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The calcium channel blocker cilnidipine selectively suppresses hypoxia-inducible factor 1 activity in vascular cells

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ABSTRACT

Calcium ion is one of the most important second messengers of cellular signal transduction including hypoxia-elicited signals. In this study, we investigated the effects of the L-type calcium channel blockers such as nifedipine, efonidipine cilnidipine, diltiazem, and verapamil, on the activity of hypoxia-inducible factor-1 (HIF-1), a key transcription factor in control of hypoxia-induced gene expression. Using the lung carcinoma cell line A549 cells, human aortic smooth muscle cells, and human umbilical vein endothelial cells, we demonstrated that cilnidipine exclusively suppressed HIF-1 activity and the expressions of downstream genes in a cell-type specific manner. We also demonstrated that cilnidipine blocked the synthesis of the HIF-1α protein not by affecting activity of the intracellular hypoxia-sensing element prolyl hydroxylases but inhibiting activity of Akt and mitogen-activated protein kinase and that the inhibition is not dependent on the effect on calcium homeostasis.

1. Introduction

Adaptation to hypoxia and maintenance of O_2 homeostasis involve a variety of cellular and systemic responses at different organizational levels in the body. Hypoxia induces a series of adaptive physiological responses including the expression of a select set of genes required for glycolysis, angiogenesis, erythropoiesis, cell proliferation, vasomotor responses, and vascular remodeling (Hirota, 2002). The transcriptional factor hypoxia inducible factor-1 (HIF-1) plays a central role in regulating the expression of these genes. HIF-1, which is a heterodimer composed of HIF-1α and HIF-1β subunits (Wang et al., 1995), binds to specific *cis*-active regulatory sequence known as hypoxia response elements (HREs) to induce the gene expression.

The balance between protein synthesis and degradation determines HIF-1α protein levels. HIF-α hydroxylases and von Hippel-Lindau tumor-suppressor protein (VHL) play an essential role in hypoxia-induced HIF-1 activation (Hirota and Semenza, 2005). In addition to these well established O_2-dependent activation mechanisms of HIF-1α, a role for changes in intracellular calcium levels in hypoxia-induced gene expression has been postulated. Increased calcium concentration has been suggested to be involved in hypoxia-induced expression of tyrosine hydroxylase gene and vascular endothelial growth factor (VEGF). It was reported that elevated calcium concentration caused by hypoxia increased extracellular signal-regulated kinase (ERK)1/2 activation and increased HIF-1 transcriptional activity without changing HIF-1α protein expression levels (Mottet et al., 2003). In contrast, Metzen et al. (1999) reported that $[Ca^{2+}]i$ did not play a key role in hypoxia-induced gene expression in Hep3B human hepatoma cells and that the membrane permeant Ca^{2+} chelator BAPTA-AM did not affect the expression HIF-1α but EGTA-AM, a closely related agent, did in Hep3B cells. Recently, it was reported that chelation of cellular calcium by BAPTA-AM caused the accumulation of HIF-1α protein, providing evidence for negative role of calcium in the regulation of HIF-1 activity (Berchner-Pfannschmidt et al., 2004). Moreover, Liu et al. (2004) reported that both the calcium ionophore A23187 and BAPTA-AM stimulated the HIF-1α protein accumulation by different molecular mechanism in HepG2 cells.

L-type voltage-gated calcium channel blockers are among the most frequently prescribed drugs for the treatment of cardiovascular disease. Calcium channel blockers are potent vasodilators, and their use in cardiovascular treatment remains largely based on the blockade of Ca^{2+} mobilization. On the other hands, calcium channel blockers modulate intracellular signaling pathways by multiple mechanisms including effects that are independent of Ca^{2+} channel blocking activity (Hirata et al., 2000; Yue et al., 2004). Moreover, calcium channel blockers have been shown to limit cardiovascular remodeling

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by regulating angiogenesis and vascular smooth muscle cell growth (Mason et al., 2003). Mice that are heterozygous for knockout allele at the *Hif1a* locus (*Hif1a*$^{+/-}$) demonstrated significantly delayed development of hypoxia-induced pulmonary hypertension compared with *Hif1a*$^{+/+}$ mice. Analysis of *Hif1a*$^{+/-}$ and *Hif1a*$^{+/+}$ mice suggested that impaired development of pulmonary hypertension associated with partial HIF-1α deficiency was due to decreased muscularization of pulmonary arterioles (Yu et al., 1999).

All these reports taken into consideration, we studied the effects of calcium channel blockers on HIF-1 activity in various types of cells and demonstrate that among the calcium channel blockers cilnidipine exclusively suppresses HIF-1 activity and the expression of downstream genes in a cell-type specific manner.

2. Materials and methods

2.1. Cell culture and reagents

The human lung epithelial-like carcinoma cell line A549 cells were maintained in RPMI supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin. Human aortic smooth muscle cells (HASMCs) and human umbilical vein endothelial cells (HUVECs) were obtained from Kurabo (Osaka, Japan). The calcium channel blocker nifedipine, efonidipine, cilnidipine, diltiazem, or verapamil was obtained from Buyer (Germany), Shionogi (Tokyo, Japan), Ajinomoto (Tokyo, Japan), Tanabe Seiyaku (Tokyo, Japan), or Eizai (Tokyo, Japan), respectively. Cycloheximide (CHX), Z-Leu-Leu-Leu-al (MG132), 1,2-*bis*-(o-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM), thapsigargin, the synthetic dihydropyridine derivative Bay K-8644, and the calcium ionophore ionomycin were from Calbiochem (San Diego, CA). Ascorbate and the iron chelator desferrioxamine were from Sigma (St. Louis, MO). Ca^{2+}-free media were prepared by supplementation with 100 μM of EGTA.

2.2. Hypoxic treatment

Cells were maintained in a multi-gas incubator (APMW-36, Astec, Japan) and were exposed to hypoxia (1% O_2–5% CO_2–94% N_2 or 5% O_2–5% CO_2–90% N_2) at 37 °C (Kasuno et al., 2004; Oda et al., 2008).

2.3. Immunoblot assays

Whole cell lysates were prepared using ice-cold lysis buffer following a protocol described previously (Kasuno et al., 2004). Aliquots of the cell lysates were fractionated by 7.5% SDS-PAGE and subjected to immunoblot assay using mouse monoclonal antibody against HIF-1α (BD Biosciences, San Jose, CA) or HIF-1β (H1b234; Novus Biologicals, Littleton, CO) at 1:1000 dilution and HRP-conjugated mouse monoclonal antibodies for mouse IgG (Amersham Bioscience, Piscataway, NJ, 1:1000 dilution). Anti-extracellular signal-regulated kinase (ERK) 3 antibody was from Santa Cruz (San Diego, CA) and used at 1:1000 dilution. Anti-β-actin antibody was from Sigma (St. Louis, MO) and used at 1:5000 dilution. For detection of phosphorylated proteins, 50-μg aliquots of cell lysate were analyzed using specific antibodies against: phosphorylated p44/42 mitogen-activated protein kinase (MAPK) (Thr-202/Tyr-204), phosphorylated Akt (Ser-473), phosphorylated S6 ribosomal protein (S6R) (Ser-235/236), and phosphorylated p70 S6 kinase (p70S6K) (Thr-389) (1:1000 dilution) (Cell Signaling Technology, Beverly, MA). Chemiluminescent signal was developed using ECL reagent (Amersham Biosciences).

2.4. Reverse transcription (RT)-PCR

The RT-PCR protocol is described elsewhere (Kasuno et al., 2004). 1 μg of total RNA was subjected to first strand cDNA synthesis. cDNAs were amplified with TaqGold™ polymerase (Roche, Manheim, Germany) in a thermal cycler with the specific primers. Thermocycling conditions were 30 s at 94 °C, 60 s at 57 °C, and 30 s at 72 °C for 25 (vascular endothelial growth factor [VEGF]), 20 (lactate dehydrogenase A [LDHA]), 25 (HIF-1A), and 20 (18S ribosomal RNA) cycles proceeded by 10 min at 94 °C.

2.5. Quantitative real-time RT-PCR

RNA was DNase-treated and purified using RNeasy (Qiagen Inc.). First-strand synthesis and real-time PCR reaction was performed using QuantiTect SYBR Green PCR Kit™ (Qiagen Inc.) following a protocol provided by the company. PCR reaction and detection was performed using Applied Biosystems 7300 real time PCR system (Foster City, CA). PCR primers were purchased from Qiagen Inc. The fold change in expression of each target mRNA relative to 18S ribosomal RNA was calculated (Kelly et al., 2003).

2.6. In vitro HIF-1α-VHL interaction assay

The protocol was described previously (Kasuno et al., 2004; Kimura et al., 2008). Glutathione S-transferase (GST)-HIF-1α (429–608) fusion protein was expressed in *E. coli* as described. Biotinylated methionine-labeled proteins were generated in reticulocyte lysates using the TNT T7 coupled transcription/translation system and Transcend Biotinylated tRNA™ (Promega). 25-μg aliquots of HASMC lysate were preincubated with cilnidipine (30 μM) or desferrioxamine (100 μM) for 30 min at 30 °C, and then 5 μg of GST-HIF-1α (429–608) was added and incubated for 30 min at 30 °C. A 5-μl aliquot of *in vitro*-translated biotinylated VHL protein was mixed with 5 μg of GST fusion protein in a final volume of 200 μL of HEB buffer [20 mM Tris (pH 7.5), 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol] and incubated for 30 min at 4 °C with rotation followed by addition of 10 μL of glutathione-Sepharose 4B beads (Amersham Bioscience) and incubation at 4 °C for 1 h. The beads were pelleted, washed in NETN buffer [150 mM NaCl, 0.5 mM EDTA, 20 mM Tris–HCl at pH 8.0, 0.5% NP-40], pelleted again, resuspended in Laemmli sample buffer, and analyzed by SDS-PAGE. Proteins were transferred to PVDF membrane and visualized using streptavidin-labeled horseradish peroxidase and ECL reagent (Amersham Bioscience).

2.7. Reporter gene assay

Reporter gene assay was performed in HASMCs as described previously (Kasuno et al., 2004). For luciferase assay, the reporter plasmid p2.1 (150 ng) kindly provided by Dr. Semenza, Johns Hopkins University (Semenza et al., 1996), which contains a 68-bp hypoxia response element (HRE) from the human enolase 1 (ENO1) gene and the control plasmid pRL-SV40 (50 ng) (Promega), containing a SV40 promoter upstream of *Renilla reniformis* luciferase coding sequences, were pre-mixed and used. After treatment, the cells were harvested and the luciferase activity was determined using the Dual-Luciferase Reporter Assay System ™ (Promega).

2.8. Cell viability

Toxicity of each CCB was excluded as judged from the 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide assay (Hansen et al., 1989).

2.9. Statistical analysis

Data were expressed as mean ± S.D. Significance tests (ANOVA with *post-hoc* test) were performed using Prism™ version 4 application.

3. Results

3.1. Effect of calcium channel blocker on HIF-1α and HIF-1β protein expression and its dependency on O_2-tension

To examine the effect of CCBs on HIF-1 activity, A549 cells, HASMCs and HUVECs were treated with or without 30 μM calcium channel blockers (nifedipine, efonidipine, cilnidipine, diltiazem, and verapamil) under non-hypoxic (20% O_2) or hypoxic (1% O_2) conditions (Fig. 1). No cell toxicity was detected judged from MTT assay (data not shown). HIF-1α protein levels were low under non-hypoxic conditions and increased markedly in response to hypoxia in all types of cell (Fig. 1, lanes 1 and 7). Calcium channel blockers affected the expression of HIF-1α protein in A549 cells neither under non-hypoxic nor hypoxic conditions (Fig. 1A). In HASMCs, 30 μM cilnidipine significantly inhibited the expression of HIF-1α under non-hypoxic conditions (Fig. 1B, lane 4), while no other calcium channel blockers exerted such inhibition. Under hypoxic conditions, 30 μM cilnidipine treatment suppressed hypoxic induction of HIF-1α protein accumulation in HASMCs (lane 10). Steady state protein expression of HIF-1β protein was not affected by exposure to hypoxia or CCBs in any types of cells. 30 μM cilnidipine also suppressed expression of HIF-1α protein in HUVECs under 20% and 1% O_2 conditions (data not shown). The suppression of HIF-1α protein expression is cilnidipine-dose dependent up to 40 μM (Fig. 1D). The suppressive ratio of HIF-1α protein expression by 30 μM cilnidipine correlates with O_2 tension. Cilnidipine suppressed HIF-1α protein expression more efficiently in normoxic conditions than in hypoxic conditions (Fig. 1E). Interestingly, cilnidipine inhibited desferrioxamine-induced HIF-1α protein accumulation under 20% O_2 conditions more efficiently compared to the accumulation under 1% O_2 in HASMCs, providing another evidence that inhibitory effect of cilnidipine is O_2 tension-dependent (Fig. 1F).

3.2. Effect of calcium channel blocker on HIF-1-dependent gene expression

We investigated the effect of calcium channel blockers on hypoxia-induced HIF-1-mediated gene expressions. The mRNA expression of genes was assayed using RT-PCR technique (Fig. 2A). In A549 cells, no calcium channel blockers affected the mRNA expression of VEGF or lactate dehydrogenase A, of which expression is under HIF-1 regulation (Fig. 2A, upper panel). In contrast, no calcium channel blockers except cilnidipine affected the basal mRNA expression of VEGF and lactate dehydrogenase A in HASMCs (Fig. 2A, lower panel). Messenger RNA expression of HIF-1α was not affected by calcium channel blockers, indicating that cilnidipine affects HIF-1α protein synthesis. Cilnidipine also suppressed hypoxia-induced endothelin 1 mRNA expression in a dose-dependent manner in HASMCs (Fig. 2B). We next investigated VEGF and HIF-1α mRNA expression by quantitative real-time RT-PCR method. Cilnidipine not only suppressed VEGF mRNA expression under normoxic conditions but also

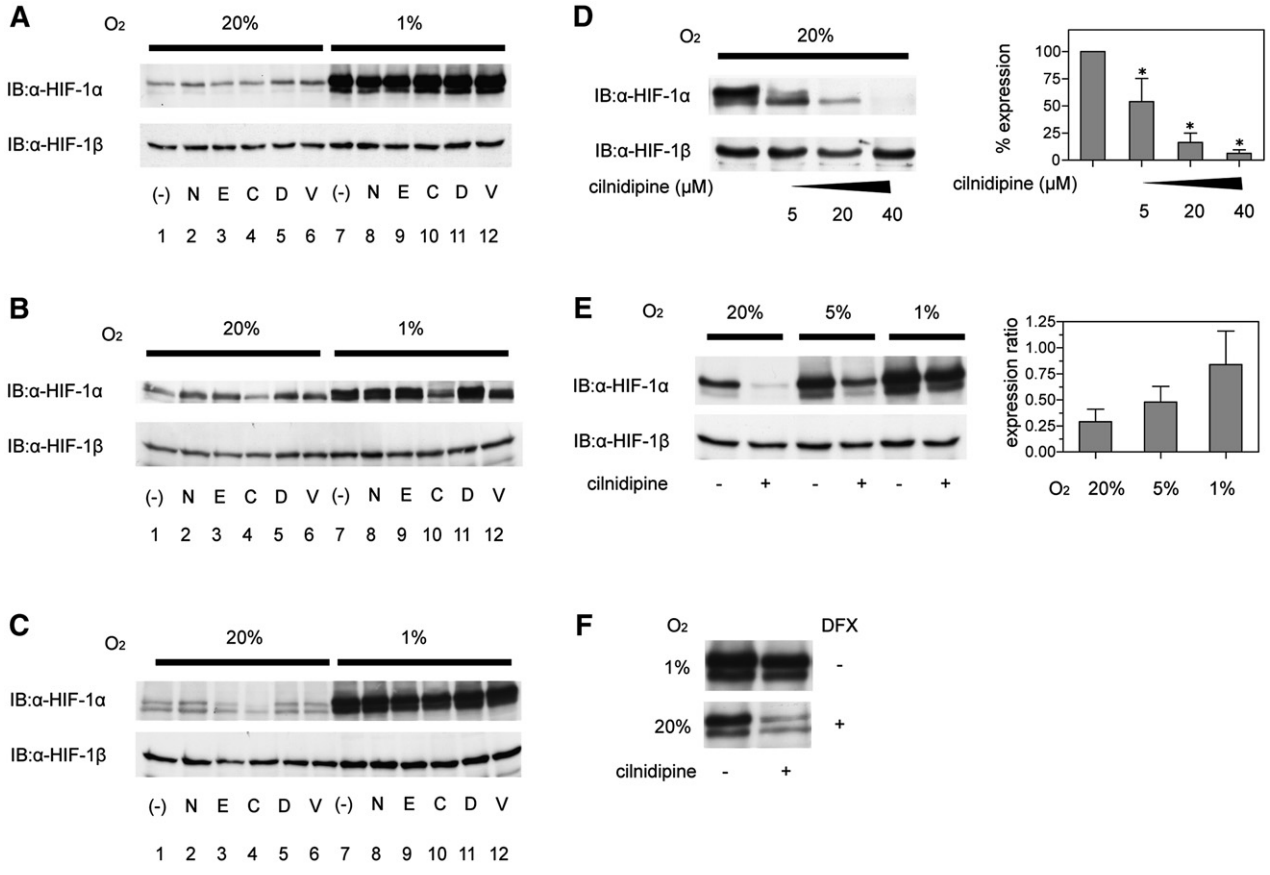

Fig. 1. Impact of CCBs on HIF-1 protein expression. Impact of dose and O_2-tension on cilnidipine-induced suppression of HIF-1α protein expression. A549 cells (A), HASMCs (B), and HUVECs (C) were exposed to 20% or 1% O_2 with or without 30 μM L-type voltage-gated calcium channel blockers for 4 h and harvested for immunoblot assays using anti-HIF-1α and -HIF-1β Abs (lysate: 100 μg, exposure time: 5 min). (−); no treatment, N; nifedipine, E; efonidipine, C; cilnidipine, D; diltiazem, and V; verapamil. Three independent experiments were performed and representative immunoblots are shown. D, HASMCs were exposed to 5, 20, or 40 μM cilnidipine under 20% O_2 conditions for 4 h (left panel). E, HASMCs were exposed to 20%, 5%, or 1% O_2 with or without 30 μM cilnidipine for 4 h. Cells were harvest for immunoblot assays using anti-HIF-1α and -HIF-1β Abs (lysate: 200 μg, exposure time: 15 min) (left panel). Three independent experiments were performed and representative immunoblots are shown (D and E). Intensity of respective bands was analyzed densitometrically and expression ratio to no treatment in 20% O_2 conditions is plotted accordingly as mean ± S.D. ($n=3$; *$p<0.05$) (D, right panel). Suppression ratio was calculated based on the band density of lane of each O_2 concentrations without cilnidipine treatment ($n=3$) (E, right panel). F, HASMCs were exposed to 1% O_2 or 100 μM desferrioxamine with or without 30 μM cilnidipine for 4 h. Cells were harvest for immunoblot assays using anti-HIF-1α. DFX; desferrioxamine.

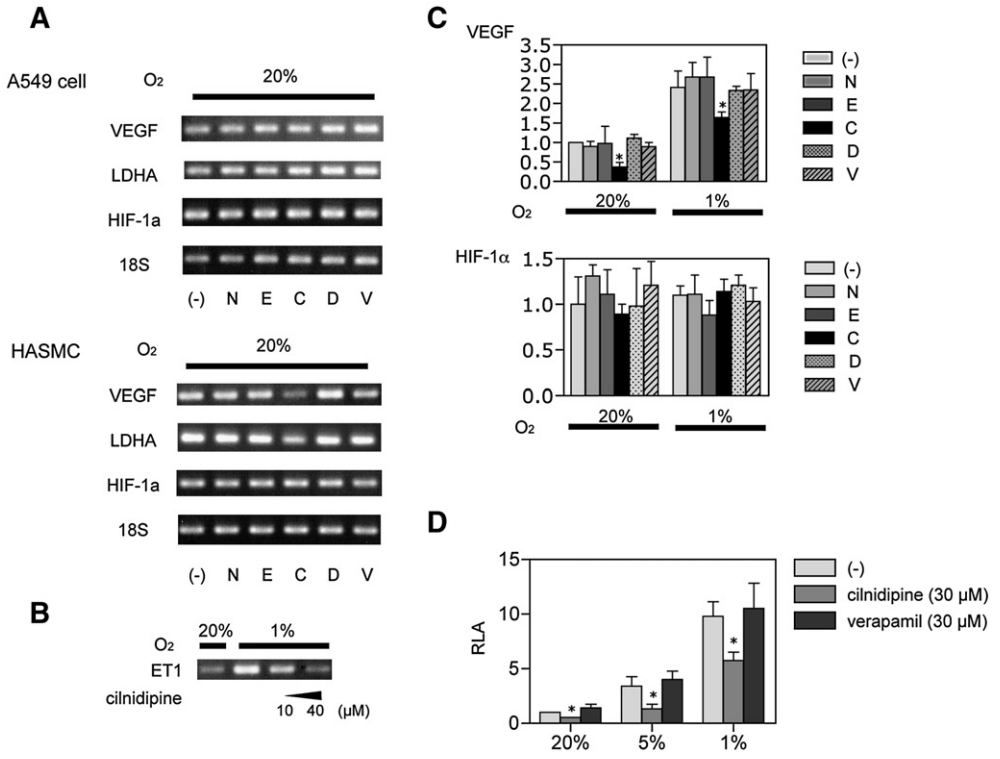

Fig. 2. Impact of CCBs on HIF-1-depenent or related genes expressions. A. A549 cells and HASMCs were exposed to 30 μM CCBs under 20% O_2 conditions for 24 h and total RNA was isolated. Expressions of mRNA of vascular endothelial growth factor (VEGF), lactate dehydrogenase A (LDHA), HIF-1α and 18S ribosomal RNA were analyzed by RT-PCR using specific primer pairs. B and C. HASMCs were exposed to 30 μM CCBs for 24 h under 20% or 1% O_2 conditions and total RNA was isolated. Expressions mRNA of endothelin 1 (ET1) was analyzed by RT-PCR using specific primer pairs (B). Expression of mRNA of VEGF and HIF-1α was analyzed by quantitative real-time PCR method. Results were shown as mean ± S.D. of six independent transfections. *$p<0.05$ compared to no treatment (C). D, HASMCs were transfected with p2.1 reporter. After 6 h incubation, cells were treated with 30 μM cilnidipine or verapamil under 20% or 1% O_2 for 18 h and harvested for luciferase assays. The relative luciferase activity (RLA) was calculated based on the value of non-hypoxic untreated cells. Results were shown as mean ± S.D. of six independent transfections. *$p<0.05$ compared to no treatment in each O_2 concentration. (−); no treatment, N; nifedipine, E; efonidipine, C; cilnidipine, D; diltiazem, and V; verapamil.

inhibited the induction under hypoxic conditions in HASMCs (Fig. 2C). The effect of cilnidipine on HIF-1 activity was also investigated in HASMCs using HRE-luciferase reporter construct, which is one of the most sensitive assay to detect gene induction. Cilnidipine but not verapamil inhibited the HRE-dependent gene expression under both normoxic and hypoxic conditions in HASMCs (Fig. 2D). The suppressive effect is O_2 tension-dependent. This is consistent with the results of RT-PCR (Fig. 2C) and Western blot (Fig. 1E).

3.3. Effect of CCBs on degradation and synthesis of HIF-1α protein

The balance between synthesis and degradation determines HIF-1α protein levels. To explore mechanisms for HIF-1 suppression by cilnidipine, we examined the effect of cilnidipine on HIF-1α accumulation induced by the protease inhibitor MG132. Exposure of HASMCs to cycloheximide (Fig. 3A, lane 2) or ascorbate (lane 3) had inhibitory effect on HIF-1α protein levels. 30 μM cilnidipine suppressed the expression of HIF-1α. Cycloheximide decreased HIF-1α protein by inhibition of protein translation whereas ascorbate, a cofactor for the prolyl hydroxylases, promoted HIF-1α protein degradation. Next, HASMCs were treated with cilnidipine, ascorbate or cycloheximide in the presence of MG132. Cilnidipine (lane 8) as well as cycloheximide (lane 5) suppressed the accumulation of HIF-1α induced by MG132 treatment. Ascorbate, which is an essential cofactor of HIFα-hydroxylation reaction (Hirota and Semenza, 2005), did not inhibit the accumulation of HIF-1α under MG132 (lane 7). These data indicate that cilnidipine inhibits HIF-1α protein accumulation by suppressing protein synthesis not by promoting protein degradation. Next, we investigated the specificity of cilnidipine effect as an inhibitor of protein synthesis. ERK3 is a protein with rapid turnover and mainly degraded by ubiquitin–proteasome system (Coulombe et al., 2003). The proteasome inhibitor MG132 induces the accumulation of ERK3 (lane 4). Cycloheximide but not cilnidipine suppressed protein expression of ERK3 (Fig. 3B, lanes 2 and 3), providing an evidence that effect of cilnidipine is HIF-1α-specifc rather than general inhibitory effect on protein translational mechanism. Next, we determined whether HIF-1α hydroxylation status was affected by cilnidipine by assaying the interaction between HIF-1α and VHL (Mahon et al., 2001). GST-fused recombinant protein of HIF-1α was treated with indicated cell lysates and the binding to *in vitro*-transcripted and translated (IVTT)-VHL was investigated. As shown in Fig. 3C, the binding between HIF-1α and VHL was detected by treatment with cell lysate (lanes 1 and 2) and cilnidipine treatment did not affect the binding (lane 3). In contrast, the iron chelator desferrioxamine abolished the interaction (lane 4). These results suggest that cilnidipine does not affect HIF-α prolyl hydroxylase-activity. Because MAPK, Akt, S6 ribosomal protein and p70 S6 kinase are critical regulators of signal transduction to the translational machinery, phosphorylation of these molecules under cilnidipine treatment was examined using phosphospecific antibodies. Cilnidipine suppressed phosphorylation of Erk1/2, Akt, S6 ribosomal protein and S6 kinase under 20% O_2 (Fig. 3D).

3.4. Impact of cilnidipine and intracellular calcium concentration on HIF-1α protein expression

To investigate the influence of intracellular calcium concentration to HIF-1α protein accumulation, HASMCs were treated with BAPTA-AM, which is a cell permeable calcium chelator (Fig. 4A), or thapsigargin, which promotes the discharge of calcium ion from intracellular stores by

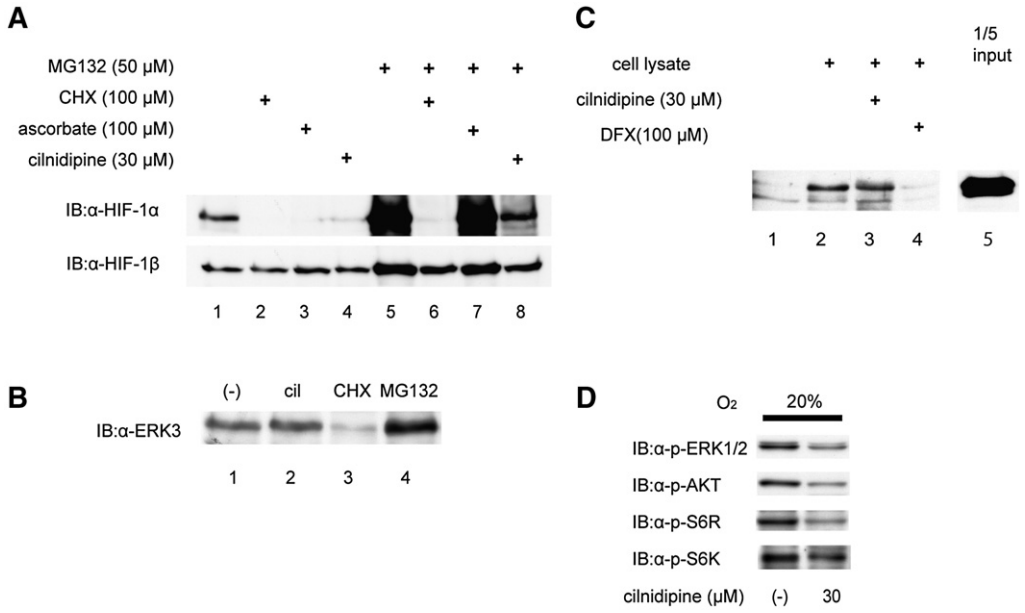

Fig. 3. Effect of cilnidipine on the degradation or synthesis of HIF-1α. A. HASMCs cells were treated with 100 μM cycloheximide, 100 μM ascorbate, or 30 μM cilnidipine with or without 50 μM MG132 in 20% O_2 conditions for 4 h. Lysate were analyzed by Western blot using anti-HIF-1α antibody. B. HASMCs cells were treated with 30 μM cilnidipine (cil), 100 μM cycloheximide, or 50 μM MG132 for 4 h. Lysate were analyzed by Western blot using anti-ERK3 antibody. C. GST-HIF-1α (429–608) fusion protein was incubated with *in vitro*-translated (IVTT-) VHL in the presence of PBS or cell lysates untreated or treated with cilnidipine or desferrioxamine (lanes 1–4). Glutathione–Sepharose beads were used to capture GST-HIF-1α (429–608) and the presence of bound VHL was detected by PAGE. One-fifth of the input biotinylated lysine-labeled IVTT-VHL protein (lane 5) was also analyzed. D. HASMCs cells were treated with 30 μM cilnidipine under 20% O_2 conditions for 4 h. Lysate were analyzed by Western blot using indicated antibodies. CHX: cycloheximide.

specific inhibition of the endoplasmic reticulum calcium-ATPase (Fig. 4B). Under non-hypoxic conditions, accumulation of HIF-1α protein was observed as early as half an hour after the treatment of BAPTA-AM and this effect peaked between 2 h and 4 h later. In contrast, thapsigargin decreased HIF-1α protein levels under non-hypoxic conditions and almost no accumulation of HIF-1α protein was observed 2 h after the

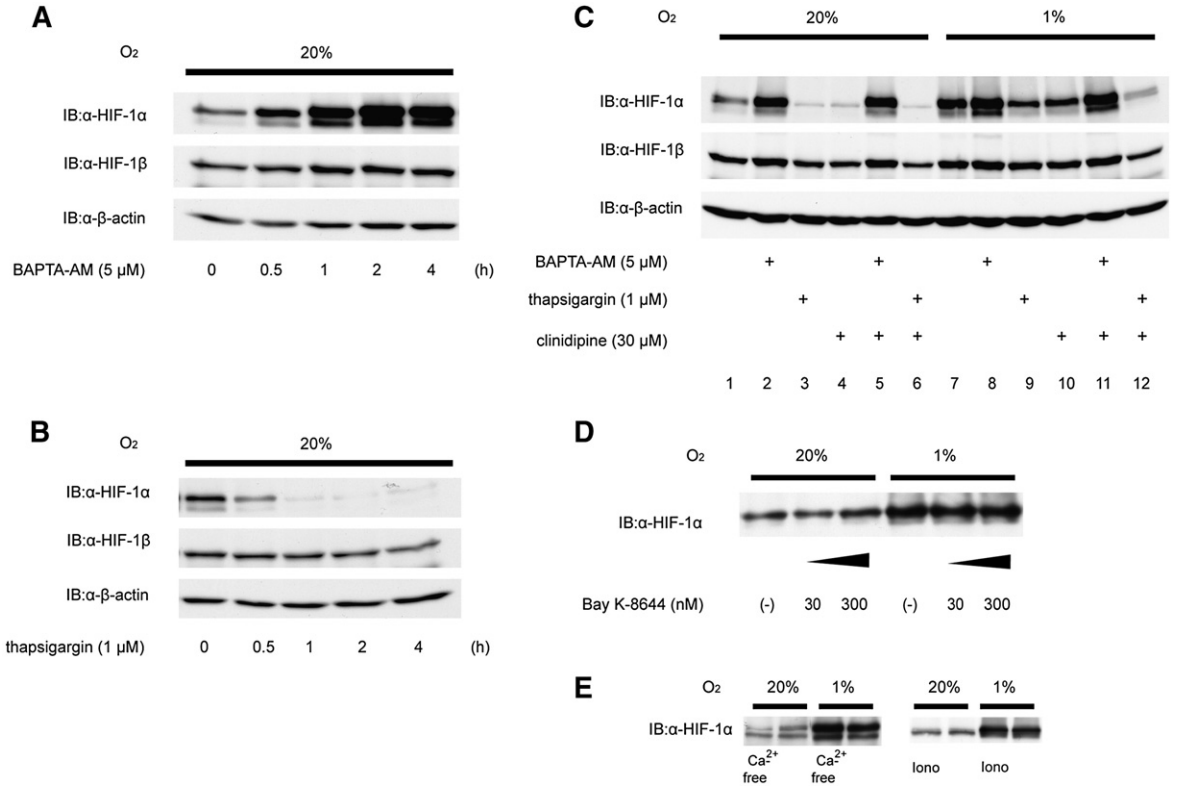

Fig. 4. Impact of calcium mobilization on HIF-1-suppressive effect of cilnidipine. HASMCs were treated with BAPTA-AM (A and B), thapsigargin (B and C), cilnidipine (B), or Bay K-8644 (D) and incubated for indicated time (A and B) or 4 h (C and D) under 20% O_2 conditions (A, B, C, and D) or 1% O_2 conditions (C and D). E. The culture media were replaced with the fresh media or Ca^{2+}-free media (left panel). Cells were treated by 2.5 μM ionomycin (iono) (right panel). Cells were exposed to 20% or 1% O_2 conditions for 4 h. Cells were harvested and lysates were subjected to Western blotting using anti-HIF-1α, HIF-1β, and β-actin Abs.

treatment. Next, we examined the effect of cilnidipine under non-hypoxic or hypoxic conditions in the presence of BAPTA-AM or thapsigargin. Co-treatment with cilnidipine and BAPTA-AM did not affect the HIF-1α protein levels under both non-hypoxic and hypoxic conditions (Fig. 4C, lanes 5 and 11). Co-treatment with cilnidipine and thapsigargin had an additive inhibitory effect on HIF-1α protein accumulation compared with the treatment with each single reagent under both 20% and 1% O_2 conditions (Fig. 4C, lanes 6 and 12). Next, the effect of the synthetic dihydropyridine derivative that acts as an active Ca^{2+} slow channel agonist Bay K-8644 was investigated. HASMCs were treated by Bay K-8644 under 20% or 1% O_2 conditions (Fig. 4D). Bay K-8664 did not affect HIF-1α accumulation under 20% or 1% O_2 conditions. Moreover, HIF-1α expression was not significantly affected in Ca^{2+}-free media and by treatment with ionomycin under both 20% and 1% O_2 conditions (Fig. 4E).

4. Discussion

In this study, we demonstrated that the calcium channel blocker cilnidipine suppresses HIF-1 activity exclusively in HASMCs in an oxygen-tension dependent manner by suppressing neosynthesis of HIF-1α protein.

Nearly all calcium channel blockers used clinically act on the L-type calcium channels and belong to three distinct classes of compounds, including dihydropyridines (e.g., nifedipine, efonidipine and cilnidipine), benzothiazepines (e.g., diltiazem), and phenylalkylamines (e.g., verapamil). We examined the effect of all the types of calcium channel blockers in this study. It is shown that cilnidipine but not nifedipine or efonidipine among DHP-type calcium channel blockers has suppressive effect on HIF-1α accumulation. Regulation of HIF-1 activity occurs via multiple mechanisms including effects on HIF-1α expression, intracellular localization of the HIF-1α:HIF-1β complex, and transcriptional activity of HIF-1α (Hirota and Semenza, 2005). Among them, regulations of HIF-1α expression level and transcriptional activity are the most crucial steps. The expression of HIF-1α is determined by balance between degradation and synthesis. In this study we indicate that cilnidipine does not affect the expression of mRNA or HIF-1α-hydroxylase activity but suppresses the translation of HIF-1α mRNA into protein (Figs. 2A and 3). Although the details of molecular process of HIF-1α synthesis are still to be elucidated, MAPK and PI3 kinase activities, which are inhibited by cilnidipine (Fig. 3D), play significant roles in determining the translational rate of HIF-1α from mRNA (Kasuno et al., 2004).

A notable finding in this study is that the effect of cilnidipine on HIF-1α expression is dependent on O_2 concentration. As shown in Fig. 1E, under 20% and 5% O_2 conditions, the inhibitory effect is prominent but under 1% O_2 condition, the effect is weaker. Lang et al. (2002) reported the presence of internal ribosomal entry site (IRES) in the HIF-1α 5'UTR promoter region. They also indicated that IRES activity is not affected by hypoxic conditions that caused a reduction in cap-dependent translation, and IRES activity was less affected by serum-starvation than was cap-dependent translation. Cilnidipine may affect IRES- and cap-dependent translation differentially.

Results in Fig. 4 strongly suggest that the suppressive effect of cilnidipine does not depend on the activity of calcium channel blockade. The calcium chelator BAPTA-AM induced HIF-1α protein expression under 20% O_2 conditions (Fig. 4A) and accordingly elevation of cytosolic calcium level by thapsigargin reduced HIF-1α protein accumulation in HASMCs (Fig. 4B) and HUVECs (data not shown). A recent study indicates that the calcium ionophore A23187 or ionomycin and thapsigargin upregulate expression of mRNA of HIF-1α via endoplasmic reticulum stress rather than a rise in intracellular calcium (Werno et al., 2008). We also demonstrated that the L-type Ca^{2+} channel agonist Bay K-8644 did not affect the HIF-1α expression (Fig. 4D) and that exposure of cells to calcium-free media did not affect expression of HIF-1α protein (Fig. 4E).

Each calcium channel blocker has individual physiochemical property. Nifedipine, efonidipine and cilnidipine have small *p*Ka value and very lipophilic. Highly lipophilic calcium channel blockers associate with cellular membranes and lipoprotein particles and modulate intracellular signaling pathway (Mason et al., 2003). The pleiotropic actions such as antioxidant properties and plasma membrane remodeling of calcium channel blockers are reported (Mason et al., 2003).

Another notable finding in this study is that cilnidipine suppresses HIF-1 activity in a cell-type specific manner. Cilnidipine selectively inhibits HIF-1 in cells from vascular smooth muscle and endothelium. Each cell may have its own HIF-1α translational system and the sensitivity to cilnidipine may be different from cells to cells. Details of the molecular mechanisms of specificity are to be clarified.

In conclusion, cilnidipine inhibits HIF-1 activity exclusively in HASMCs and HUVECs. Because HIF-1 is reported to play a critical role in the development of pulmonary hypertension by regulating pulmonary vascular remodeling (Semenza et al., 2000; Yu et al., 1999), the inhibitory effect of cilnidipine on HIF-1 may explain the therapeutic role of cilnidipine on cardiopulmonary disorders.

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