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Cyclic AMP Represses the Hypoxic Induction of Hypoxia-inducible Factors in PC12 Cells

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Hypoxia-inducible factor 1 (HIF-1) is a master regulator for hypoxic activation of genes for angiogenesis, hormone synthesis, glycolysis and cell survival. In addition to hypoxic stimulus, various effectors and reagents were reported to affect HIF-1 activity. Here, we show that cyclic AMP (cAMP) down-regulates the HIF-1 activity in pheochromocytoma PC12 cells but not in Hep3B and HeLa cells. Hypoxia response element-dependent reporter activity was decreased by the addition of dibutyryl cAMP. Expression of protein kinase A (PKA) catalytic α-subunits repressed the HIF-1 activity. HIF-1α and HLF (HIF-2α or EPAS1) protein levels were decreased by the treatment with dibutyryl cAMP. Although CREB was served as a negative factor for the HIF-1 activity, it may not be a major PKA target in the cAMP-dependent HIF-α repression pathway. Induction of hypoxia responsive genes was suppressed by dibutyryl cAMP. Our results provide additional insight into a regulatory mechanism of hypoxic response.

Key words: cyclic AMP, HIF-1α stabilization, hypoxia, PC12 cells, protein kinase A.

Abbreviations: cAMP, cyclic AMP; $CoCl_2$, cobalt chloride; CRE, cAMP response element; db, dibutyryl; HIF, hypoxia-inducible factor; HRE, hypoxia response element; IPAS, inhibitory PAS domain protein; PKA, protein kinase A; PHD, HIF prolyl hydroxylase; PKI, PKA inhibitor 14-22 Amide; TH, tyrosine hydroxylase; VEGF, vascular endothelial growth factor.

Hypoxia elicits a wide range of adaptive responses. In response to hypoxia, cells adapt by changing the transcription of genes involved in angiogenesis, erythrocyte production, cell survival and metabolism (*1*). Most of the genes that are activated during hypoxia are critically regulated by hypoxia-inducible factor-1 (HIF-1) and -2 (HIF-2). HIF is composed of an oxygen-regulated HIF-α subunit (HIF-1α and HLF) and the constitutively expressed Arnt (or HIF-1β) (*1*, *2*). HLF is also called HIF-2α or EPAS1. In normoxia, HIF-α subunits are hydroxylated by HIF prolyl hydroxylases (PHDs) at two conserved proline residues present in the oxygen-dependent degradation domain, recognized by the von Hippel-Lindau protein, ubiquitinated and degraded by the 26S proteasome (*2*). The turnover of HIF-1α protein in normoxia is very rapid and it stabilizes and accumulates at hypoxia due to lack of oxygen. The accumulated HIF-1α translocates into the nucleus, dimerizes with Arnt, binds to the specific hypoxia response elements (HREs) on DNA and activates transcription by recruiting CBP/p300 transcriptional coactivators (*1*).

It is estimated that 2.6% of the total genes are regulated by the activation of HIF-1 (*3*). Of these target genes, genes for various factors important for signal transduction pathways are included. MKP1, an important terminator of ERK activation, has been identified as a hypoxia-inducible gene (*4*). Hypoxia activates the PKCδ and ζ isozymes in many cell types (*5–7*). These finding strongly suggest that HIFs can influence critical cellular phenomena such as cell differentiation and growth through modifying relevant signalling pathways.

On the other hand, several signalling factors were found to affect HIF-1 activity via direct or indirect interactions with HIF-1α. For example, ERK, which is activated by extracellular signals such as epidermal growth factor, phosphorylates several serine and threonine residues in the N-terminal activation domain of HIF-1α, leading to activation of its transactivation activity (*8–10*). In addition to this direct activation through phosphorylation, ERK activates HIF-1α through phosphorylation of p300 (*11*). It is also reported that ERK phosphorylates specific serine residues (serine 641 and serine of HIF-1α resulting in promoting accumulation in the nucleus by inhibiting nuclear export (*12*). PKCδ increases the protein stability and transcriptional activity of HIF-1α (*7*). These studies of the relationship between HIF-1 and other signalling pathways are worthwhile in understanding the hormone synthesis, tumourigenesis and cancer therapy.

In this article, we report that cyclic AMP (cAMP) inhibits HIF-1 activity induced by hypoxia or cobalt chloride ($CoCl_2$) and this repression is a consequence of activation of protein kinase A (PKA).

MATERIALS AND METHODS

Cell Culture and DNA Transfection—Cells were obtained from the Cell Resource Center for Biomedical

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Research, Tohoku University. Rat pheochromocytoma PC12 cells were maintained in RPMI-1640 medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum in collagen IV-coated dishes (BD Biosciences, San Jose, CA) under 5% CO_2 at 37°C, and transferred every 3 days. The cells were grown for 24 h before transfection in DMEM. Transfection was performed using the lipofection reagent (TransFast, Promega, Madison, WI) according to the manufacturer's protocol. After incubation with plasmids for 1 h, cells were further incubated in serum-supplemented DMEM for 12 h (for western blotting) or 20 h (for luciferase assay) with or without reagents such as dibutyryl (db) cAMP. cAMP and PKA Inhibitor 14-22 Amide (PKI) were obtained from Sigma-Aldrich (St. Louis, MO) and Calbiochem (San Diego, CA), respectively. Hep3B and HeLa cells were grown in DMEM and MEM, respectively, with 10% fetal bovine serum, and transfected by the calcium–phosphate precipitation method. The assay of luciferase activity was described previously (*13*)

Plasmid—Construction of the HRE-driven luciferase reporter plasmid was described previously (*14*). The luciferase reporter with four copies of cAMP response element (CRE) was constructed by inserting four copies of the sequence (5′-GATC GCC TGA CGT CAT TGA CCA-3′) between the *Xho*I and *Bgl*II sites of the pGL3-promoter plasmid. Mouse PKA catalytic α-subunit cDNA was ligated into pBos-3Myc.

Western Blotting—Nuclear extracts were prepared as described previously (*14*). A total of 10 μg of protein was loaded on an 8% SDS–polyacrylamide gel. HIF-1α and HLF was detected using the ECL Western Kit (GE Healthcare, Little Chalfont, UK) with an anti-HIF-1α and HLF monoclonal antibodies (Novus Biologicals, Littleton, CO).

RT-PCR—Total RNA was prepared from cultured cells and cDNA was synthesized. PCR was performed as described previously (*13*). Bands were quantified by using Image J software. The primers of IPAS, HIF-3α, tyrosine hydroxylase (TH) are described previously (*13*). Primer sequences used for β-actin are 5′-TAG GCA CCA GGG TGT GAT GG-3′ (forward) and 5′-TCC AGG GAC GAA GAG GAT GC-3′ (reverse), and sequences for vascular endothelial growth factor (VEGF) are 5′-GAA CTT TCT GCT CTC TTG GGT GCA C-3′ (forward) and 5′-CTT TCT TTG GTC TGC ATT CAC ATC TGC-3′ (reverse).

Measurement of Alteration of the Intracellular cAMP Level—The assay was performed using the cAMP-Glo™ Assay kit (Promega) according to the manufacturer's protocol.

Statistical Analysis—Data are given as mean ± SD, with the number of the experiments indicated. Statistical significance was established by Student's *t*-tests.

RESULTS AND DISCUSSION

Inhibition of HRE-dependent Reporter Activity by cAMP—In order to examine the effect of cAMP on hypoxic gene expression, a luciferase reporter plasmid containing HREs in the promoter (Fig. 1A) was introduced into cultured cells. A chemical mimetic of hypoxia, $CoCl_2$, was used to cause hypoxic response of cells. As shown in Fig. 1B, addition of db cAMP, a membrane-permeable derivative of cAMP, to the culture medium repressed the reporter activity by ~60% in PC12 cells. This inhibition was similarly observed in PC12 cells exposed to hypoxia (Fig. 1C). On the other hand, no inhibition of the activity was found in Hep3B and HeLa cells (Fig. 1D).

Since cAMP activates PKA, we examined the effect of PKA on the reporter activity by expressing a constitutively active catalytic α-subunit of PKA in PC12 cells. As shown in Fig. 2A, over-expression of PKA catalytic subunit repressed the $CoCl_2$-induced reporter activity. In accordance with the result, addition of a PKA inhibitor (PKI) restored the reporter activity in the presence of cAMP, although the inhibitor exhibited no effect on the $CoCl_2$-induced reporter activity (Fig. 2B). These results strongly suggest that the inhibitory effect of cAMP was mediated by the activation of PKA.

Repression of Hypoxic Induction of HIF-1α and HLF Proteins by cAMP—It is well-known that HIF-1 activity is mainly regulated by oxygen-dependent changes in protein stability of HIF-1α and HLF when cells are exposed to hypoxia (*2*). Effect of cAMP on HIF-1α and HLF protein levels was investigated by western blotting using their specific antibodies. As shown in Fig. 3A, HIF-1α protein levels augmented by the $CoCl_2$ treatment were repressed by the addition of db cAMP. Induction of HLF protein was similarly inhibited by the treatment (Fig. 3B). These results suggest that inhibition of the reporter activity by cAMP can be attributed to decreased levels of HIF-1α and HLF proteins in PC12 cells. In contrast, no reduction of HIF-1α and HLF protein levels by cAMP was observed in Hep3B cells (Fig. 3C and D). We examined HIF-1α and HLF protein levels in PC12 cells treated with db cAMP in hypoxia. Similar to the $CoCl_2$ treatment, reduced protein levels of HIF-1α and HLF were found in PC12 cells exposed to hypoxia (Fig. 3E and F). As a repressive factor against HIF-1α and HLF activity, inhibitory PAS domain protein (IPAS), which is an alternative splicing product of HIF-3α (*15*), is reported (*16*), and it is expressed to cancel the reactive oxygen species-dependent hypoxic induction of HIF-1α and HLF in PC12 cells (*13*). In order to examine the possible involvement of IPAS in the repression by cAMP in PC12 cells, the expression level of IPAS was determined. As shown in Fig. 3G, IPAS mRNA levels remained unchanged regardless of the treatment with db cAMP or $CoCl_2$, suggesting no involvement of IPAS in the inhibition of HIF activity by cAMP.

PKA activates a transcription factor, CREB, by direct phosphorylation (*17*). Previous reports have shown that CREB is capable to bind to HRE (*18*). This unusual binding of CREB, which may compete with HIF-1 and HIF-2, might repress the HRE-dependent reporter activity. Therefore, we expressed CREB in PC12 cells to examine its effect on the HRE-dependent reporter activity. As shown in Fig. 4A, CREB strongly inhibited the HRE-driven reporter activity. Moreover, further addition of db cAMP synergistically reduced the reporter activity to the basal level, which is similar to the activity of the reporter plasmid without HRE sequences (Fig. 4A).

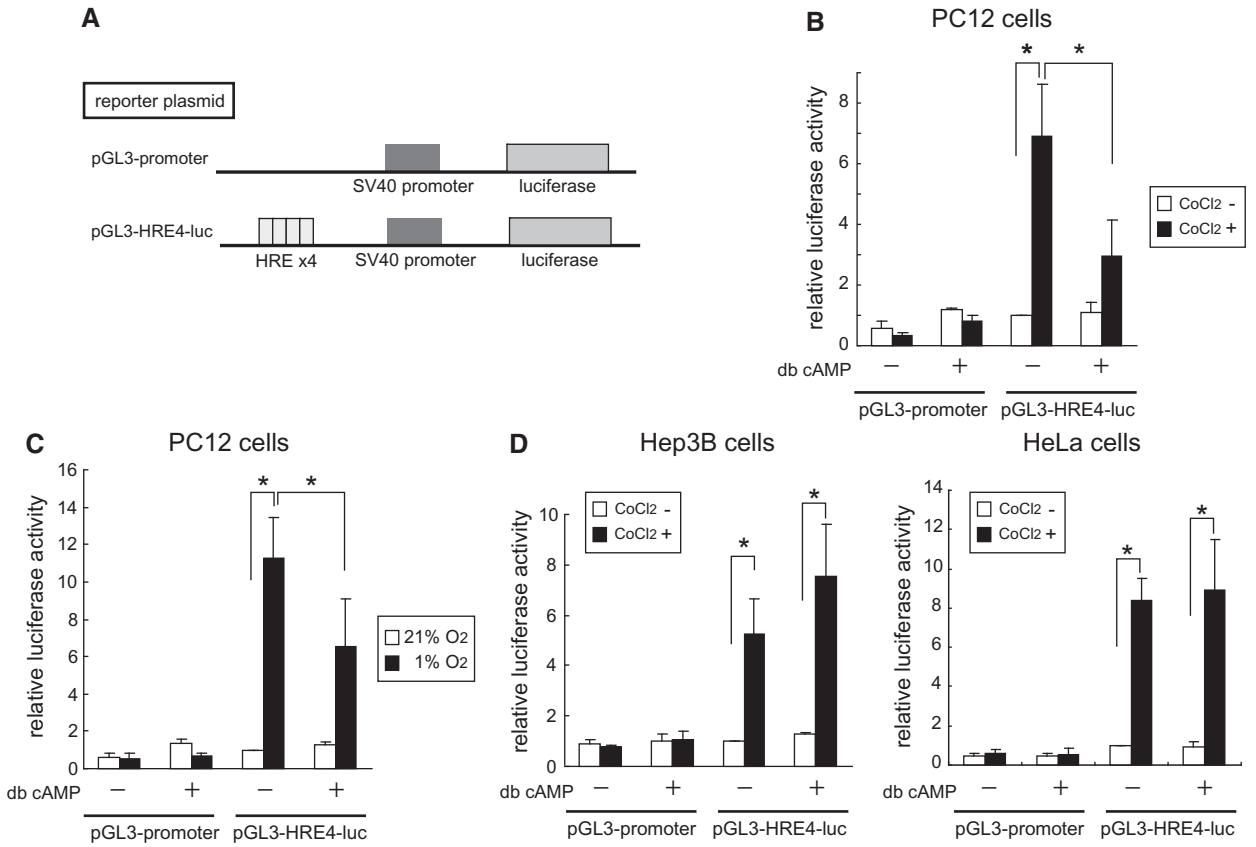

Fig. 1. **Inhibition of HRE-dependent reporter activity by cAMP in PC12 cells.** (A) Schematic representation of reporter plasmids. (B and C) db cAMP (100 μM) inhibited the HRE-dependent reporter activity in PC12 cells treated with $CoCl_2$ (100 μM) or exposed to sustained hypoxia. (D) db cAMP did not inhibit the $CoCl_2$-induced reporter activity in Hep3B and HeLa cells. $^*P < 0.05$ for indicated comparison. Data shown in bar graphs are average ± SD of three independent experiments.

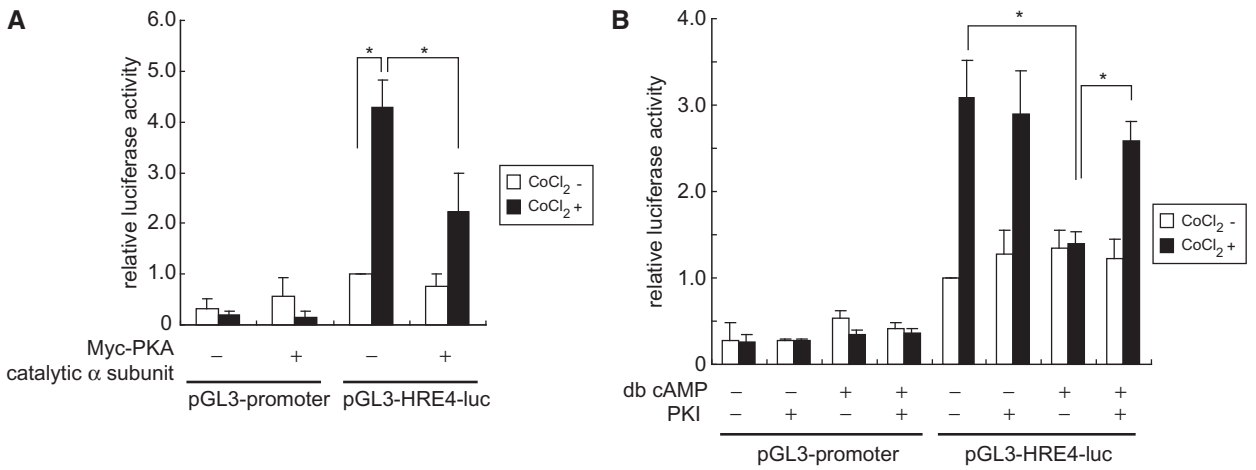

Fig. 2. **The inhibitory effect of cAMP was mediated by PKA.** (A) PKA catalytic α subunit blocked the HRE-driven reporter activity in PC12 cells. (B) PKI (10 μM) recovered the reporter activity in the presence of cAMP. $^*P < 0.05$ for indicated comparison. Data shown in bar graphs are average ± SD of three independent experiments.

Next, we examined whether $CoCl_2$-induced hypoxia regulates cAMP–PKA pathway. Addition of $CoCl_2$ did not affect the level of cAMP in PC12 cells significantly (Fig. 4B). Moreover, we examined the effect of $CoCl_2$-induced hypoxia on the CREB activity in PC12 cells using a reporter plasmid containing CREs in the promoter (Fig. 4C). As shown in Fig. 4D, the reporter activity induced by cAMP was repressed in response to

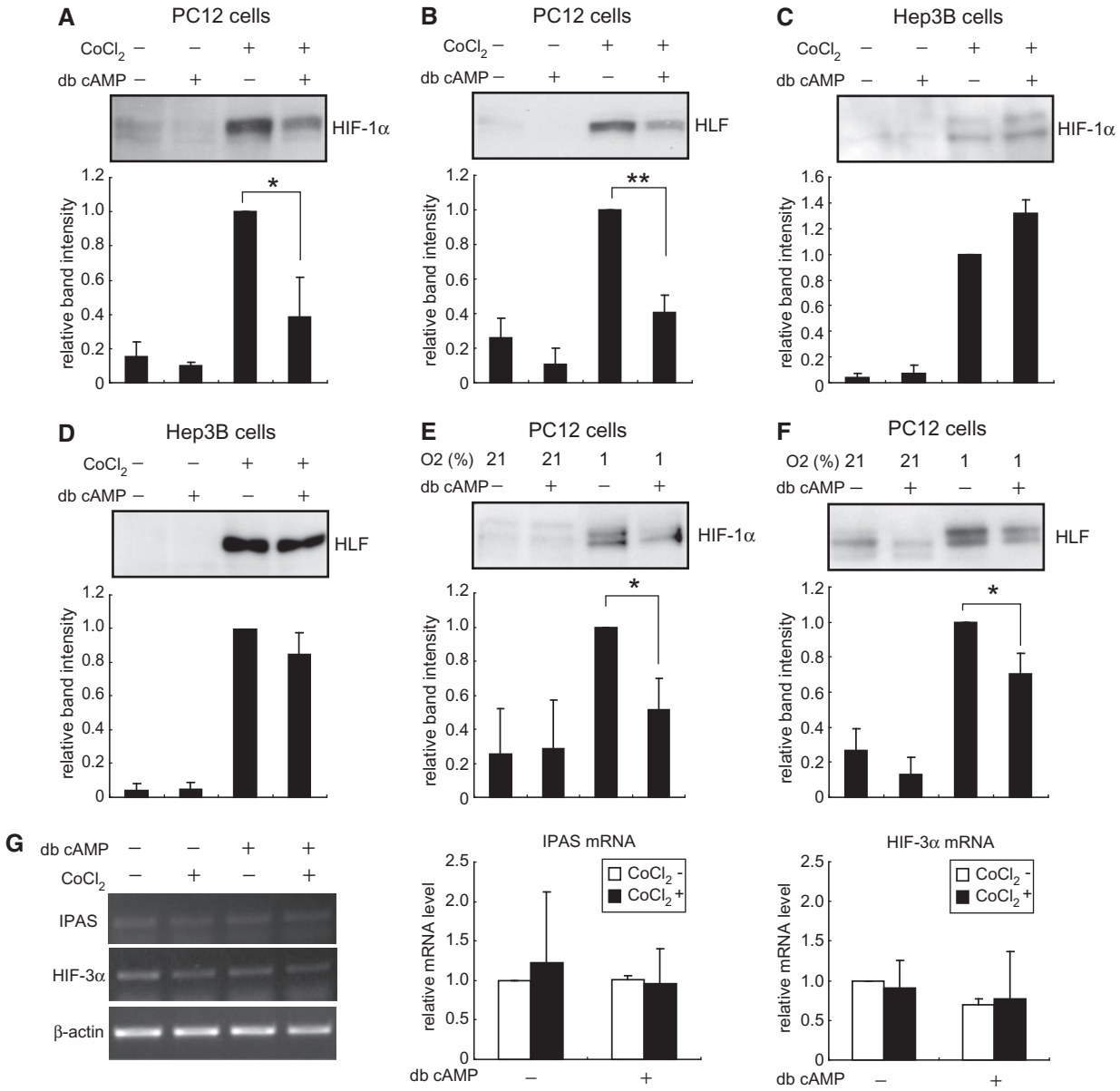

Fig. 3. **Repression of hypoxic induction of HIF-1α and HLF proteins by cAMP.** (A and B) HIF-1α and HLF protein in $CoCl_2$-treated PC12 cells. Each relative band intensity is shown below. (C and D) HIF-1α and HLF protein in $CoCl_2$-treated Hep3B cells. (E and F) HIF-1α and HLF protein in hypoxia-exposed PC12 cells. (G) *IPAS* gene expression in $CoCl_2$-treated PC12 cells in the presence of cAMP. $^{*}P < 0.05$ and $^{**}P < 0.01$ for indicated comparison. Data shown in bar graphs are average ± SD of three independent experiments.

$CoCl_2$, confirming previous results that CREB is degraded under the hypoxic conditions (*19*). All taken together, these observations suggest that although CREB was served as a negative factor for the HIF-1 activity, it may not be a major PKA target in the cAMP-dependent HIF-α repression pathway in PC12 cells.

Finally, we examined the effect of db cAMP treatment on hypoxia-induced expression of HIF target genes (Fig. 5). TH is reported to have HRE and CRE sites in its promoter. The $CoCl_2$-induced upregulation of TH was repressed by the addition of db cAMP. Likewise, the inducible expression of VEGF was decreased by the addition of db cAMP. Taken together, these results suggest that cAMP–PKA pathway affect cell response to hypoxia.

The mechanism by which PKA activation resulted in reduction of HIF-1α and HLF protein levels is presently unknown. PKA might directly phosphorylate HIF-1α and HLF, leading to accelerated degradation of the proteins. In HIF-1α and HLF sequences, many sites with motifs (R-X-S/T and R-R/K-X-S/T) that are recognized by PKA are present over the proteins, especially in the HLH-PAS domains (data not shown). Alternately, a modulation

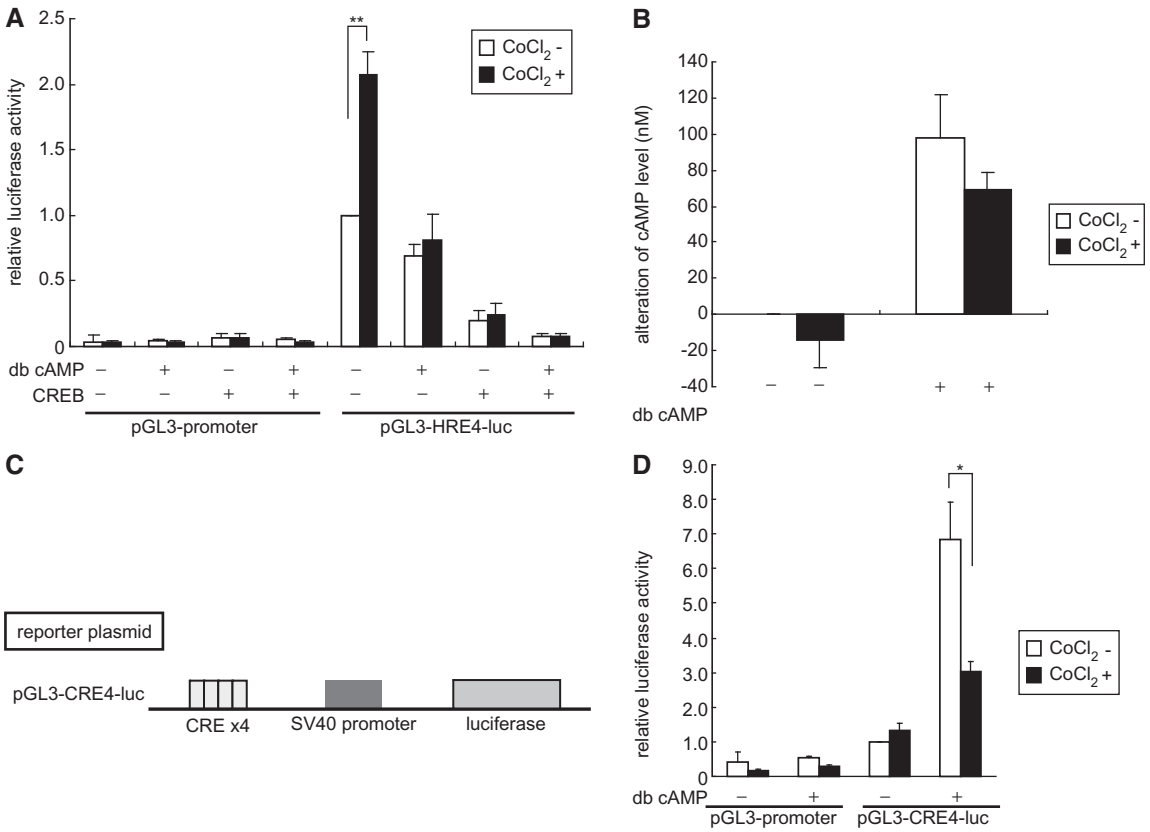

Fig. 4. **The effect of CREB on the $CoCl_2$-induced hypoxic response in PC12 cells.** (A) db cAMP and over-expression of CREB inhibited the HRE-driven reporter activity synergistically. (B) Alteration of the intracellular level of cAMP. PC12 cells were treated with db cAMP and $CoCl_2$ for 30 min. The cells were washed with PBS twice, and the assay was performed. The value of cells without db cAMP and $CoCl_2$ was set to 0 nM. Data shown in bar graphs are average ± SD of three independent experiments. (C) Schematic representation of CRE-dependent reporter plasmids. (D) The CRE-driven reporter activity induced by cAMP was repressed in the treatment with $CoCl_2$, $^{*}P < 0.05$ and $^{**}P < 0.01$ for indicated comparison. Data shown in bar graphs are average ± SD of three independent experiments.

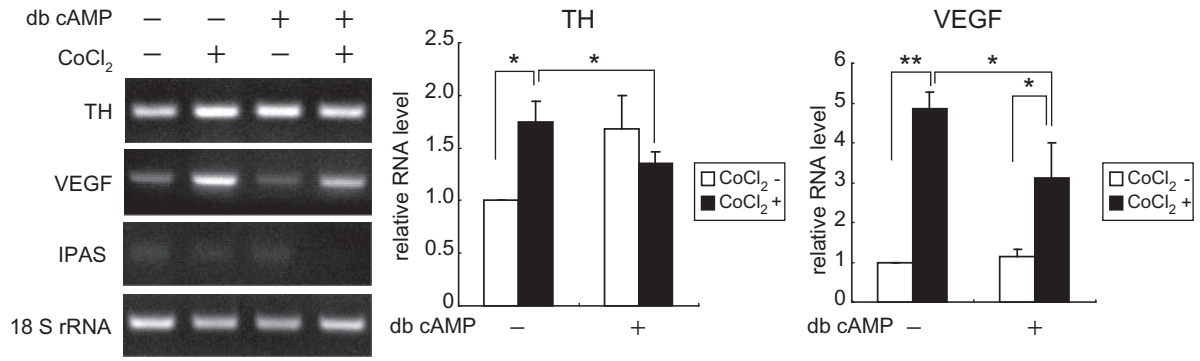

Fig. 5. **The effect of db cAMP treatment on hypoxia-induced expression of HIF target genes.** The $CoCl_2$-induced upregulation of TH and VEGF was repressed by the addition of db cAMP. PC12 cells were treated with db cAMP and $CoCl_2$ for 6 h. Data were normalized to 18S rRNA and the value of cells without db cAMP and $CoCl_2$ was set to 1. $^{*}P < 0.05$ and $^{**}P < 0.01$ for indicated comparison. Data shown in bar graphs are average ± SD of three independent experiments.

factor(s) may be present that is activated by PKA phosphorylation and in turn modifies HIF-1α and HLF proteins to decrease their stability. In the experiments shown in Fig. 1, the inhibitory effect of cAMP was only shown in PC12 cells but not in other cells, suggesting that the mechanism of direct phosphorylation of HIF-1α and HLF by PKA seems less likely. Recently, in addition to PHDs, several factors are found to increase or decrease HIF-1α activity. RACK1 competes with Hsp90 for binding to HIF-1α, resulting in decreasing its

level (*20*). A recent report has shown that the mammalian septin family member, SEPT9 variant 1, whose expression levels in cells are variable, binds and stabilizes HIF-1α by preventing the interaction of HIF-1α with RACK1 (*21*, *22*). PKA may modulate the activity of these factors which are involved in protein stabilization of HIFs in PC12 cells. p53 also known to repress HIF-1-dependent transcription (*23*). Further investigation of the molecular mechanism including the elucidation of the possible factor that is activated by PKA is necessary and studies along this line are in progress.

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CONFLICT OF INTEREST

None declared.

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