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Journal of Cellular Biochemistry 117:647–658 (2016)

Journal of Cellular Biochemistry

Protein Kinase C Isoforms Differentially Regulate Hypoxia-Inducible Factor-1α Accumulation in Cancer Cells

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ABSTRACT

Hypoxia-inducible factor-1α (HIF-1α) is one of the key transcription factors that mediate adaptation to hypoxia. Despite increasing evidence implicating the PKC family as potential modulators of HIF-1α, the molecular mechanisms of PKC isoform-dependent HIF-1α activity under hypoxic conditions have not been systematically elucidated in cancer cell lines. Here, we collectively investigated how each isoform of the PKC family contributes to HIF-1α accumulation in the human cervical cancer cell line HeLa. Among the abundant PKC isoforms, blockade of either PKCα or PKCδ was found to substantially reduce HIF-1α accumulation and transcriptional activity in hypoxic cells. Knockdown of PKCδ resulted in a reduction of HIF-1α mRNA levels, whereas the HIF-1α mRNA level was unchanged regardless of PKCα knockdown. Upon searching for the downstream effectors of these kinases, we found that PKCα controls HIF-1α translation via AKT-mTOR under hypoxic conditions. On the other hand, one of the well-known transcriptional regulation pathways of HIF-1α, nuclear factor-κB (NF-κB) is identified as a downstream effector of PKCδ. Taken together, our findings provide insights into the roles of PKC isoforms as additional, discrete modulators of hypoxia-stimulated HIF-1α accumulation through different signaling pathways. J. Cell. Biochem. 117: 647–658, 2016.

KEY WORDS: PKCα; PKCδ; HIF-1α; HYPOXIA; mTOR; NF-κB

Hypoxia-inducible factor-1 (HIF-1) regulates physiological responses to hypoxia [Semenza, 2000]. In particular, HIF-1 plays crucial roles in cancer development by enhancing cell growth, survival, and angiogenesis [Semenza, 2013]. HIF-1 is composed of two subunits: the HIF-1α subunit, which is oxygen sensitive; and the HIF-1β subunit, which is constitutively expressed. Various post-translational modifications including hydroxylation, acetylation, and phosphorylation are known to regulate the stability of HIF-1α. Importantly, under normoxic condition, HIF-1α hydroxylated by prolyl hydroxylases (PHDs) is rapidly degraded via the pVHL-mediated ubiquitin–proteasome pathway [Ivan et al., 2001]. On the other hand, HIF-1α stabilized under hypoxia interacts with coactivators such as p300/CBP, thereby activating its transcriptional activity [Lando et al., 2002]. Although the signaling pathways modulating hypoxic activation of HIF-1α are not fully understood, current models of the accumulation of HIF-1α under hypoxic conditions suggest that the decreased oxygen levels directly attenuate the activity of the PHDs, thereby blocking hydroxylation of the HIF-1α protein [Hirsila et al., 2003; Yee Koh et al., 2008; Greer et al., 2012]. Impaired hydroxylation of HIF-1α interferes with its binding to pVHL, leading to its stabilization.

Despite the prominent role of hydroxylation in HIF-1α stability, other intracellular signaling pathways have been reported to be involved in HIF-1α activation upon hypoxia. These signals include diacylglycerol kinase, small GTPases, mitochondrial reactive oxygen species, and phosphatidylinositol 3-kinase (PI3K)/AKT [Zhong et al., 2000; Zundel et al., 2000; Hudson et al., 2002; Turcotte et al., 2003]. In particular, several studies have discovered that protein kinases act as active regulators of HIF-1α [Hirota and Semenza, 2001; Shemirani and Crowe, 2002; Mylonis et al., 2006; Flugel et al.,

Grant sponsor: Multi-omics Program by Korean Ministry of Science, ICT and Future Planing; Grant sponsor: KIST; Grant number: 2E25270.

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Manuscript Received: 18 May 2015; Manuscript Accepted: 14 August 2015
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 18 August 2015
DOI 10.1002/jcb.25314 • © 2015 Wiley Periodicals, Inc.

2007]. For example, the modification of HIF-1α by the protein kinase ERK has been shown to affect the nuclear translocation of HIF-1α and the recruitment of p300/CBP, resulting in downregulation of VEGF expression through diminished HIF-1α transactivation [Mylonis et al., 2006]. Additionally, activation of GSK-3β leads to degradation of HIF-1α and further modulation of its target gene expression [Flugel et al., 2007].

Among various protein kinases, previous studies have suggested that PKC may modulate the HIF pathway. Some of these studies describe the role of PKC isoforms under "normoxic" conditions. For example, PKCζ transactivates HIF-α by boosting its interaction with p300 in renal cancer [Datta et al., 2004]. Allergic inflammation is activated by PKCδ via the PI3K/AKT/mTOR/HIF pathway in animal models and human bronchial epithelial BEAS-2B cells [Choi et al., 2013]. On the other hand, under "hypoxic" conditions, PKCα and PKCδ have been reported to be activated [Baek et al., 2001; Hui et al., 2006]. PKCα activated by Ca^{2+} ion enhances translation of HIF-1α via the mTOR pathway in PC12 and HEK293 cells [Baek et al., 2001]. The translocation of PKCδ into the cellular membrane causes the transcriptional activation of HIF-1α under hypoxic conditions in RIF cells [Baek et al., 2001]. However, the role of each PKC isoform and its subsequent signaling pathways in modulating HIF-1α activity under hypoxia has not been collectively investigated in cancer cells.

In this study, we delineate the link between PKC and HIF-1α in hypoxic cancer cells particularly focusing on the specific contributions of individual PKC isoforms to HIF-1α accumulation by using small interfering RNA (siRNA)-mediated silencing in HeLa human cervical cancer cells. Among abundant PKC isoforms, we show that the PKCα and PKCδ isoforms modulate hypoxia-induced HIF-1α accumulation in HeLa cells as well as in PC3 and HT1080 cancer cell lines. We also demonstrate that PKCα regulates HIF-1α translation via the AKT-mTOR pathway and that nuclear factor-κB (NF-κB) is a critical component that contributes to the transcription of HIF-1α downstream of PKCδ.

MATERIALS AND METHODS

MATERIALS

Fetal bovine serum (FBS) and DMEM were obtained from Life Technologies (Grand Island, NY). Gö6976, LY294002 and IKK inhibitor II were obtained from Calbiochem (La Jolla, CA). GF109203X, rottlerin, PF-4708671, bovine serum albumin (BSA) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies specific for PKCα, PKCι, PKCζ, phospho-PKCα, phospho-PKCδ, phospho-mTOR, mTOR, phospho-p70S6K, p70S6K, phospho-S6, phospho-AKT, AKT, and phospho-IκB-α were obtained from Cell Signaling Technology (Danvers, MA). Antibodies specific for PKCδ and PKCη were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody was purchased from Sigma–Aldrich, and anti-HIF-1α antibody was from BD Bioscience (San Jose, CA).

CELL CULTURE AND HYPOXIC CONDITIONS

The human cervical cancer cell line HeLa purchased from the American Type Culture Collection (Manassas, VA) was maintained in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 units/ml) at 37°C under an atmosphere of 5% CO_2. The human prostate cancer PC3 and fibrosarcoma HT1080 cell lines maintained in RPMI medium were obtained from the Korean Cell Line Bank (Korea). To generate hypoxic conditions, cells were incubated in 5% CO_2 with 1% O_2 balanced with N_2 in a hypoxic chamber (Model APM-30D, Astec Korea, Seoul, Korea) at 37°C.

RNA INTERFERENCE

Scrambled siRNAs for control measurements, as well as PKC isoform-specific siRNAs were purchased from Dharmacon (Lafayette, CO). The following siRNAs were used: PKCα-directed siRNA [ON-TARGETplus SMARTpool (L-003523)]; PKCδ-directed siRNA [ON-TARGETplus SMARTpool (L-003524)]; PKCη-directed siRNA [ON-TARGETplus SMARTpool (L-004655)]; PKCι-directed siRNA [ON-TARGETplus SMARTpool (L-004656)]; and PKCζ-directed siRNA [ON-TARGETplus SMARTpool (L-003526)]. The siRNAs were introduced into cells in Opti-MEM (GIBCO, Grand Island, NY) using Lipofectamine RNAiMAX reagent (Invitrogen, South Washington, DC) as instructed. Scrambled siRNAs were utilized for all of the control measurements. To evaluate the knockdown efficiency, the mRNA level of each gene was analyzed by real-time PCR after 48 h.

CELL EXTRACTS AND WESTERN BLOT ANALYSIS

For western blot analysis, treated HeLa cells were washed with ice-cold PBS and scraped in 150 μl of 2× sample buffer (20 mM Tris–HCl pH 6.8, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM DTT, 2% SDS, 20% glycerol). The cell lysates were sonicated. Proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes. Each membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 and incubated with primary antibodies overnight at 4°C. Blots were developed with a peroxidase-conjugated secondary antibody, followed by visualization using enhanced chemiluminescence (ATTO KOREA, Daejeon, Korea).

LUCIFERASE REPORTER ASSAY

Luciferase reporter assays were performed as previously described [Cho et al., 2008]. Briefly, cells were seeded on 6-well plates at a density of 2×10^5 cells/well. Cells were transfected with 1.6 μg of the hypoxia reporter plasmid $p(HRE)_4$-Luc containing four copies of erythropoietin HRE or of the NF-κB luciferase reporter gene [Lee et al., 1996] and 0.4 μg of plasmid pCHO110 for β-galactosidase using Lipofectamine 2,000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 h of incubation, cells were transferred to a hypoxic chamber for 18 h. The Luciferase Assay System (Promega, Fitchburg, WI) was utilized to measure the luciferase activity, which was normalized by the total protein concentrations determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA).

SEMI-QUANTITATIVE REVERSE TRANSCRIPTION (RT)-PCR AND REAL-TIME QUANTITATIVE PCR ANALYSIS

For gene expression analysis, total RNA was purified using an RNeasy Mini Kit (QIAGEN, Germantown, MD). From the purified RNA, cDNA

was synthesized using a TOPscript™ cDNA Synthesis kit (Enzynomics, Daejeon, Korea). The primers for human HIF-1α and α-tubulin were purchased from Bioneer Inc. (Daejeon, Korea). The following primers were used: HIF-1α (P190030) and α-tubulin (P204937). The primers for human VEGF, synthesized by Bioneer, were 5′-TCGGGCCTCCGAAACCATGA-3′ (forward) and 5′-CCTGGTGAGAGATCTGGTTC-3′ (reverse) [Uthoff et al., 2002]. The detailed protocol for PCR included 25 cycles of denaturation at 94°C for 45 s, annealing at 54°C (HIF-1α, α-tubulin) or 59°C (VEGF) for 45 s, and elongation at 72°C for 45 s.

For quantitative real-time PCR analysis, total RNA (2 μg) purified as above was reverse-transcribed using the TOPscript™ cDNA Synthesis kit (Enzynomics), and 1 μl of cDNA was analyzed by real-time PCR using the Power SYBR Green kit (Applied Biosystems, Foster City, CA). The primers for the human PKC isoforms (α, β, γ, δ, ε, η, θ, ι, and ζ), HIF-1α and α-tubulin were purchased from Bioneer Inc. (Daejeon, Korea). The primers used are as follows: PKCα (P312739); PKCβ (P324297); PKCγ (P322618); PKCδ (P279370); PKCε (P277339); PKCη (P184366); PKCθ (P157186); PKCι (P143696); PKCζ (P186939); HIF-1α (P190030); and α-tubulin (P204937). To amplify and quantify the mRNA of the PKC isoforms and α-tubulin control, the following conditions were used: pre-incubation at 46°C for 30 min, an initial melt at 95°C for 10 min, and 40 cycles of amplification (95°C for 15 s, 60°C for 60 s) with 5 μM of each primer. The mRNA expression level was normalized to that of α-tubulin and compared using the $2^{-\Delta\Delta C}{}_{T}$ method [Livak and Schmittgen, 2001].

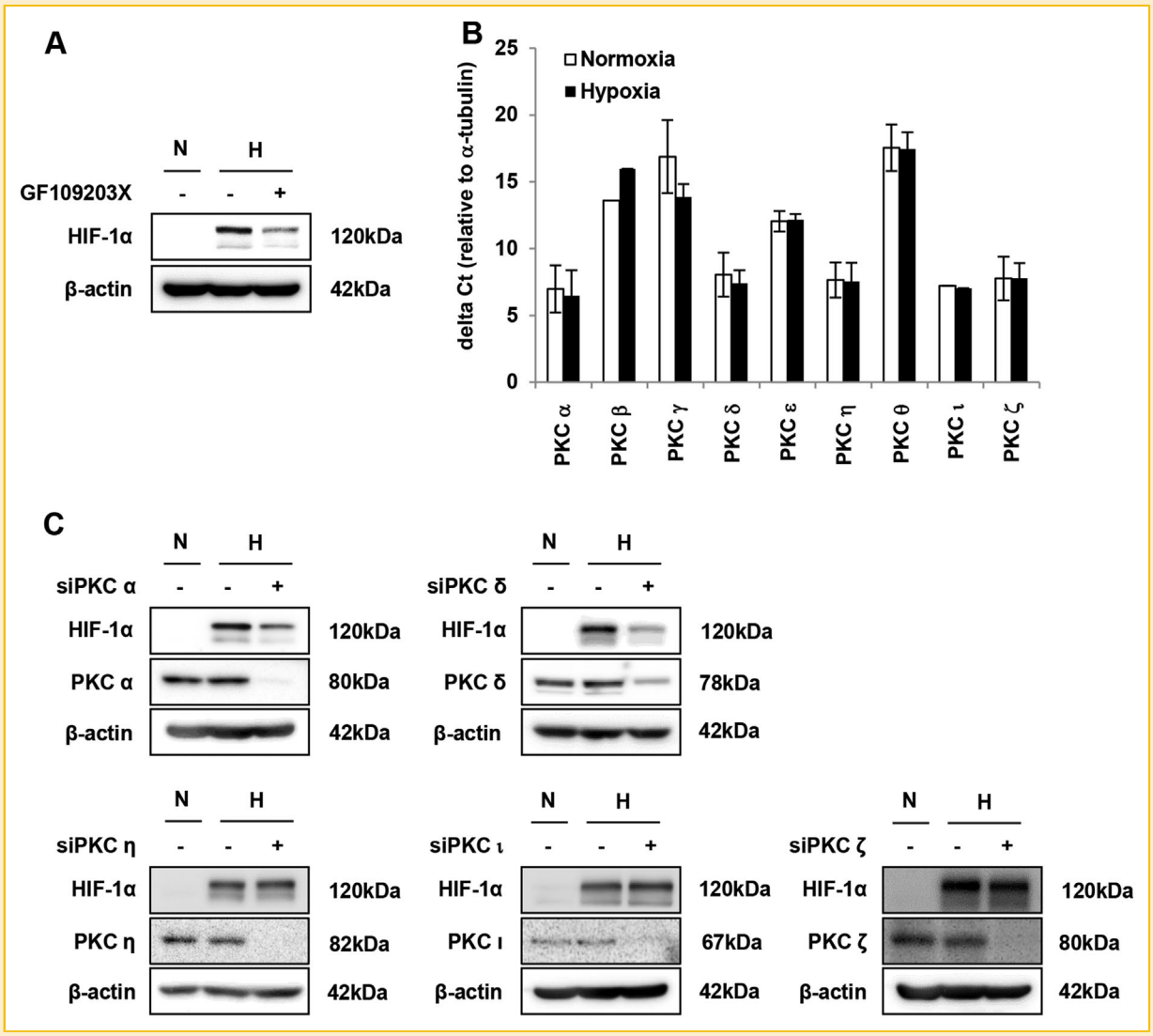

Fig. 1. PKCα and PKCδ control HIF-1α accumulation in HeLa cells. A: HeLa cells were pretreated with the broad PKC inhibitor GF109203X (10 μM) for 30 min and then exposed to hypoxic conditions for 18 h. HIF-1α protein expression was evaluated using western blotting (N, normoxia; H, hypoxia). B: mRNA levels of PKC isoforms (α, β, γ, δ, ε, η, θ, ι, and ζ) were measured by quantitative real-time PCR analysis, and the ΔCt values were normalized to α-tubulin from samples harvested 18 h after the induction of hypoxia. C: HeLa cells were transfected with control siRNAs or siRNAs directed against PKCα, δ, η, ι, and ζ. After 48 h, the transfected cells were exposed to hypoxic conditions for 18 h. The protein levels of both HIF-1α and PKC were evaluated using western blotting (N, normoxia; H, hypoxia).

DATA ANALYSIS AND STATISTICS

The data represent three independent experiments. The results are presented as the means ± Standard deviation (S.D.). Statistical analyses were performed with Student's *t*-test using SigmaPlot 8.0 software. *P*-values less than 0.05 were considered significant.

RESULTS

PKCα AND PKCδ ARE INVOLVED IN HYPOXIA-INDUCED HIF-1α ACCUMULATION IN CANCER CELLS

To explore the possible regulation of HIF-1α expression by PKC under hypoxic conditions, HeLa cells were subjected to a broad PKC inhibitor GF109203X [Lee and Stern, 2000] (Fig. 1A). The PKC inhibitor dramatically reduced HIF-1α expression upon hypoxic insult, implying that PKC likely regulates HIF-1α accumulation. On the basis of such finding, we investigated the extent to which PKC isoforms contribute to HIF-1α accumulation. We first determined the abundance of PKC isoforms in HeLa cells by using quantitative real-time PCR analysis. The relative gene expression levels were estimated by calculating the ΔCt values for each PKC isoform. The Ct values for the PKC isoforms were normalized to the α-tubulin Ct values. Because ΔCt is inversely correlated with the gene expression level, a lower ΔCt value indicates a greater amount of gene transcript. As shown in Figure 1B, the mRNA levels of PKCα, PKCδ, PKC*η*, PKCι, and PKCζ were much greater than those of the other PKC isoforms regardless of the oxygen concentration. Therefore, we further investigated whether these five abundant PKC isoforms, PKCα, PKCδ, PKC*η*, PKCι, and PKCζ, affected HIF-1α accumulation under hypoxic conditions. Knockdown of PKCα and PKCδ by siRNAs markedly decreased hypoxia-induced HIF-1α accumulation in HeLa cells, but knockdown of PKC*η*, PKCι and PKCζ (Fig. 1C) did not. The depletion of either PKCα or PKCδ did not affect cell viability (data not shown). Consistent with the observations in HeLa cells, decreased HIF-1α accumulation was similarly observed in hypoxic PC3 and HT-1080 cells treated with GF109203X (Fig. 2A) and both PKCα and PKCδ were activated under hypoxic conditions (Fig. 2B). Also, the HIF-1α level stabilized by hypoxia was reduced upon knockdown of either PKCα or PKCδ in HT1080 cells (Fig. 2C). Taken together, these results pointed out that PKCα and PKCδ are involved in hypoxia-induced HIF-1α accumulation in cancer cells.

SUPPRESSING PKCα OR PKCδ PHOSPHORYLATION USING PHARMACOLOGICAL INHIBITORS SIGNIFICANTLY ATTENUATES HIF-1α ACCUMULATION IN HYPOXIC HELA CELLS

Next, we investigated whether phosphorylated PKCα and PKCδ were involved in HIF-1α accumulation upon hypoxic treatment [Rahman et al., 2001; Nakashima, 2002; Rybin et al., 2003]. Under normoxic conditions, we found that both PKCα and PKCδ were partially phosphorylated at Ser657 and Thr505, respectively (Fig. 3). Treatment with Gö6976 (100 nM) for PKCα inhibition [Aaltonen and Peltonen, 2010], or rottlerin (5 μM) for PKCδ inhibition [Lee et al., 2007], markedly inhibited the phosphorylation of PKCα and PKCδ, respectively, without affecting their total levels (Fig. 3). Compared with normoxia, hypoxia increased the phosphorylation of both

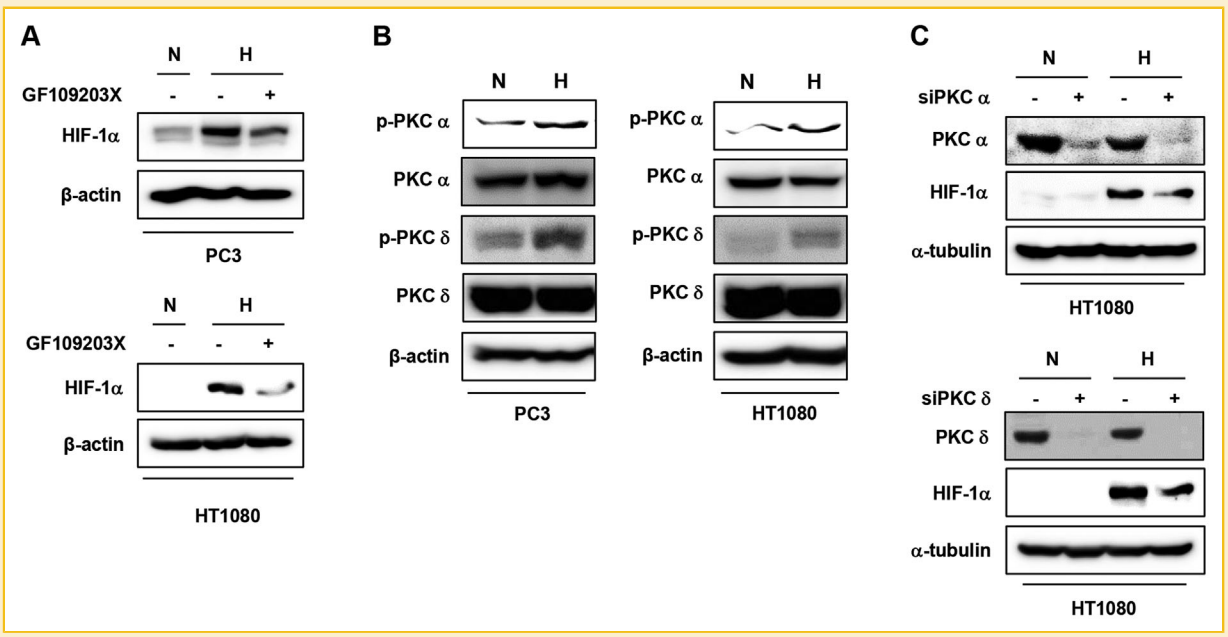

Fig. 2. Both PKCα and PKCδ control HIF-1α accumulation in various cancer cells. A: PC3 and HT1080 cells were pretreated with the broad PKC inhibitor GF109203X (10 μM) for 30 min and then exposed to hypoxic conditions for 18 h. HIF-1α protein expression was evaluated using western blotting (N, normoxia; H, hypoxia). B: PC3 and HT1080 cells were exposed to hypoxic conditions for 18 h. The expression levels of p-PKCα (Ser657), PKCα, p-PKCδ (Thr505), and PKCδ were evaluated by western blotting (N, normoxia: H, hypoxia). C: HT1080 cells were transfected with control siRNAs or siRNAs directed against PKCα and PKCδ. After 48 h, the transfected cells were exposed to hypoxic conditions for 18 h. The protein level of HIF-1α was evaluated using western blotting (N, normoxia; H, hypoxia).

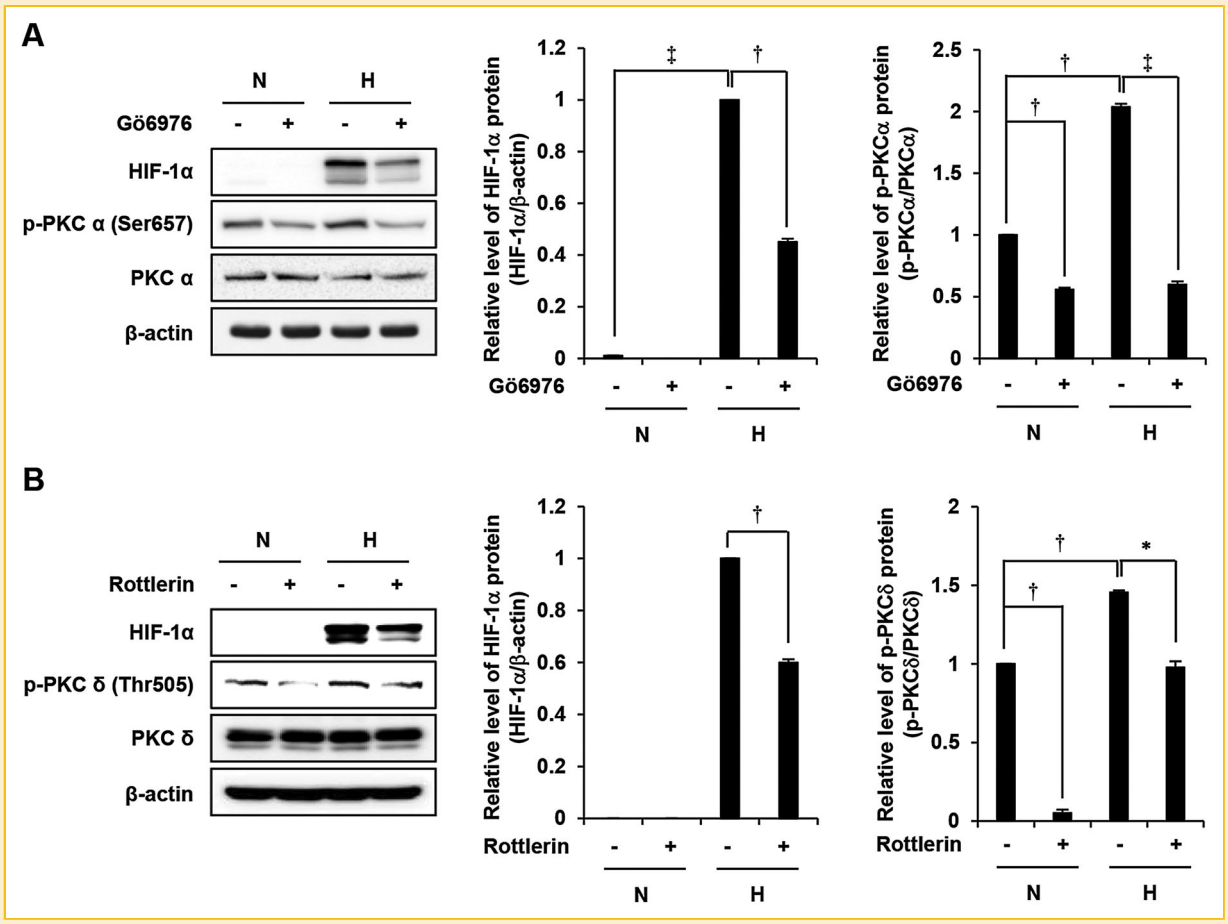

Fig. 3. Pharmacological suppression of PKCα or PKCδ with specific pharmacological inhibitors significantly attenuates hypoxia-induced HIF-1α accumulation in HeLa cells. A: HeLa cells were pretreated with Gö6976 (100 nM) for 30 min and then exposed to hypoxic conditions for 18 h. The expression levels of HIF-1α, p-PKCα (Ser657) and endogenous PKCα were evaluated by western blotting (N, normoxia; H, hypoxia). The protein levels of HIF-1α relative to β-actin and p-PKCα (Ser657) relative to PKCα were calculated. $^{*}P < 0.05$, †$P < 0.01$, ‡ $P < 0.005$. B: HeLa cells were pretreated with rottlerin (5 μM) for 30 min and then exposed to hypoxic conditions for 18 h. The expression levels of HIF-1α, p-PKCδ (Thr505), and endogenous PKCδ were evaluated by western blotting (N, normoxia; H, hypoxia). The protein levels of HIF-1α relative to β-actin and p-PKCδ (Thr505) relative to PKCδ were calculated. $^{*}P < 0.05$, †$P < 0.01$, ‡ $P < 0.005$.

PKCs. Furthermore, both inhibitors attenuated the hypoxia-induced HIF-1α accumulation in HeLa cells (Fig. 3), which was consistent with the decrease in phosphorylated PKCs. Based on these observations, we concluded that the activation of PKCα or PKCδ by phosphorylation contributes to the upregulation of hypoxia-induced HIF-1α accumulation in HeLa cells.

PKCα AND PKCδ ENHANCE THE TRANSCRIPTIONAL ACTIVITY OF HIF-1α

Stabilized under hypoxic conditions, the HIF-1α protein can activate a specific set of genes by association with the hypoxia response element (HRE) in the promoter region. To determine the effects of PKCα and PKCδ on HIF-1α transactivation, the activity of a luciferase reporter construct containing four repeats of the HRE was measured in HeLa cells after treatment with PKCα- or PKCδ-directed siRNAs. Knockdown of either PKCα or PKCδ barely changed the luciferase activity level in normoxia, but significantly attenuated the hypoxia-induced increase in HRE activity (Fig. 4A). These changes in HIF-1-mediated transcriptional activity were further confirmed by determining the hypoxia-induced expression of VEGF, a target of HIF-1. The mRNA level of VEGF under hypoxic conditions decreased after treatment with the PKCα- or PKCδ-directed siRNAs in HeLa cells (Fig. 4B), indicating that both PKCα and PKCδ contributed to the activation of HIF-1α-mediated transcription.

PKCδ REGULATES HIF-1α TRANSCRIPT LEVELS

The expression level of HIF-1α under hypoxic conditions can be actively regulated at three different levels: transcription, translation, and modulation of its degradation. To reveal the detailed mechanism by which PKCα and PKCδ regulate HIF-1α accumulation, thereby regulating its transcriptional activity, we first investigated whether HIF-1α protein expression was regulated through its mRNA level. As shown in Figure 5A, reducing PKCα phosphorylation by exposing cells to Gö6976 did not affect the HIF-1α mRNA level under hypoxic conditions. Similarly, knockdown of PKCα by a PKCα-directed siRNA

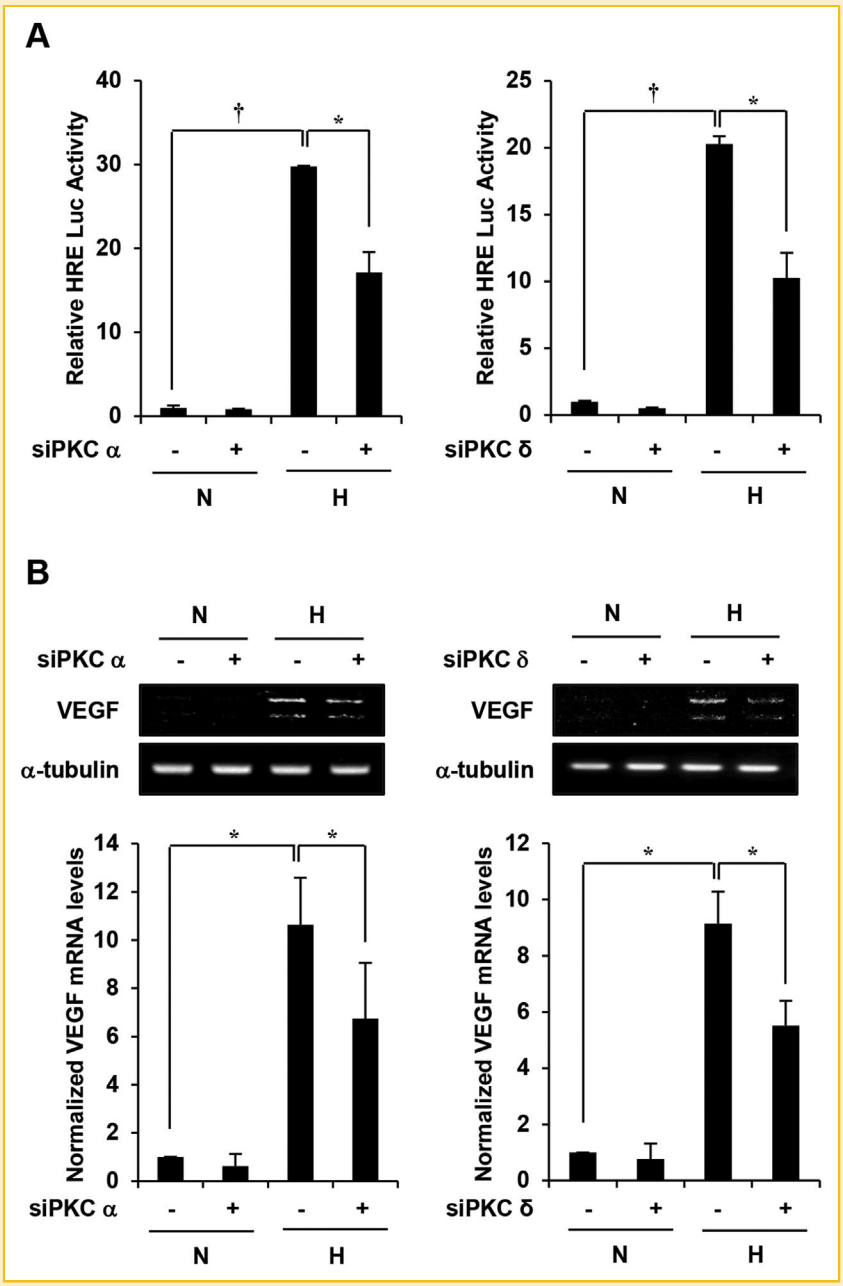

Fig. 4. Both PKCα and PKCδ enhance the transactivation activity of HIF-1α. A: HeLa cells were transfected with siRNAs targeting PKCα (left panel), PKCδ (right panel) or control SiRNAs for 24 h and then transfected with the HRE-Luc reporter plasmid for 24 h. The treated cells were exposed to hypoxic conditions for 18 h, harvested, and then subjected to a relative luciferase activity assay (N, normoxia; H, hypoxia). All quantitative data are presented as the mean ± S.D. of three independent experiments. $^{*}P < 0.05$, $\dagger P < 0.01$, $\ddagger P < 0.005$. B: HeLa cells were transfected with siRNAs targeting PKCα (left panel) or PKCδ (right panel) for 48 h and then exposed to hypoxic conditions for 18 h. The mRNA level of VEGF was determined by semi-quantitative RT-PCR (N, normoxia; H, hypoxia).

in HeLa cells did not alter the HIF-1α mRNA level under hypoxic conditions (Fig. 5B). In contrast, inhibition of PKCδ activity with either rottlerin or specific siRNA decreased the level of HIF-1α mRNA regardless of oxygen availability (Fig. 5C, D), although it should be noted that rottlerin exhibits some inappropriate, off-target effects [Soltoff, 2007]. Therefore, it is likely that PKCδ regulates HIF-1α expression at the transcriptional level, whereas PKCα may exploit a different signaling pathway.

PKCα ENHANCES THE TRANSLATION OF HIF-1α DURING HYPOXIA VIA PI3K-AKT-mTOR PATHWAY

Since the PI3K-AKT-mTOR pathway has been implicated in regulating HIF-1α translation [Semenza, 2009; Dodd et al., 2015], we examined the possibility of cross-talk between PKCα and the PI3K-AKT-mTOR pathway by observing the effects of PKCα knockdown on the activity of PI3K-AKT-mTOR and on the mTOR-dependent phosphorylation of p70S6 kinase (p70S6K). Although a recent study describes 4E-BP1, a

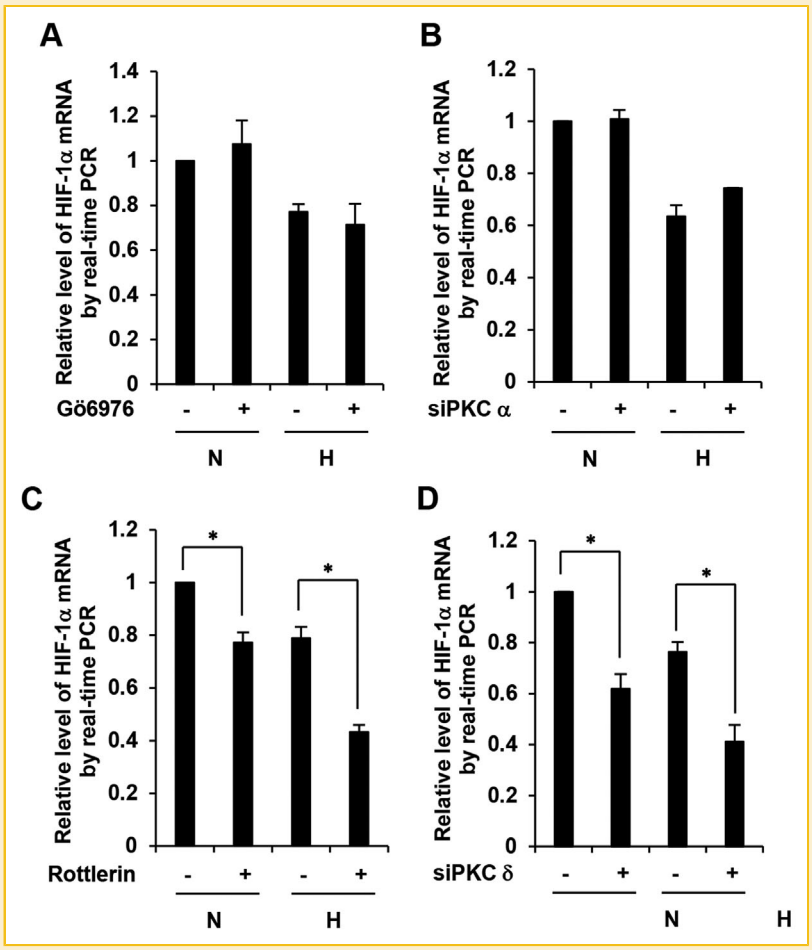

Fig. 5. PKCδ regulates the HIF-1α transcript level. A: HeLa cells were pretreated with Gö6976 (100 nM) for 30 min and then exposed to hypoxic conditions for 18 h. The level of HIF-1α mRNA was determined by real-time RT-PCR (N, normoxia; H, hypoxia). B: HeLa cells were transfected with PKCα or control siRNAs for 48 h and then exposed to hypoxic conditions for 18 h. The level of HIF-1α mRNA was determined by real-time PCR (N, normoxia; H, hypoxia). C: HeLa cells were pretreated with rottlerin (5 μM) for 30 min and then exposed to hypoxic conditions for 18 h. The level of HIF-1α mRNA was determined by real-time RT-PCR (N, normoxia; H, hypoxia). D: HeLa cells were transfected with PKCδ-directed or control siRNAs for 48 h and then exposed to hypoxic conditions for 18 h. The level of HIF-1α mRNA was determined by real-time PCR (N, normoxia; H, hypoxia). All quantitative data are presented as the mean ± S.D. from three independent experiments. $^{*}P < 0.05$, †$P < 0.01$, ‡ $P < 0.005$.

downstream of mTOR can also regulate the translation of HIF-1α [Dodd et al., 2015], we specifically focused on the phosphorylation of p70S6K, because it plays an important role in maintaining active translation by phosphorylating ribosomal S6 protein (S6) [Hay and Sonenberg, 2004]. As shown in the left panel of Figure 6A, we found that siRNA-mediated PKCα knockdown inhibited the hypoxia-induced phosphorylation of AKT, while the level of AKT protein remained unchanged. Although hypoxia inhibited mTOR activity by decreasing the phosphorylation of mTOR and p70S6K, PKCα knockdown further inhibited the phosphorylation of mTOR and p70S6K compared with the control cells (Fig. 6A, left panel). We also found that the phosphorylated form of S6 was diminished by knockdown of PKCα (Fig. S1A). To further reveal the role of S6 phosphorylation on HIF-1α translation, we treated cells with PF-4708671, a specific inhibitor of p70S6K [Pearce et al., 2010]. Cells treated with PF-4708671 did not show any change of HIF-1α protein accumulated under hypoxic conditions (Fig. S1B). Such findings suggest that the p70SK6/S6 pathway might not be directly involved in HIF-1α translation.

Next, we examined whether AKT signaling influences the mTOR pathway by applying the PI3K inhibitor LY294002 (30 μM) to HeLa cells under hypoxic conditions. LY294002 inhibited the phosphorylation of mTOR and p70S6K (Fig. 6B, left panel). Furthermore, the cells pre-treated with LY294002 showed decreased expression of HIF-1α (Fig. 6B, right panel), which correlated with the decrease in AKT phosphorylation. Under this condition, we confirmed that both the HIF-1α mRNA level and the PKCα activity remained unchanged (Fig. 6C). Therefore, these results suggest that the induction of HIF-1α translation in hypoxia requires PKCα-dependent stimulation of the PI3K-AKT-mTOR pathway.

MECHANISTIC CONSIDERATION OF THE ACTION OF PKCδ ON HYPOXIA-INDUCED HIF-1α ACCUMULATION IN HELA CELLS

Based on the results shown in Figure 5, we explored the signaling pathway of PKCδ-dependent HIF-1α transcription in more detail. First, we further observed that PKCδ did not affect the phosphorylation of AKT or mTOR (Fig. 7A). It has been shown that NF-κB

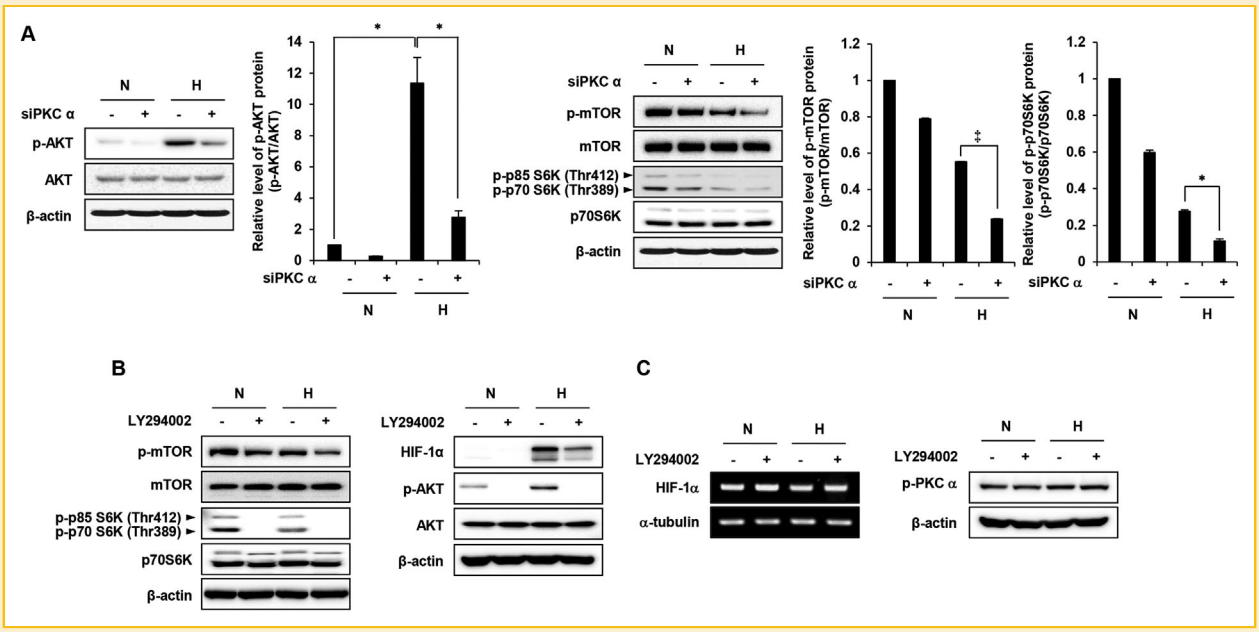

Fig. 6. PKCα is involved in HIF-1α translation during hypoxia. A: HeLa cells were transfected with PKCα-directed or control siRNAs for 48 h, and then exposed to hypoxic conditions for 18 h. The cell lysates were subjected to western blotting (N, normoxia; H, hypoxia) for p-AKT/AKT (left) and for p-mTOR, mTOR, p-p70S6K, and p70S6K (right). The protein levels of HIF-1α relative to β-actin, p-mTOR relative to mTOR and p-p70S6K relative to p70S6K were calculated. $^{*}P < 0.05$, †$P < 0.01$, ‡ $P < 0.005$. B: HeLa cells pretreated with LY294002 (30 μM) for 30 min were exposed to hypoxic conditions for 18 h. The cell lysates were subjected to western blotting (N, normoxia; H, hypoxia) for p-mTOR, mTOR, p-p70S6K, and p70S6K (left), as well as p-AKT/AKT and HIF-1α (right). C: HeLa cells were treated as described in (B). The HIF-1α mRNA level was determined by semi-quantitative RT-PCR (left, N, normoxia; H, hypoxia). The p-PKCα (Ser657) expression level was determined by western blotting (right, N, normoxia; H, hypoxia).

activation is a crucial component of the transcriptional response to hypoxia. NF-κB was previously demonstrated to bind to the HIF-1α promoter, resulting in the transcriptional regulation of HIF-1α [van Uden et al., 2008]. Therefore, we investigated whether PKCδ regulates hypoxia-induced NF-κB activation. Western blot analysis revealed that depletion of PKCδ by RNAi moderately inhibited the phosphorylation of the NF-κB inhibitor IκB-α (Fig. 7B), whose phosphorylation by the IκB kinase complex leads to NF-κB activation [Baud and Karin, 2009]. Consistent with these observations, PKCδ depletion significantly attenuated the activity of the NF-κB-dependent luciferase reporter gene (Fig. 7C). Lastly, we explored whether NF-κB contributes to the HIF-1α accumulation resulting from hypoxia. When HeLa cells were pretreated with the NF-κB inhibitor IKK inhibitor II before exposed to hypoxia, HIF-1α expression was attenuated under the hypoxic condition (Fig. 7D, left panel). Furthermore, the NF-κB inhibitor reduced the HIF-1α mRNA levels in both normoxia and hypoxia (Fig. 7D, right panel), indicating that NF-κB activation is downstream of PKCδ and regulates HIF-1α transcription.

DISCUSSION

The central regions of solid tumors frequently display severe hypoxia, and hypoxic areas have been recognized as a main target for clinical studies and treatment strategies owing to their high resistance to chemotherapy and radiation. Specifically, given the critical role of HIF-1α in hypoxia, tremendous efforts have been made to reveal the signaling pathways involved in HIF-1α regulation. On the other hand, PKC has been linked to cancer development and progression because its activators often serve as tumor promoters [Griner and Kazanietz, 2007]. PKC is generally classified into three subfamilies based on its structure and allosteric requirements [Parker and Murray-Rust, 2004], and its isoforms generally display changes in activation balance in a variety of human cancers [Garg et al., 2014] despite a rather surprising recent identification of cancer-associated PKC mutations as tumor suppressors [Antal et al., 2015]. While overexpression of PKC isoforms is closely correlated with tumor aggressiveness, their expression patterns and functions depend on the type of cancer cells [Urtreger et al., 2012]. We have shown here that HeLa cervical cancer cells express five PKC isoforms including PKCα, δ, η, ι, and ζ more than the any other isoforms (Fig. 1B). Previously, PKC was found to be significantly overexpressed in cancer samples compared with adjacent normal cervical tissues [Yu et al., 2011]. However, distinctly from our observations, the levels of PKCα, δ, θ, and ζ were particularly elevated, implying context-specific expression of PKC isoforms.

In cancer, PKC isoforms play critical roles in cell proliferation, survival, invasion, migration, apoptosis, angiogenesis, and anti-cancer drug resistance. Expression of more than one isoform of PKC in most cancer cells suggests that individual isoforms have unique rather than overlapping functions, and therefore different PKC isoforms are expected to have distinctive effects on cancer progression. Activation of PKCα has been associated with malignant phenotype by increasing cell proliferation and migration as well as

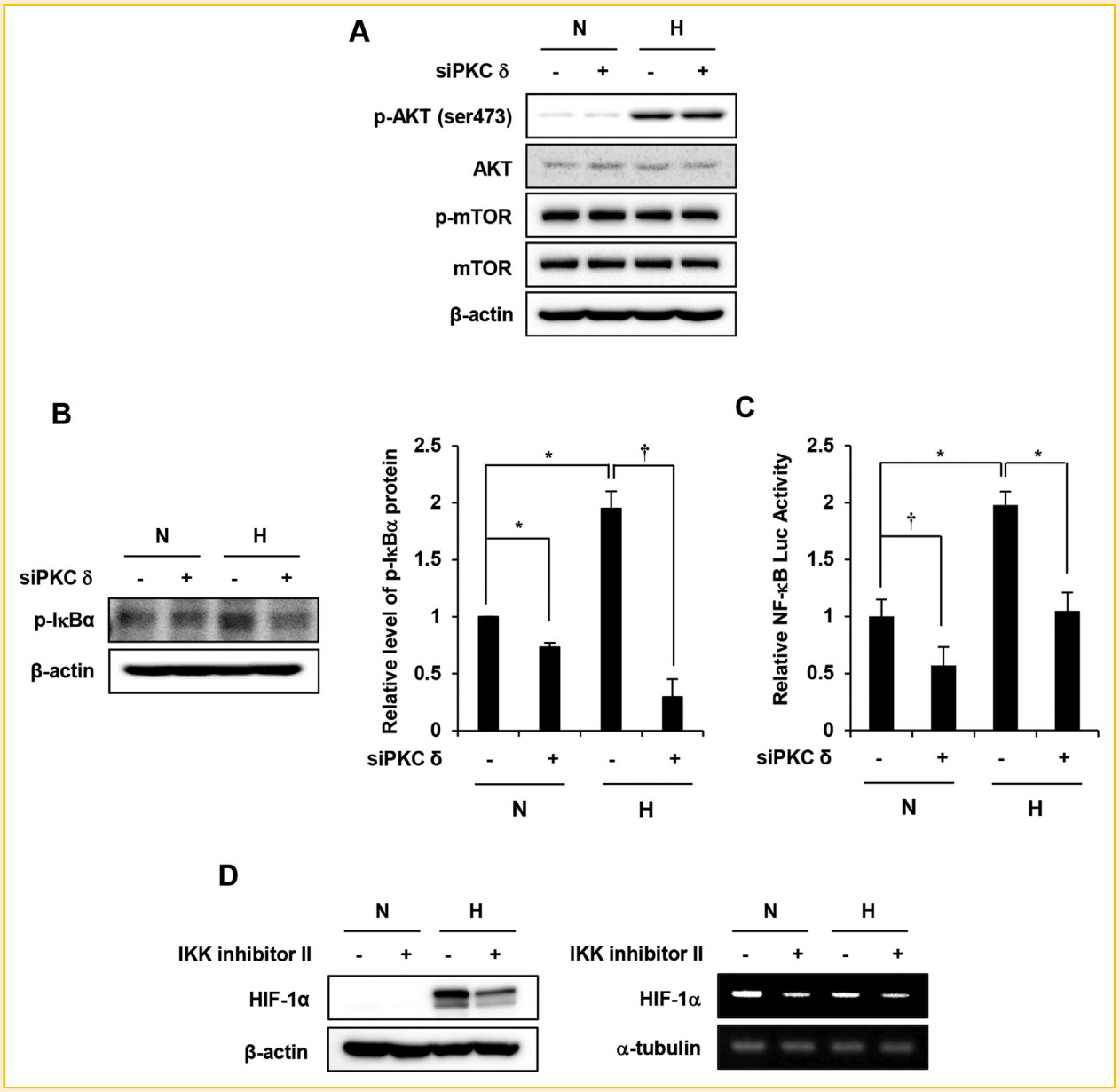

Fig. 7. NF-κB mediates the effect of PKCδ on hypoxia-induced HIF-1α accumulation in HeLa cells. A: HeLa cells were transfected with PKCα-directed or control siRNAs for 48 h, and then exposed to hypoxic conditions for 18 h. The cell lysates were subjected to western blotting for p-AKT, AKT, p-mTOR, and mTOR (N, normoxia; H, hypoxia). B: HeLa cells treated as described in (A) were subjected to western blotting for p-IκBα (N, normoxia; H, hypoxia). The protein level of p-IκBα relative to β-actin was calculated. C: HeLa cells were transfected with PKCδ-directed siRNAs for 24 h and then transfected with the NF-κB-Luc reporter plasmid for 24 h. The transfected cells were exposed to hypoxic conditions for 18 h. The cells were then harvested and assayed for the relative luciferase activity (N, normoxia; H, hypoxia). All quantitative data are presented as the mean ± S.D. of three independent experiments. $^{*}P < 0.05$. D: HeLa cells were treated with IKK inhibitor II (10 μM) for 30 min and then exposed to hypoxic conditions for 18 h. The cell lysates were subjected to western blotting for HIF-1α (left, N, normoxia; H, hypoxia). The HIF-1α mRNA levels were determined by semiquantitative RT-PCR (right, N, normoxia; H, hypoxia).

inhibiting apoptosis, but PKCβ and PKCδ are thought to produce opposing outcomes depending on cancer types [Urtreger et al., 2012]. Interestingly, our present study shows that two different isoforms, PKCα and PKCδ, likely play important roles in hypoxic stabilization of HIF-1α via distinct signaling pathways.

Previous studies have shown the involvement of the PI3K-AKT-mTOR pathway in regulating HIF-1α expression under hypoxic conditions [Zhong et al., 2000; Hudson et al., 2002; Zhou et al., 2004; Dodd et al., 2015]. The regulation of PKCα on PI3K-AKT-mTOR pathway, on the other hand, has been described. PKCα has previously been shown to participate in the regulation of AKT [Partovian and Simons, 2004]. Furthermore, calcium-dependent activation of PKCα and mTOR induces HIF-1α protein synthesis in PC12 cells upon exposure to hypoxia [Hui et al., 2006]. Consistent with the previous reports, we observed that siRNA-mediated PKCα knockdown inhibited the hypoxia-induced phosphorylation of AKT

and of components of the mTOR signaling pathway, such as p70S6K and mTOR itself (Fig. 6A). Although general protein synthesis is mainly hampered by hypoxia [Arsham et al., 2003; Brugarolas et al., 2003, 2004], such inhibition is incomplete, and some mRNAs including HIF-1α are translated through the mTOR pathway in a cap dependent manner [Yee Koh et al., 2008]. Consistent with the result obtained by depleting PKCα, the PI3K inhibitor LY294002 attenuated the phosphorylation of mTOR and p70S6K, as well as the hypoxic accumulation of HIF-1α (Fig. 6B). These data imply that the induction of HIF-1α expression by hypoxia requires the activation of the PKCα-AKT-mTOR pathway in HeLa cells. As a downstream of mTOR, Dodd et al. have described that three distinct downstream targets, 4E-BP1, p70S6K, and STAT3 are all involved in HIF-1α regulation [Dodd et al., 2015]. In specific, both 4E-BP1 and p70S6K regulate HIF-1α translation, while STAT3 controls transcription level of HIF-1α protein. Although we found that both p70S6K and S6 proteins were dephosphorylated by knockdown of PKCα (Fig. 6B and Fig. S1A), the amount of HIF-1α accumulated under hypoxia was not changed by a specific p70S6K inhibitor, PF-470861 (Fig. S1B). In this regard, it is likely that HIF-1α accumulation is achieved by PKCα via mTOR-4E-BP1 pathway, rather than mTOR-p70S6K. However, other possibility such as HIF-1α stabilization by AMPK activated via PF-4708671 cannot be excluded [Vainer et al., 2014].

In contrast to our data, PKCδ, not PKCα regulates HIF through the mTOR pathway under normoxic condition in response to the airway inflammation [Choi et al., 2013]. These inconsistent results emphasize that different PKC isoforms can play the same role in regulating HIF pathway depending on the experimental conditions. Also, the discrepant observations may likely be attributed to utilizing different cell lines and oxygen availability.

Our study also provides a mechanistic insight into how PKCδ acts on regulating HIF-1α. In accordance with the involvement of NF-κB as the downstream mediator (Fig. 7), it has been previously suggested that NF-κB is a direct modulator of HIF-1α [van Uden et al., 2008] and that PKCδ modulates the activity of NF-κB [Cummings et al., 2004; Satoh et al., 2004]. We also found that PKCδ knockdown suppressed NF-κB activation (Fig. 7B, C), resulting in decreased HIF-1α mRNA level regardless of oxygen availability (Fig. 5D) due to the absolute necessity of basal canonical NF-κB signaling for HIF expression [Rius et al., 2008]. Furthermore, the specific inhibitor of IKKα/β kinase attenuated hypoxia-induced HIF-1α accumulation by reducing the HIF-1α mRNA level (Fig. 7D). However, the detailed mechanism responsible for the potential link between PKCδ and NF-κB requires further clarification.

In summary, we firmly establish a role for the PKC isoforms PKCα and PKCδ in the hypoxia-induced accumulation of HIF-1α in tumor cells, which provides important insight into the protein kinase-dependent accumulation of HIF-1α in cancer cells. Since we have observed the similar results in three different cell lines representing cervical cancer (HeLa), prostate cancer (PC3), and fibrosarcoma (HT1080), hypoxic environments in these solid tumors likely induce PKC isoform-dependent HIF-1α accumulations. As summarized in Figure 8, our data reveal that the PKCα-AKT-mTOR and the PKCδ-NF-κB cascades lead to hypoxia-induced HIF-1α accumulation in cancer cells. Because of the insignificant changes in HIF-1α levels upon siRNA knockdown, we did not attempt to investigate the effects of the other abundant PKC isoforms, including PKC*η*, PKCι, and PKCζ. Thus, we cannot entirely exclude the possibility that these isoforms also act to regulate the function of HIF-1α by modulating its transcriptional activity. Further elucidation of hypoxic behaviors of these isoforms in relation to HIF-1α might augment our understanding of the mechanisms controlling the transcriptional activity of HIF-1α.

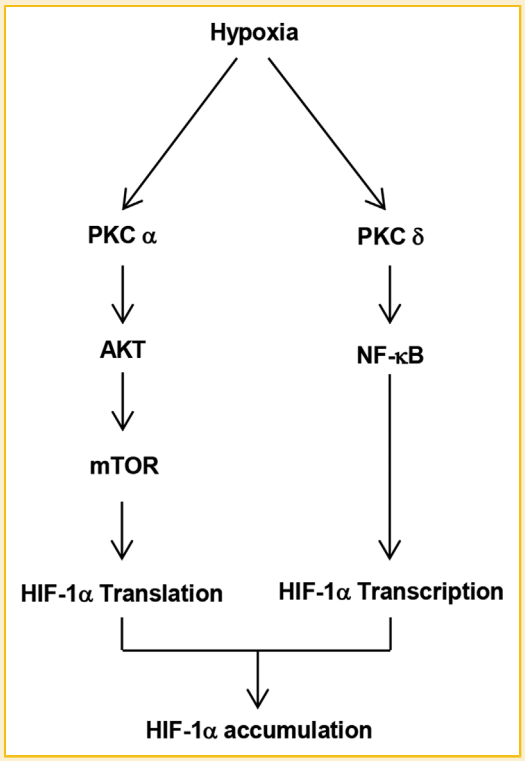

Fig. 8. A proposed schematic of the signaling pathways that regulate hypoxia-induced HIF-1α accumulation through two PKC isoforms; PKCα and PKCδ. PKCα regulates HIF-1α translation through the AKT-mTOR pathway, while PKCδ is involved in HIF-1α transcription via NF-κB.

ACKNOWLEDGMENT

This work was supported by the Multi-omics Program funded through NRF by the Korean Ministry of Science, ICT and Future Planning, and by a KIST grant (2E25270). H. Kim is the recipient of a post-doctoral fellowship from the National Research Council of Science and Technology.

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