used to induce tube formation (Bussolati *et al.*, 2001; Chen *et al.*, 2005; Matou *et al.*, 2005), hypoxia alone was used in this study. In the control, HUVECs on a Matrigel substratum displayed high motility and differentiated into well-defined network-like structures in 24 h (Fig. 4C). However, their angiogenic function was significantly diminished in the presence of vitexin, as shown by the reduced width and length of the endothelial network-like structures. Treatment with 2 μM of vitexin yielded incomplete, broken network-like structures, and treatment with 20 μM resulted in further inhibition. Quantification of tube formation rate by counting the number of tubes revealed that vitexin at 0.2, 2, and 20 μM reduced tube formation by 61.9%, 42.9%, and 40.6%, respectively, compared to the control group.

Inhibition of *in vitro* migration and invasiveness by vitexin To determine the effect of vitexin on cell migration, we analyzed the ability of PC12 cells to migrate through the transwells of Boyden chambers. As seen in Fig. 5A, PC12 cells migrated to the lower chamber in response to fibronectin, a chemoattractive factor. However, the presence of vitexin (2 μM) in the lower chamber significantly inhibited migration. The effect of vitexin on cell invasion was also examined using Matrigel as extracellular matrix component. Quantification of invading cells by staining and dye extraction showed that 20 μM of vitexin inhibited invasion by approximately 66% (Fig. 5B).

Involvement of JNK in the inhibition of HIF-1α by vitexin To elucidate the mechanism by which vitexin inhibits HIF-1α levels, we analyzed the effect of vitexin on

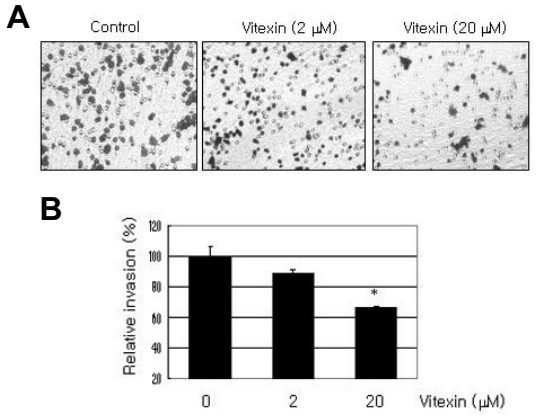

Fig. 5. Effects of vitexin on migration and invasion by PC12 cells. PC12 cells (1×10^5 cells) were added to Matrigel-coated upper wells of Boyden chambers and allowed to migrate towards lower wells filled with 1% FBS-DMEM containing 5 μg/ml of fibronectin and vitexin (2 and 20 μM). Cells were incubated for 24 h and stained with 0.1% crystal violet. For the migration assay, tissue culture plates were prepared without Matrigel. Photographs of migrated PC12 cells (× 100) were taken (A). Relative invasion rate was analyzed with cells on the lower side by dissolving the cell bound crystal violet in 10% acetic acid and subsequent spectrophotometric analysis at 540 nm (B). Data were normalized to the control group and an asterisk (*) indicates a significant difference ($P < 0.05$) compared with the control. *bars*., ± SE.

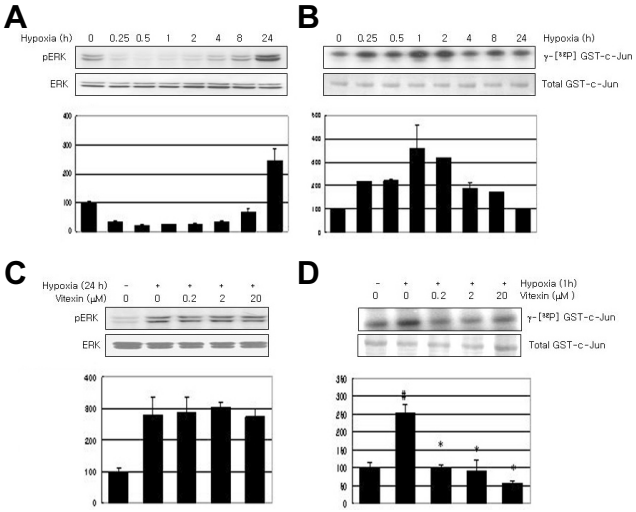

Fig. 6. Effects of vitexin on ERK and JNK activities in PC12 cells under hypoxia. PC12 cells were incubated under hypoxic conditions for the indicated times. For analysis of the activation rate of ERK, whole cell lysates (30 μg) were fractionated by 10% SDS-PAGE, and the activity of ERK was assayed by Western blot analysis using a rabbit anti-phospho ERK antibody and normalized with the levels of ERK (A). The activity of JNK was measured by immunoprecipitation and *in vitro* kinase assays with GST-c-Jun beads as described in **Materials and Methods**. Total GST-c-Jun proteins were used to normalize the activity (B). Vitexin (0.2–20 μM) was added for 24 h (C) and 1 h (D) and the activities of ERK and JNK, respectively, were assayed. The graphs represent the relative activities of ERK and JNK (%) compared to a normoxic control. The asterisk (*) in D indicates a significant difference ($P < 0.05$) compared to the positive control ($^{\#}$). *Bars*: ± SE.

the activities of MAP kinases (p38 kinase, ERK, and JNK) in PC12 cells under hypoxia. As shown in Figs. 6A and 6B, hypoxic conditions induced ERK activation 2.5 fold at 24 h and JNK activation 3.5 fold at 1 h. However, the activity of p38 kinase was unchanged (data not shown). Vitexin at concentrations from 0.2 to 20 μM did not alter the activity of ERK in PC12 cells (Fig. 6C), but reduced the hypoxia-induced activation of JNK (Fig. 6D). To determine whether JNK signaling pathways are involved in the inhibition of HIF-1α, PC12 cells were treated with SP600125, a JNK- specific inhibitor, and incubated under hypoxia. Treatment of cells with 40 μM of SP600125 decreased the level of HIF-1α (Fig. 7A). To confirm the effect of JNK on HIF-1α, expression vectors containing the cDNAs for HA-JNK and SEK1 wt or a domininant negative mutant were transiently transfected into PC12 cells as described in Materials and Methods. As shown in Fig. 7B, blockade of the JNK pathway resulted in a significant decrease in the protein level of HIF-1α, suggesting that vitexin inhibits the formation of HIF-1α protein at least in part via the JNK pathway in the PC12 cells.

Discussion

HIF-1 consists of a constitutively expressed subunit (HIF-1β) and an oxygen-regulated subunit (HIF-1α). The stability and activity of the α subunit of HIF are regulated by posttranslational modifications such as hydroxylation, ubiquitination, acetylation, and phosphorylation (Ke and Costa, 2006; Lee *et al*., 2002; Mylonis *et al.*, 2006). Overexpression of HIF-1 has been found in various cancers (Vaupel, 2004), rheumatoid arthritis (Hollander *et al.*, 2001), psoriasis (Kwon *et al.*, 2004) and hemangioblastoma (Flamme *et al.*, 1998), and targeting HIF-1 could represent a novel approach to cancer therapy.

Recently many novel therapeutic agents inhibiting the activity of HIF-1α have been discovered by screening natural products such as plant extracts (Hodges *et al.*, 2004; Hossain *et al.*, 2005; Zhou *et al.*, 2005). Flavonoids are especially well known to suppress tumorigenesis by inhibiting HIF-1α (Fang *et al.*, 2005; Hasebe *et al.*, 2003; Osada *et al.*, 2004). In the present study we demonstrated that vitexin reduced the protein level of HIF-1α in PC12 cells. Down-regulation of HIF-1α by vitexin altered the expression of HIF-1α-inducible genes such as those encoding VEGF, aldolase A, enolase 1, GAPDH and growth

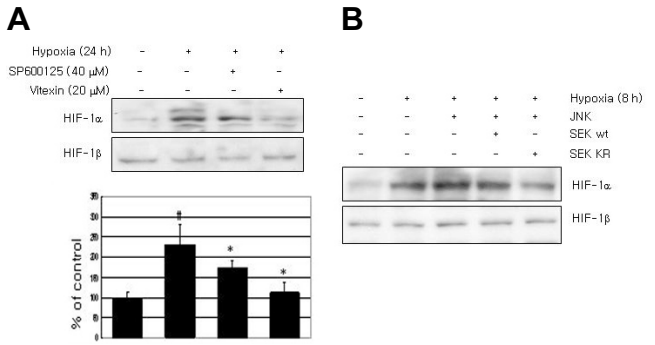

Fig. 7. Involvement of JNK in the regulation of HIF-1α in PC12 cells. PC12 cells were treated with 40 μM SP600125 or 20 μM vitexin and incubated under hypoxia for 24 h (A). Expression vectors containing cDNAs for HA-JNK and SEK1 wt or a SEK1 dominant negative mutant were cotransfected as indicated (B). After transfection, cells were incubated for 8 h and nuclear proteins were collected for detection of HIF-1α. The nuclear protein levels of HIF-1α were analyzed by Western blot analysis using an antibody against HIF-1α and compared to the levels of HIF-1β. The graph represents the relative level of HIF-1α protein (%) compared to the normoxic control, and an asterisk (*) indicates a significant difference ($P < 0.05$) compared with the positive control ($^{\#}$). *Bars*: ± SE.

factors such as TGF-β1 and PDGFα. The expression of various growth factors such as TGF-β1 and PDGFα may be essential for tumor pathology (Bos *et al.*, 2005). Under hypoxic conditions cells show a variety of responses, such as activation of signaling pathways that regulate angiogenesis and cell proliferation; changes in energy metabolism are another important adaptation to hypoxia. Because oxidative phosphorylation is impaired in hypoxic conditions several glycolytic enzymes must be induced to maintain the basal level of ATP required for cell survival (Semenza *et al.*, 1994). Therefore, the inhibitory action of vitexin on the expression of GAPDH, aldolase, enolase probably may promote cell death in a hypoxic environment.

Angiogenesis is both complex and dynamic and requires the proliferation of endothelial cells from preexisting blood vessels, breakdown of the extracellular matrix, and migration of the endothelial cells (Stetler-Stevenson, 1999). The continued growth of tumors requires persistent blood vessel formation, and inhibition of angiogenesis can result in tumor dormancy. Over expression of HIF-1α in cancers could lead to activation of oncogenes like bcl-2 and inactivation of tumor suppressor genes like p53, and result in high rate of metastasis and malignancy (Zhong *et al.*, 1999). Vitexin inhibited the production of VEGF and tube formation by HUVECs, implying that it possess an inhibitory effect on angiogenesis. Furthermore, vitexin attenuated metastatic potential by inhibiting the migration and invasion in PC12 cells.

The effects of hypoxia on the mitogen-activated protein kinase (MAPKs) activities in PC12 cells are well characterized. PC12 cells are oxygen-sensitive cells that are useful for studying the effects of hypoxia (Beitner-Johnson *et al.*, 2002; Conrad *et al.*, 1999; Hou *et al.*, 2003; Tabakman *et al.*, 2004). In particular, the activation of MAPKs is a function of the degree of hypoxia. For instance, ERK and p38 kinase were activated in 5% of oxygen (Beitner-Johnson *et al.*, 2002; Conrad *et al.*, 1999) whereas JNK remained unchanged. However, all three were activated under anoxia (Hou *et al.*, 2003; Tabakman *et al.*, 2004). Our data indicate that hypoxic stress leads to activation of JNK and ERK without alteration of p38 kinase activity in PC12 cells in 1% oxygen. In the present study vitexin inhibited the hypoxia-induced activation of JNK, but not that of ERK. The significant attenuation of HIF-1α by SP600125, a JNK inhibitor, implies that JNK is involved in modulating HIF-1α. However, there are no reports that JNK directly phosphorylates HIF-1α, whereas nuclear ERK is known to phosphorylate it (Mylonis *et al*., 2006).

To see whether the JNK pathway was involved in the differential effect of vitexin in the different cell lines we have treated HepG2 cells with vitexin and H_2O_2. Vitexin affected the activation of JNK but not that of ERK, suggesting that it inhibits the JNK pathway not only in PC12 cells but also in other cell lines (data not shown). The data in Fig. 2 point to a cell-type specific effect of vitexin, since it inhibited HIF-1α only in PC12 cells not in HOS nor HepG2 cells. Interestingly, PD98059 (an ERK inhibitor) or SP600126 (a JNK inhibitor) alone did not significantly inhibit HIF-1α in HepG2 cells (manuscript in preparation), implying that JNK may have an indirect effect on HIF-1α via some modulatory factors positioned between JNK and HIF-1α. In addition, the differential effect of vitexin on the different cell types may depend on the types of modulators downstream of the JNK pathway.

The data presented here suggest a molecular mechanism by which vitexin decreases the protein level of HIF-1α and point to possible benefits of vitexin as an antimetastatic agent. However, the discrepancy between the extents by which vitexin decreases HIF-1α level and the expression levels of its target genes must be considered. Transactivation of target genes by HIF-1α is cell-type specific, and it is not to be expected that the same battery of genes reported here would be transactivated by HIF-1α in every cell line. Furthermore, the data presented here do not distinguish between direct and indirect regulation of the identified target genes by HIF-1α (Krishnamachary *et al.*, 2003). Nevertheless our results indicate that vitexin affects multiple steps in the complex process of angiogenesis and invasion by inhibiting HIF-1α.

In summary, vitexin attenuated the protein level of HIF-1α in PC12 cells under hypoxia, leading to a reduction of hypoxia-induced responses such as angiogenesis, metastasis and invasion *in vitro*. The angiogenesis and metastasis frequently observed in tumors might be blocked by vitexin

via inhibition of HIF-1α. Since it may suppress a number of genes induced by HIF-1α, vitexin could prove a potent therapeutic agent against cancers and other HIF-1α-related diseases.

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