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PKC-δ inhibitors sustain self-renewal of mouse embryonic stem cells under hypoxia *in vitro*

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Abbreviations: GF, GF 109203X; HIF-1α, hypoxia-inducible factor-1α; LIFR, LIF-specific receptor; mESCs, mouse embryonic stem cells; STAT, signal transducers and activators of transcription

Abstract

Under hypoxia, mouse embryonic stem cells (mESCs) lose their self-renewal activity and display an early differentiated morphology mediated by the hypoxia-inducible factor-1α (HIF-1α). Previous studies have demonstrated that PKC-δ is activated by hypoxia and increases the protein stability and transcriptional activity of HIF-1α in human cancer cells. Furthermore, activation of PKC-δ mediates cardiac differentiation of ESCs and hematopoietic stem cells. However, the role of PKC-δ in hypoxia-induced early differentiation of mESCs remains largely unknown. Here, we show the inhibition of PKC-δ activity prevents the early differentiation of mESCs under hypoxia using PKC-δ inhibitors, GF 109203X and rottlerin. Reduction of PKC-δ activity under hypoxia effectively decreased HIF-1α protein levels and substantially recovered the expression of LIF-specific receptor (LIFR) and phosphorylated-STAT3 in mESCs. Furthermore, PKC-δ inhibitors aid to sustain the expression of self-renewal markers and suppress the expression of early differentiation markers in mESCs under hypoxia. Taken together, these results suggest that PKC-δ inhibitors block the early differentiation of mESCs via destabilization of HIF-1α under hypoxia.

Keywords: anoxia; embryonic stem cells; hypoxia-inducible factor 1, α subunit; protein kinase C-δ; rottlerin

Introduction

The mESCs derived from the inner cell mass of blastocysts have been used for the research fields including molecular mechanism and cell-fate in early mammalian development, because of their potentials to generate any type of cell (Evans and Kaufman, 1981). In the presence of the leukemia inhibitory factor (LIF), which is a cytokine of the IL-6 family, mESCs have the ability to self-renew (Niwa *et al*., 1998); however, when LIF is absent or when appropriate differentiation agents are added, mESCs differentiate into many types of cells, including endothelial cells (Li *et al*., 2007), adipocytes (Bost *et al*., 2002), neurons (Schrenk-Siemens *et al*., 2008), and smooth muscle cells (Du *et al*., 2004; Yu and Thomson, 2008). In a related signaling pathway, LIF binds to the LIFR with low-affinity, followed by heterodimerization with glycoprotein 130. This high-affinity complex activates the Janus kinase (JAK) and the signal transducer and activator of transcription 3 (STAT3) signaling pathways (Ernst *et al*., 1999; Tighe and Gudas, 2004). In mESCs, STAT3 is an important substrate for activated JAKs and is considered as a sufficient key regulator of the LIF-induced self-renewal of mESCs (Matsuda *et al*., 1999).

During early mammalian development, embryos and their tissues are generally exposed to pO_2 values (~5% O_2, or hypoxia) that are lower than atmospheric pO_2 values (20% O_2, or normoxia) (Land, 2004; Powers *et al*., 2008). As the embryo grows, oxygen requirements may be markedly increased, while blood circulation remains inadequate until vascularization begins after implantation (Gassmann *et al*., 1996; Simon and Keith, 2008); thus, the early embryo undergoes a transient hypoxia. Hypoxic regions in normal embryo development (Lee *et al*., 2001) and even after a

well-developed vascularization (Powers *et al*., 2008) include reproductive tract (Masuda *et al*., 2000), the microvasculature (Intaglietta *et al*., 1996), and the ocular vitreous (Shui *et al*., 2006). Hypoxia induces many cellular processes mainly mediated by the hypoxia-inducible factor-1 (HIF-1) (Lee *et al*., 2004; Peyssonnaux *et al*., 2007). HIF-1 is composed of two subunits: an oxygen-insensitive β unit (HIF-1β) and a hypoxia-regulated α subunit (HIF-1α) (Fedele *et al*., 2002). Under normoxic conditions, the HIF-1α subunit is rapidly ubiquitinated and degraded through the pVHL-E3 ligase pathway, which is triggered by the oxygen-dependent hydroxylation of proline residues in HIF-1α (Semenza, 2001); however, under hypoxia this degradation pathway is blocked and HIF-1α becomes stable, forms a heterodimer with HIF-1β, and interacts with coactivators (such as p300/CBP) to bind to hypoxia responsive elements (HREs) in oxygen-regulated genes and modulate their transcriptional activity (Wang *et al*., 1995). The stability of HIF-1α is critically regulated by many posttranslational modifications, including hydroxylation (Berra *et al*., 2003), acetylation (Bae *et al*., 2004), and phosphorylation (Wei and Yu, 2007). These modifications are mediated by interaction of HIF-1α with several proteins, including PHD, HDAC, ARD-1, pVHL, and p300/CBP (Jeong *et al*., 2002).

PKC is a heterogenous family of phospholipid-dependent kinases. PKC isoforms sub-divided into three groups based on mechanism of activation, expression pattern, and structure (Min *et al*., 2008). The classical PKC isoforms (α, β, and γ), contain domains conferring regulation to the secondary messengers, diacylglycerol (DAG) and Ca^{2+} and require both for full activation. The novel isoforms (δ, ε, θ, η) are Ca^{2+}-insensitive whereas the atypical isoforms (ζ , λ/ι) are not respond to DAG or Ca^{2+}. The serine/threonine kinase PKC-δ is one of the most extensively studied novel PKC subfamily and plays a key regulatory role in a variety of cellular functions, including apoptosis, as well as cell growth and differentiation (Junttila *et al*., 2003). Previous studies have identified that PKC-δ is translocated into cellular membrane and mediates the transcriptional activation of HIF-1 under hypoxia in RIF cells (Baek *et al*., 2001). After that, we also reported that activated PKC-δ modulates the stability of HIF-1 and enhances its transcriptional activity under hypoxic conditions in HeLa cells (Kim *et al*., 2004). In addition, PKC-δ mediates cardiogenesis of ESCs and its activation requires the cardiac differentiation of hematopoietic stem cells (Junttila *et al*., 2003; Koyanagi *et al*., 2009). Therefore, it is possible that PKC-δ and its inhibitors may affect the differentiation in mESCs under hypoxia by regulation of HIF-1α stability.

Previously, we demonstrated that HIF-1α may act as a negative regulator of the LIFR-STAT3 pathway in hypoxic mESCs (Jeong *et al*., 2007). Accordingly, another research group reported that hypoxia promotes a limited early differentiation of mESCs in the presence of LIF (Powers *et al*., 2008). Based on these results, we examined the role of PKC-δ inhibitors in the hypoxia-induced differentiation of mESCs. Our data show that PKC-δ inhibitors destabilize HIF-1α protein and block the downregulation of LIFR-STAT3 signaling during hypoxia-induced differentiation of mESCs, which in turn sustains the self-renewal of mESCs under hypoxia. Accordingly, our results suggest an inhibitory effect of PKC-δ inhibitors in the hypoxia-induced early differentiation of mESCs via destabilization of HIF-1α.

Results

PKC-δ inhibitors downregulate the protein expression of HIF-1α in mouse embryonic stem cells (mECS) under hypoxia

PKC-δ stabilize the HIF-1α protein and its inhibitors destabilize HIF-1α under hypoxic conditions in human cervical adenocarcinoma (HeLa) cells and human fibrosarcoma (HT1080) cells (Lee *et al*., 2007). Since, however, the effect of PKC-δ and their specific inhibitors varies depending on the dose, the duration of the treatment, and the types of the treated cells, we examined the role of PKC inhibitors on the hypoxia-induced differentiation of mESCs. For this, we cultured mESCs together with the indicated PKC-δ inhibitors under both normoxic (20% O_2) and hypoxic (1% O_2) culture conditions in the presence of LIF. Rottlerin is a specific inhibitor of PKC-δ and GF 109203X is a broad inhibitor of PKC. We first evaluated the effect of these inhibitors on HIF-1α expression by western blot analysis. HIF-1α protein levels were increased under hypoxia when compared with normoxia and were markedly decreased after treatment with the PKC inhibitors, rottlerin (Rot) and GF 109203X (GF) (Figure 1A). The activity of PKC inhibitors was confirmed by the reduction of phosphorylated PKC-δ (p-PKC-δ, at the threonine 505 residue) levels. In contrast to protein level, HIF-1α mRNA expression did not change in CCEs treated with inhibitors under hypoxia (Figure 1B). These results suggest that PKC-δ inhibitors destabilize HIF-1α protein levels under hypoxia in mESCs.

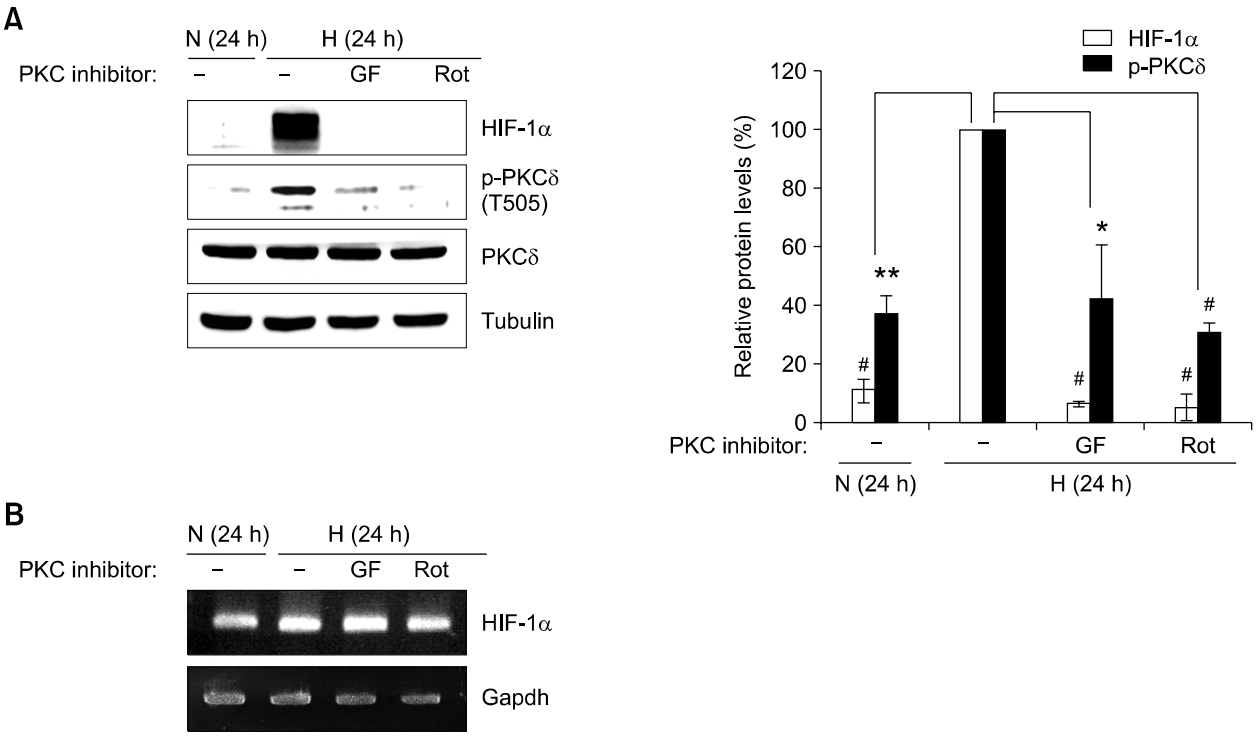

Figure 1. PKCδ inhibitors decreased HIF-1α protein levels under hypoxic conditions in mESCs. (A and B) CCE cells were treated with 5 μM GF 109230X (GF) and 5 μM rotttlerin (Rot), and were then exposed immediately to hypoxia for 24 h. (A) HIF-1α, phosphorylated PKC-δ (p-PKCδ, at threonine 505 residue), and endogenous PKC-δ were examined using western blot analysis. Graph represents mean values ± S.D. (n = 3). *, $P < 0.05$; **, $P < 0.01$; #, $P < 0.001$. (B) The expression level of HIF-1α mRNA was examined using RT-PCR. N, normoxia; H, hypoxia. Tubulin and gapdh were used as internal controls. Results are representative of three independent experiments.

PKC-δ inhibitors block the attenuation of LIFR-STAT3 pathway under hypoxia

Our previous data clearly suggest that HIF-1α binds to reverse HREs (rHREs) of the LIFR promoter, which leads to a downregulation of LIFR-STAT3 signaling in mESCs under hypoxia (Jeong *et al*., 2007). To examine the effect of PKC-δ inhibitors on LIFR-STAT3 signaling, we cultivated mESCs in the presence of LIF with two kinds of PKC-δ inhibitors, rotttlerin and GF under hypoxic conditions. As indicated, expression of LIFR and phosphorylated-STAT3 was downregulated under hypoxia, whereas treatment with PKC-δ inhibitors effectively blocked the hypoxia-induced reduction of LIFR and phosphorylated-STAT3, but not of total STAT3 levels (Figure 2A). Importantly, rottlerin markedly upregulated phosphorylated-STAT3 under hypoxia to expression levels similar to those of the control (normoxia). We further confirmed the effect of PKC-δ inhibitors on the hypoxia-induced differentiation of mESCs using immunofluorescent staining of LIFR and phosphorylated-STAT3. Undifferentiated mESCs cultured under normoxia showed an abundant expression of LIFR in the cytosol and of phosphorylated-STAT3 in the nucleus, whereas the expression of these proteins was decreased under hypoxia (Figure 2B). Interestingly, treatment of mESCs with PKC-δ inhibitors under hypoxia sustained the expression of LIFR and phosphorylated-STAT3; therefore, these results suggest that PKC-δ inhibitors may maintain LIFR-STAT3 signaling under hypoxia.

Maintenance of self-renewal activity in mESCs treated with PKC-δ inhibitors

Based on the effect of PKC-δ inhibitors on LIFR-STAT3, RT-PCR was conducted to access the state of mESCs. Rex1 and fgf4 are represented markers for mESC stemness and self-renewal activity, whereas fgf5 and STAT5a are related to the early differentiation of mESCs (Jeong *et al*., 2007). Expression levels of rex1 and fgf4 were decreased under hypoxia, whereas treatment with PKC-δ inhibitors blocked this suppression of the rex1 and fgf4 (Figure 3). In contrast to self-renewal markers, expression levels of fgf5 and STAT5a were increased under hypoxia, whereas treatment with PKC-δ inhibitors blocked the increase in fgf5

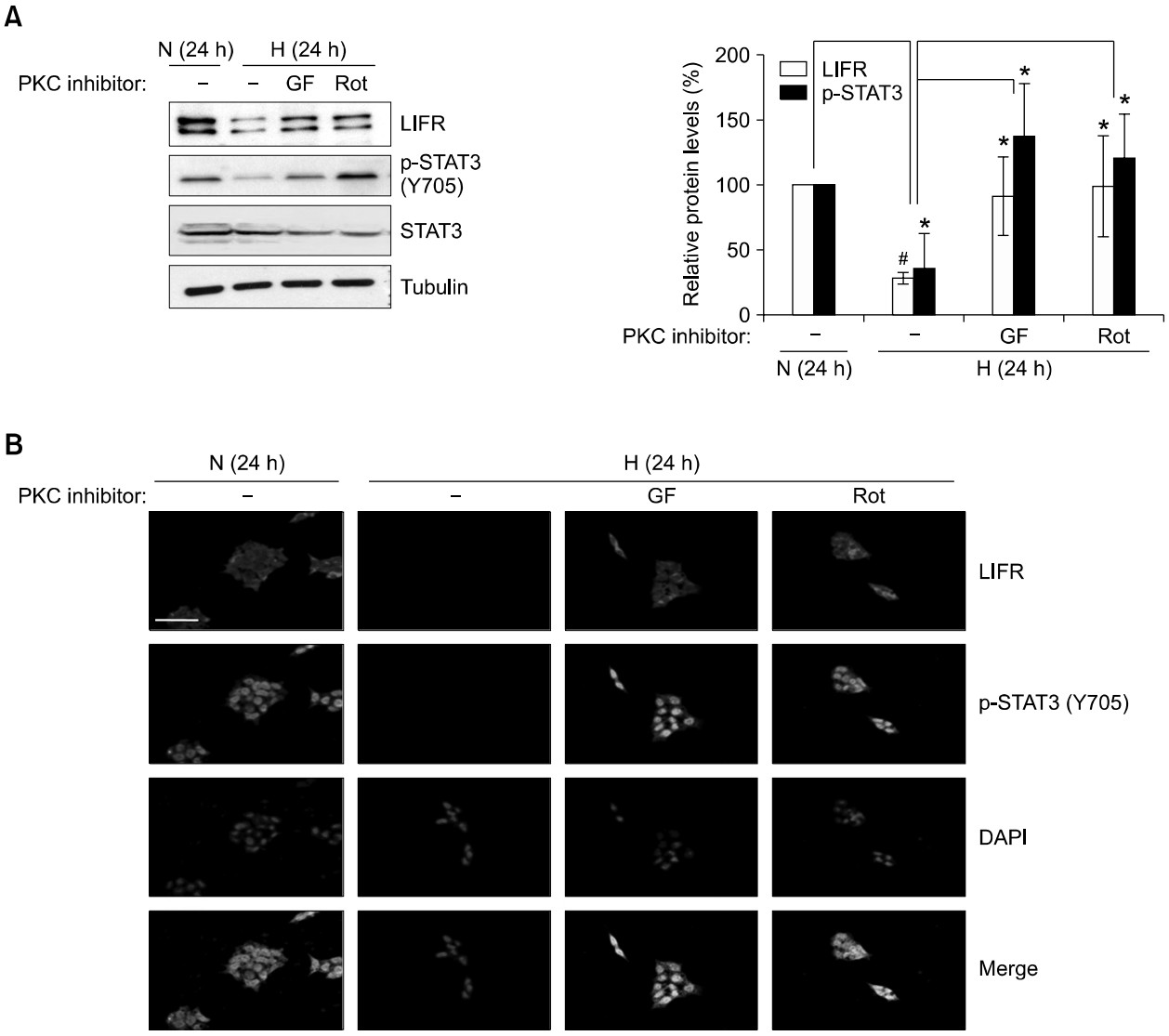

Figure 2. PKCδ inhibitors blocked the down-regulation of LIF-STAT3 pathway under hypoxia in mESCs. (A) CCE cells were treated with 5 μM GF and 5 μM rotttlerin (Rot), and were then exposed immediately to normoxia (N) or hypoxia (H) for 24 h. Western blot analysis of LIF receptor (LIFR), phosphorylated-STAT3 (p-STAT3, at tyrosine 705 residue), and endogenous STAT3 in CCE cells treated with PKC inhibitors. Tubulin was used as internal control. Graph represents mean values ± S.D. (n = 3). *, $P < 0.05$; #, $P < 0.001$ (B) Immunofluorescent staining with LIFR (red) and phosphorylated-STAT3 (at tyrosine 705 residue, green) of cells treated for 24 h with 5 μM GF and 5 μM rotttlerin and grown under normoxic conditions (N), hypoxic conditions (H) in the presence of LIF. Nuclei are stained with DAPI (blue). Scale bar is 50 μm.

and STAT5a expression levels. These results demonstrate that PKC-δ inhibitors maintain the self-renewal state of mESCs and block the early differentiation of mESCs under hypoxia.

Next, we confirmed the state of mESCs by alkaline phosphatase (AP) which is a most widely used stem cell marker. When fixed mESCs are stained with AP, undifferentiated cells appear brown or purple in compact colonies, whereas differentiated cells become colorless. AP staining showed many purple colonies in normoxic group and appeared colorless under hypoxia (Figure 4A). However, AP-positive violet colonies were markedly increased after treatment with the PKC inhibitors, rottlerin and GF 109203X (GF). As Figure 4B shown, when the data are normalized for normoxic group, hypoxia decreased the AP activity (~30% as compared with normoxia) (Figure 4B). But, undifferentiated colonies were distinctly increased after treatment with the PKC inhibitors, rottlerin and GF 109203X (GF) (about 2-fold increase as compared with hypoxia). Thus, these results suggest that blockade of HIF-1α by PKC-δ inhibitors may maintain AP activity under hypoxia.

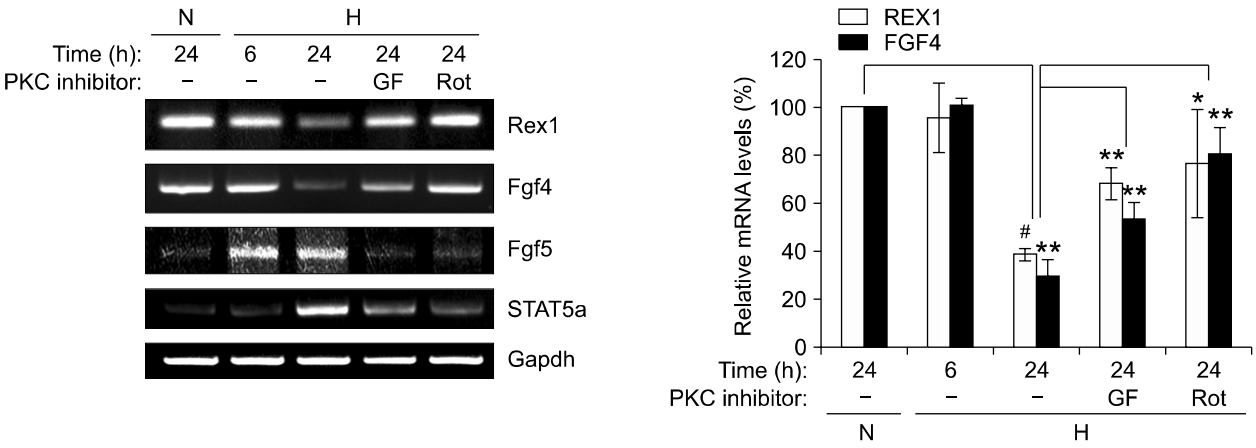

Figure 3. PKCδ inhibitors maintained the self-renewal and blocked the early differentiation of mESCs under hypoxia. CCE cells were treated with 5 μM GF and 5 μM rottlerin (Rot), and were then exposed immediately to normoxia (N) or hypoxia (H) for 24 h. The fate of mESCs was determined using self-renewal markers (rex1 and fgf4) and early differentiation markers (fgf5 and STAT5a) expression levels using RT-PCR. Gapdh was used as an internal control. Results are representative of three independent experiments. Graph represents mean values ± S.D. (n = 3). *, $P < 0.05$; **, $P < 0.01$; #, $P < 0.001$.

Taken together, these results suggest that PKC-δ inhibitors may sustain the stemness of mESCs and suppress the early differentiation of mESCs under hypoxia.

Discussion

Hypoxia affects many cellular responses, including angiogenesis, glycolysis, apoptosis, and differentiation, via the regulation of HIF-1α (Maltepe *et al*., 2005); therefore, regulation of HIF-1α may be an important step in many aspects of cellular regulation, especially in cell-fate decisions (Simon and Keith, 2008). It has been reported that, among the PKC isoforms, PKC-δ is activated under hypoxia and regulates HIF-1α stability. Accordingly, rottlerin (a specific inhibitor for PKC-δ) decreases HIF-1α protein levels in HeLa cells and HT 1080 cells, and so does GF 109203X (a general inhibitor of PKCs) (Lee *et al*., 2007). In the present study, we described the inhibitory role of PKC-δ inhibitors for HIF-1α-mediated differentiation of mESCs under hypoxia.

In LIFR-STAT3 pathway, Stat3 is activated by phosphorylation at Tyr705, which induces dimerization, nuclear translocation and DNA binding. Transcriptional activation seems to be regulated by phosphorylation at Ser727 through the MAPK or mTOR pathways (Tighe and Gudas, 2004). We here demonstrated that under hypoxic conditions PKC-δ inhibitors block the down-regulation of LIFR expression and maintain phosphorylation of STAT3 (at a 705 tyrosine residue) signaling which is a crucial for self-renewal activity of mESCs. These studies cannot exclude the possibility that PKC-δ inhibitors may directly modulate the LIFR-STAT3 pathway, as well as destabilizing the HIF-1α stability. However, other studies have investigated that rottlerin does not affect both STAT1 Tyr^{701} and STAT3 Tyr^{705} phosphorylation in human peripheral blood monocytes (Bhattacharjee *et al*., 2006). Hence, taken together, we concluded that PKC-δ inhibitors may not regulate the LIFR-STAT3 via direct phosphorylation of STAT3, but mediate the destabilization of HIF-1α protein, resulting in maintenance of pluripotency in mESCs.

Earlier we also conducted experiments that stability of HIF-1α is significantly modulated by many proteins, including PHD, HDAC, ARD-1, pVHL, and p300/CBP (Jeong *et al*., 2002; Lee *et al*., 2004; Kim *et al*., 2007). For example, it has been reported that class I and II HDACs deacetylate and stabilize HIF-1α protein under hypoxia (Qian *et al*., 2006). In addition, HDAC inhibitors suppress the deacetylating activity of HDAC and induce acetylation of HIF-1α, which leads to the destabilization of HIF-1α (Kong *et al*., 2006). Thus, a study of the relationship between HIF-1α and those proteins in the differentiation of mESCs under hypoxia is worthwhile to gain an understanding about the molecular mechanism in early mammalian development.

In summary, the present study collectively provided evidence that PKC-δ inhibitors have a negative effect on HIF-1α stability in mESCs. Furthermore, PKC-δ inhibitors prevented the attenuation of LIFR-STAT3 pathway, which led to inhibition of the differentiation of these cells under hypoxia. Taken together, these results suggest that

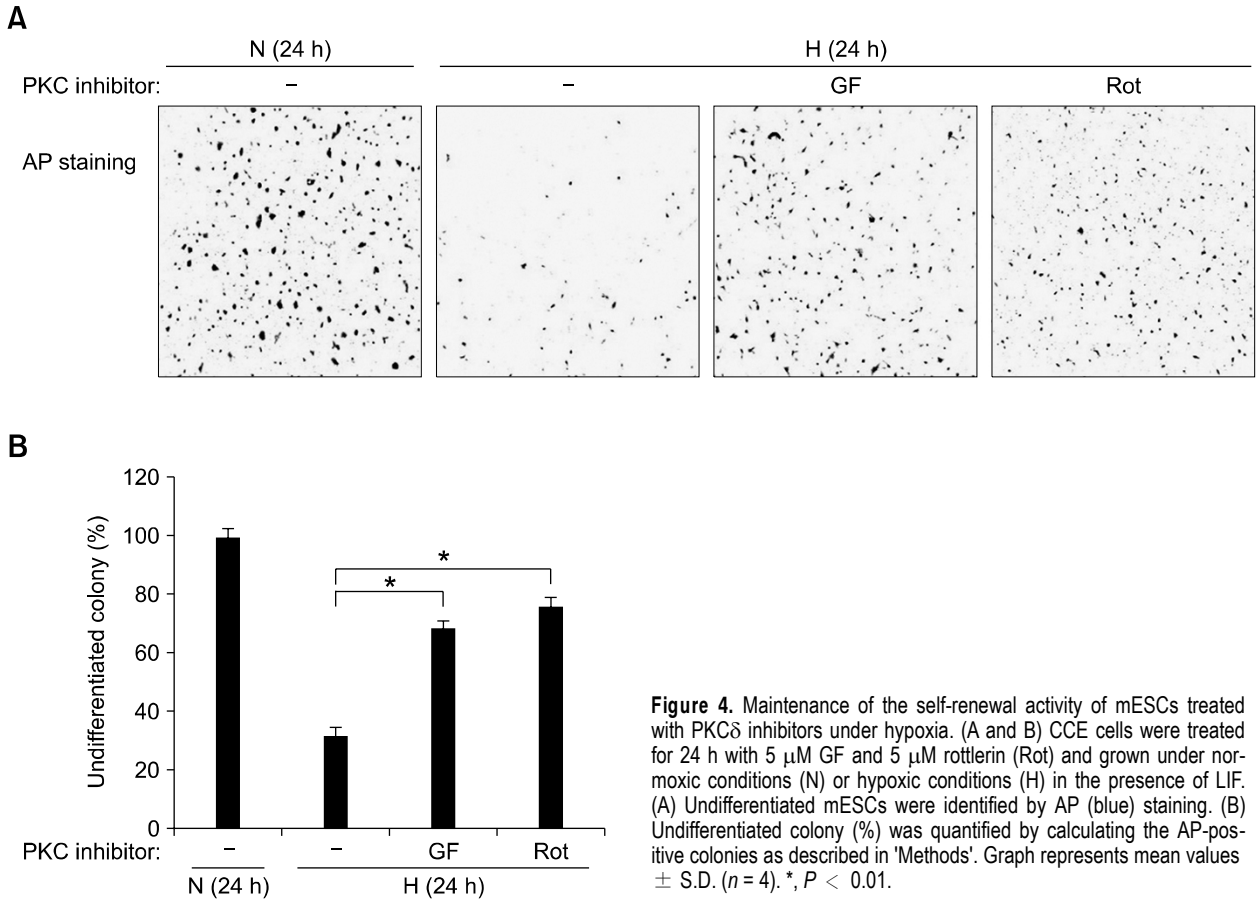

Figure 4. Maintenance of the self-renewal activity of mESCs treated with PKCδ inhibitors under hypoxia. (A and B) CCE cells were treated for 24 h with 5 μM GF and 5 μM rottlerin (Rot) and grown under normoxic conditions (N) or hypoxic conditions (H) in the presence of LIF. (A) Undifferentiated mESCs were identified by AP (blue) staining. (B) Undifferentiated colony (%) was quantified by calculating the AP-positive colonies as described in 'Methods'. Graph represents mean values ± S.D. ($n = 4$). *, $P < 0.01$.

inhibitors of PKC-δ suppress the hypoxia-induced differentiation of mESCs via downregulation of HIF-1α stability and may be important for the understanding of the fundamental mechanisms underlying physiologic events during early mammalian embryogenesis.

Methods

Cell culture

mESC lines, CCEs cultured under feeder-free conditions were maintained in knockout-DMEM (Invitrogen, Grand Island, NY) containing 15% defined serum replacement (Knockout SR™; Invitrogen, Grand Island, NY), 1 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, and 1,000 U/ml LIF (ESGRO; Chemicon, Temecula, CA). For hypoxic conditions, CCE cells were incubated at 1% O_2 level with 5% CO_2 balanced with N2 in a hypoxic chamber (Forma Scientific, Marietta, OH) with an interior temperature of 37°C.

Reagents

Rottlerin and GF 109203X were purchased from Sigma-Aldrich (St Louis, MO).

RT-PCR analysis and primers

Total RNA from cells was isolated using Trizol reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions and quantified by spectrophotometer (NanoDrop, Nyxor Biotech). First-stranded cDNA was synthesized with 5 μg of each DNA-free total RNA and oligo-$(dT)_{16}$ primer by *Moloney* murine leukemia virus reverse transcriptase (Promega, Madison, WI). One microlitre of cDNA was amplified by PCR using 1.25 U of ExTaq DNA polymerase kit (Takara, Madison, WI). The primers used had the following sequences: fgf4 forward, 5'-TACTGCAACGTGGGCATCGGA-3'; reverse, 5'-GTGGGTTACCTTCATGGTAGG-3'; rex1 forward, 5'-CGTGTAACATACACCATCCG-3'; reverse, 5'-GAAATCCTCTTCCAGAAT-GG-3'; fgf5 forward, 5'-ATGAGCCTGTCCTT-GCTC-3'; reverse, 5'-GTCTGTACTTCACTGGGC-3'; STAT5a forward, 5'-GCTGTATCCG-TCACATTCTG-3'; reverse, 5'-CCACTGGATCAGCTCGTCGT-3'; gapdh forward, 5'-AAC-GGGAAGCCCATCACC-3'; reverse, 5'-CAGCCTTGGCAGCACCAG-3'. The PCR products were separated on 1.2-1.5% agarose gels and visualized by ethidium bromide staining under a UV transilluminator.

Western blot analysis

Cells were harvested and the pellets were immediately frozen in liquid Nitrogen. After thawing, the cell pellet lysed in lysis buffer (20 mM Tris-HCl (pH 7.5); 150 mM NaCl; 1 mM Na_2EDTA; 1 mM EGTA; 1% Triton; 2.5 mM sodium pyrophosphate; 1 mM beta-glycerophosphate; 1 mM Na_3VO_4; 1 μg/ml leupeptin) followed by centrifugation for 30 min at 15,000 rpm and protein concentration was determined by the BCA assay (Sigma, St. Louis, MO) and protein extracts were resolved in SDS-PAGE gels and transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The protein-bearing membrane was blocked with 5% skim milk and probed with specific primary antibodies to HIF-1α (Cayman Chemical), LIFR, STAT3, and PKC-δ (Santa Cruz Biotechnology, CA), tyr-705-phosphorylated STAT3 and thr-505-phosphorylated PKC-δ (Cell Signaling Technology, Beverly, MA), and α-tubulin (InnoGenex, San Ramon, CA), followed by incubation with secondary HRP-conjugated antibodies to mouse or rabbit IgG (Pierce, Rockford, IL). Antibody detection was performed by standard procedures using ECL Plus reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunofluorescence

Cells were fixated with 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 15 min and directly permeabilized with 0.1% Triton X-100 in PBS for 20 min. Then, cells were blocked with 5% BSA in PBS-T for 1 h at room temperature (RT). Next, anti-LIFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-phosphorylated STAT3 antibody (Cell Signaling Technology, Beverly, MA) were applied overnight at 4°C and washed three times with PBS-T. Then, cells were incubated with secondary Alexa 488/546-conjugated IgG (Molecular Probes, Inc.). Nuclear counterstaining was performed using DAPI (Molecular Probes, Inc.). Fluorescence staining was evaluated using a fluorescence microscope (Carl Zeiss, Germany)

Alkaline Phosphatase Assay

The cells were fixed with 4% paraformaldehyde for 2 min at RT. Staining for alkaline phosphatase was performed using a diagnostic kit (Sigma) following protocols provided by the manufacturer. The ratios of AP-positive colonies were scored as previously decribed (Jeong, Lee *et al*., 2007).

Acknowledgments

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