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Ca^{2+}/Calmodulin Kinase-dependent Activation of Hypoxia Inducible Factor 1 Transcriptional Activity in Cells Subjected to Intermittent Hypoxia*

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Intermittent hypoxia (IH) occurs in many pathological conditions. However, very little is known about the molecular mechanisms associated with IH. Hypoxia-inducible factor 1 (HIF-1) mediates transcriptional responses to continuous hypoxia. In the present study, we investigated whether IH activates HIF-1 and, if so, which signaling pathways are involved. PC12 cells were exposed to either to 20% O_2 (non-hypoxic control) or to 60 cycles consisting of 30 s at 1.5% O_2, followed by 4 min at 20% O_2 (IH). Western blot analysis revealed significant increases in HIF-1α protein in nuclear extracts of cells subjected to IH. Expression of a HIF-1-dependent reporter gene was increased 3-fold in cells subjected to IH. Although IH induced the activation of ERK1, ERK2, JNK, PKC-α, and PKC-γ, inhibitors of these kinases and of phosphatidylinositol 3-kinase did not block HIF-1-mediated reporter gene expression induced by IH, indicating that signaling via these kinases was not required. In contrast, addition of the intracellular Ca^{2+} chelator BAPTA-AM or the Ca^{2+}/calmodulin-dependent (CaM) kinase inhibitor KN93 blocked reporter gene activation in response to IH. CaM kinase activity was increased 5-fold in cells subjected to IH. KN 93 prevented IH-induced transactivation mediated by HIF-1α, and its coactivator p300, which was phosphorylated by CaM kinase II *in vitro*. Expression of the HIF-1-regulated gene encoding tyrosine hydroxylase was induced by IH and this effect was blocked by KN93. These observations suggest that IH induces HIF-1 transcriptional activity via a novel signaling pathway involving CaM kinase.

Sleep-disordered breathing with recurrent apnea is a major cause of morbidity and mortality in the United States population, affecting an estimated 18 million people (1). In this condition, transient repetitive apnea (cessation of breathing) results in periodic hypoxemia (decreased arterial PO_2). In severely affected patients, the frequency of apneas may exceed 60 episodes per hour and blood hemoglobin saturation of O_2 can be reduced to as low as 50%. Patients with chronic intermittent hypoxia (IH)[1] caused by sleep apnea have a greatly increased risk for the development of systemic hypertension (2, 3). Rats exposed to chronic IH also develop systemic hypertension (4). However, rats in which the carotid bodies have been denervated show no increase in blood pressure in response to IH (5). Studies in humans and rodents suggest that the carotid body, which is the primary chemoreceptor for detecting changes in arterial PO_2, mediates reflex increases in the activity of the sympathetic nervous system that result in elevated blood pressure (6). Chronic IH induces long lasting sensory excitation of the carotid body (7), which in turn may contribute to increased sympathetic tone and systemic hypertension.

The transcriptional activator hypoxia inducible factor 1 (HIF-1) plays an essential role in O_2 sensing by the carotid body (8). HIF-1 is a global regulator of oxygen homeostasis that controls multiple key developmental and physiological processes (9, 10). Over 60 HIF-1 target genes have been identified, including those encoding erythropoietin (*EPO*) and vascular endothelial growth factor (*VEGF*). HIF-1 is a heterodimeric protein that is composed of a constitutively expressed HIF-1β subunit and an O_2-regulated HIF-1α subunit. Analysis of *Hif1a*$^{+/-}$ mice that were heterozygous for a knock-out allele at the locus encoding HIF-1α revealed that their carotid bodies were markedly impaired in the ability to sense and/or respond to hypoxia (8). Carotid body histology was normal, including the presence of glomus cells, which perform the O_2-sensing function of the carotid body, and *Hif1a*$^{+/-}$ carotid bodies responded normally to cyanide, indicating a specific defect in O_2 sensing (8).

HIF-1 activity is induced under conditions of continuous hypoxia as a result of a decreased rate of O_2-dependent proline hydroxylation, ubiquitination, and proteasomal degradation of the HIF-1α subunit (11–14). HIF-1α transcriptional activity is also regulated via O_2-dependent arginine hydroxylation that blocks coactivator recruitment (15). However, less is known about the effect of IH on HIF-1 activity. Recent studies suggest that HIF-1-regulated gene expression is activated by repeated cycles of hypoxia and reoxygenation. Thus, exposure of mice to

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[1] The abbreviations used are: IH, intermittent hypoxia; ERK, extracellular signal-regulated kinase; JNK, Jun N-terminal kinase; CaM, calmodulin; CaMK, CaM kinase; HIF, hypoxia inducible factor; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione *S*-transferase; PI3K, phosphatidylinositol kinase; TH, tyrosine hydroxylase; EPO, erythropoietin; VEGF, vascular endothelial growth factor; DMOG, dimethyloxalylglycine; MAP, mitogen-activated protein; HRE, hypoxia response element; β-gal, β-galactosidase.

an IH protocol (6% O_2 for 6 min, followed by 21% O_2 for 6 min, repeated for a total of 5 cycles) was shown to induce expression of erythropoietin (EPO), which mediated cardiac protection against ischemia-reperfusion injury (16). EPO expression immediately following IH and cardiac protection 24 h later were not induced in *Hif1a*$^{+/-}$ mice, demonstrating critical involvement of HIF-1 in these responses. Clinical studies indicate that recurrent sleep apnea is associated with increased serum levels of EPO and VEGF (17–20).

To further evaluate the effect of IH on HIF-1 activity, we have utilized PC12 rat pheochromocytoma cells. PC12 cells share many properties with glomus cells of the carotid body including O_2-regulated neurotransmitter release (21) and expression of tyrosine hydroxylase, the rate-limiting enzyme for catecholamine production (22). In this study, we demonstrate that HIF-1α protein expression and HIF-1 transcriptional activity are induced in PC12 cells subjected to IH via signal transduction pathways that are distinct from those involved in the response to continuous hypoxia.

EXPERIMENTAL PROCEDURES

Cell Culture—PC12 cells (original clone from Dr. L. Green) were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% horse serum, 5% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) under 90% air and 10% CO_2 at 37 °C. Prior to all experiments, the cells were placed in antibiotic-free medium and serum-starved for 16 h to avoid any confounding effects of serum on HIF-1 activity. In the experiments involving treatment with drugs, cells were preincubated for 30 min with either drug or vehicle.

Exposure to Intermittent Hypoxia—Cell cultures were exposed to alternating cycles consisting of 1.5% O_2 for 30 s followed by 20% O_2 for 4 min at 37 °C in a 12 × 12 × 7 inch Lucite chamber. The chamber was equilibrated with gases at a flow rate of 2.4 liter/min by timer-controlled solenoid valves. O_2 levels were monitored by an electrode (Lazar) placed in the tissue culture medium and by an O_2 analyzer (Beckman LB2) placed in the chamber. 45 s was required for ambient O_2 to reach 1.5% O_2 during de-oxygenation and 50 s for return to 20% O_2 during re-oxygenation.

Chemicals—All chemicals and reagents were of analytical grade and obtained from Sigma unless otherwise mentioned. Dimethyloxalylglycine (DMOG) was a gift from Dr. P. Ratcliffe (University of Oxford).

Plasmids—The following plasmids used in the present study have been described previously: p2.1 (23), pGalA (24), pG5E1bLuc, pCaMKII-WT, and pCaMKII-290 (25). pRSV-LacZ was from the American Type Culture Collection.

Preparation of Nuclear Extracts—Cells were lysed in hypotonic solution (10 mM KCl, 10 mM HEPES, 1 mM PMSF, pH 7.8) in the presence of a protease inhibitor mixture (Roche Applied Science) as described previously (26). The disrupted cells were centrifuged at 16,000 × *g*, and the pellet was resuspended in a solution containing 400 mM KCl, 20 mM HEPES, 25% (v/v) glycerol, 0.2 mM EDTA, and 1.5 mM $MgCl_2$ at pH 7.8 to lyse the nuclear membrane. The suspension was centrifuged and proteins in the supernatant were quantified by protein assay kit (Bio-Rad).

Preparation of Membrane Fractions—Membrane fractions were prepared as described previously (27). Cells were washed twice with phosphate-buffered saline containing 2 mM EDTA and incubated on ice with extraction buffer (20 mM Tris, 2 mM EDTA, 5 mM EGTA, 10 mM mercaptoethanol, 0.1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin) for 10 min. Cell lysates were homogenized and centrifuged at 500 × *g* for 10 min. The supernatant was recovered and centrifuged at 100,000 × *g* for 60 min to separate the plasma membrane from the cytosolic fraction. The plasma membrane fractions were resuspended in a buffer containing 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 5 mM mercaptoethanol, 0.1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml aprotinin.

Immunoblot Assays—Nuclear protein extracts (25 μg) were fractionated by 7.5% polyacrylamide-SDS gel electrophoresis and transferred to a polyvinylpyrrolidone difluoride membrane (Immobilon-P, Millipore, Bedford, MA). The membrane was blocked with Tris-buffered saline (TBS-T) containing 5% nonfat milk at 4 °C overnight. Membranes were incubated with anti-HIF-1α mouse monoclonal antibody H1α67 (28) at 1:500 dilution of stock (Novus Biologicals) in TBS-T containing 3% nonfat milk. Membranes were treated with goat anti-mouse secondary antibody conjugated with horseradish peroxidase (Santa Cruz; dilution 1:2000) in TBS-T containing 3% nonfat milk. Immune complexes on the membrane were visualized using enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). The membranes were exposed to Kodak XAR films. Similar procedures were employed for immunoblot analysis of phosphorylated and unphosphorylated forms of calcium-calmodulin-dependent protein kinase (CaMK) II with appropriate antibodies (Chemicon; dilution 1:1000); phosphorylated forms of the MAP kinase/extracellular signal-regulated kinase (ERK) 1 and 2 (Cell Signaling; 1:1000 dilution); and protein kinase C (PKC) isoforms (Transduction Laboratories; 1:1000 dilution).

Transient Transfection and Reporter Gene Assays—Cells were transfected with plasmid DNA using Lipofectamine (Invitrogen, Life Technologies, Inc.) reagent as described previously (29). Briefly, cells were plated in 35-mm tissue culture plates at a density of 1×10^5 cells/plate in growth medium containing serum. After 24 h, cells were washed twice with 5 ml of serum-free medium. The DNA-liposome mixture was prepared using Lipofectamine (10 μg), 1 μg of the desired plasmid (unless otherwise specified) and 0.25 μg of pRSV-LacZ (internal control for determining the transfection efficiency) in 2 ml of serum-free medium. The total amount of DNA transfected per plate was held constant by addition of pUC19 carrier DNA. Cells were incubated in the DNA-liposome mixture for 4 h followed by addition of 2 ml of serum-containing medium. After 24 h, cells were washed with serum-free medium and 4 ml of serum-free growth medium was added. After 18 h, cells were exposed either to 20% O_2 or IH and then harvested in 200 μl of reporter lysis buffer (Tropix). The cell lysate was centrifuged at 10,000 × *g* for 10 min to remove debris. 20 μl of cell lysate was mixed with 100 μl of buffer containing luciferin. Relative luminescent light units were recorded in a Berthold luminometer. For measurement of β-galactosidase (β-gal) activity, 10 μl of cell lysate was incubated with 100 μl of the reaction mixture containing β-galactam^{TM} (Tropix) as substrate for 30 min at room temperature. Following incubation, 150 μl of luminescence enhancer (Tropix) was added to the reaction mixture and the resulting luminescence was measured. Protein analysis was performed using a protein assay kit (Bio-Rad). We verified that all reporter gene assays were in the linear range.

Probe Preparation—DNA oligonucleotides were synthesized complementary to nucleotides 902–959 and 1435–1489 of rat tyrosine hydroxylase (TH) mRNA (30), end-labeled using T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP, purified using QuickSpin Sephadex G25 columns (Roche Applied Science), and quantitated by liquid scintillation counting.

Northern Blot Hybridization—Total RNA was isolated from PC-12 cells using the RNAeasy kit (Qiagen), and aliquots (10 μg) were fractionated by formaldehyde-agarose-ethidium gel electrophoresis. RNA was transferred to a nitrocellulose membrane (Gene Screen Plus, Schleicher & Schuell) and UV-cross-linked (model FB UVXL-1000, Fisher Scientific). Following prehybridization at 42 °C for 4 h, 5×10^6 cpm of ^{32}P-labeled oligonucleotide probe was added with fresh hybridization buffer (containing a final concentration of 5 SSPE, 5× Denhardt's solution, 25% deionized formamide, 1% SDS, and 5 μg/ml salmon sperm DNA) and incubated with rotation at 42 °C for 16 h. The membrane was washed at room temperature twice for 15 min in 2× SSC solution containing 0.1% SDS and then washed twice in 1× SSC containing 0.1% SDS at 50 °C for 15 min. The membrane was then exposed to x-ray film for autoradiography. The probe was stripped off the membrane by boiling in 0.1× SSC containing 1% SDS for 30 min with shaking. The stripped membrane was re-probed with ^{32}P-labeled rat β-actin cDNA. The relative amounts of β-actin mRNA were determined by optical density measurements and were used to normalize the expression levels of TH mRNA.

GST Fusion Protein Expression and Purification—Plasmid pGEXP300TD (generously provided by J. Caro, Jefferson Medical College, Philadelphia) was transformed into BL21-competent cells (Invitrogen), which were cultured in LB medium supplemented with 50 μg/ml of ampicillin. Expression of the fusion protein was induced by the addition of 0.1 mM isopropyl β-thiopyranoside (Promega), and purification was essentially the same as described previously (31). Briefly, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 1% Triton X-100, 5 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM PMSF, protease inhibitor mix). The cellular fraction was centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was mixed with a 50% solution of glutathione agarose beads and washed three times in lysis buffer. The fusion protein was eluted with 50 mM Tris-HCl (pH 8.0) containing 5 mM reduced glutathione at 4 °C for 20 min. After centrifugation, the supernatant were collected, and the protein concentration was determined using a protein assay kit (Bio-Rad).

In Vitro Kinase Assay—CaM kinase II (CaMK II) activity was measured as described previously (32). Briefly, cells (5×10^6) were lysed in buffer containing 25 mM HEPES, pH 7.4, 150 mM NaCl, 2.5 mM $MgCl_2$,

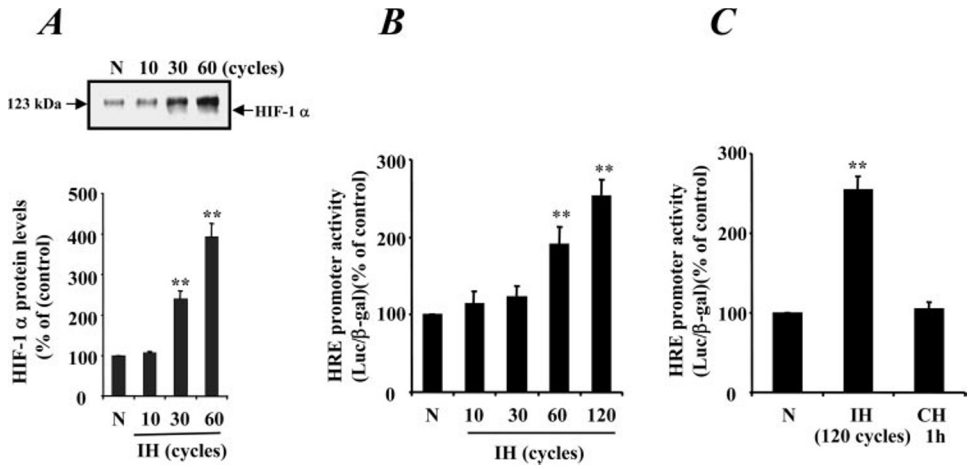


FIG. 1. **Activation of HIF-1 by IH in PC12 cells.** *A*, immunoblot analysis of HIF-1α protein expression. Cells were exposed to 20% O_2 (N) or to 10–60 cycles of IH (30 s at 1.5% O_2 alternated with 4 min at 20% O_2). Western blot assay was performed on nuclear extracts with an anti-HIF-1α antibody. *Top panel*, representative HIF-1α immunoblot. *Bottom panel*, densitometric analysis of HIF-1α expression. *B*, activation of HRE reporter gene expression by IH. PC12 cells were cotransfected with p2.1, containing an HRE upstream of SV40 promoter and luciferase coding sequences, and pRSV-LacZ, containing RSV promoter and β-gal coding sequences. Transfected cells were exposed to either 20% O_2 or 10–120 cycles of IH. *C*, comparison of IH with continuous hypoxia. Cells cotransfected with p2.1 and pRSV-LacZ were exposed to 120 cycles of IH or to 1 h of continuous hypoxia (CH; 1.5% O_2). Mean and S.D. are shown. **, $p < 0.01$ compared with control (*N*).

0.1 mM EDTA, 0.5 mM dithiothreitol, 20 mM glycerophosphate, 1 mM sodium orthovanadate, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, and 1% Nonidet P-40. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min, and protein content was quantified using a protein assay kit (Bio-Rad). The standard curve for the kinase assay was constructed according to the protocols suggested by the manufacturer (CaMK II Assay kit, Upstate Biotechnology). The reaction mixture was 30 μl of solution containing 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP, 125 μM autocamtide (peptide substrate KKALRRQETVDAL), and 400 ng of calmodulin. Activities of other serine/threonine kinases such as protein kinase A and protein kinase C were inhibited by mixture of inhibitor peptides (0.1 μM). The reaction was initiated by adding 10 μl of cell supernatant (equal amount of protein). The reaction mixture was incubated at 30 °C for 15 min, 10-μl aliquots were spotted on p81 phosphocellulose paper. After 3 min, the paper was washed with 0.75% phosphoric acid for 10–15 times and then washed with acetone five times for 5 min each. The radioactivity was counted by scintillation counter. For demonstrating direct phosphorylation of p300 by CaMK II, an *in vitro* kinase assay was performed by a modification of the protocol described by Sang *et al.* (31). Purified GST or GST-p300TD fusion protein was incubated at 30 °C for 10 min with recombinant CaMK II (Upstate Biotechnology) in the presence of 10 μCi of [γ-^{32}P]ATP in CaMK buffer. The reaction was stopped by adding an equal volume of 2× Laemmli sample buffer and heating at 95 °C for 3 min. After fractionation by 4–20% continuous gradient SDS-PAGE (Bio-Rad), the gel was stained with Coomassie Blue R250 (Sigma), destained, and dried before autoradiography.

Data Analysis—The data are expressed as mean ± S.D. from 3–5 independent experiments each run in triplicate. Statistical analysis was performed by analysis of variance (ANOVA) and *p* values less than 0.05 were considered significant.

RESULTS

IH Induces HIF-1 Activity—As described under "Experimental Procedures," we established a cell culture system in which PC12 cells were exposed to IH consisting of alternating cycles of hypoxia (1.5% O_2 for 30 s) and re-oxygenation (20% O_2 for 4 min). Under these conditions the expression of HIF-1α protein increased in a dose-dependent manner as the duration of IH was increased from 10 to 30 to 60 cycles (Fig. 1*A*). HIF-1α protein levels were unaffected by exposing cells to alternating cycles of normoxia instead of hypoxia (data not shown). To demonstrate an increase in HIF-1 transcriptional activity, PC12 cells were transfected with reporter gene p2.1, in which the expression of firefly luciferase was driven by a HIF-1-dependent hypoxia response element (HRE) upstream of an SV40 promoter (23). HRE-dependent transcriptional activity was induced by IH although, relative to the induction of HIF-1α protein expression, the response was not as dramatic and required an increased number of cycles (Fig. 1*B*). Transcription of a co-transfected reporter gene in which β-gal expression was driven by a Rous sarcoma virus (RSV) promoter was not induced by IH. 120 cycles of IH, which represents a total of 60 min of hypoxic exposure, induced HRE-dependent transcription, whereas 60 min of continuous hypoxia did not (Fig. 1*C*).

MAP kinase, PKC, and PI 3-kinases Are Not Required for IH-induced HIF-1 Activation—Exposure of PC12 cells to continuous hypoxia induces membrane depolarization, increased intracellular Ca^{2+} levels, and increased activity of phosphatidylinositol 3-kinase (PI3K), $p42^{ERK2}$, and $p44^{ERK1}$ (33). We therefore investigated the involvement of major intracellular signal transduction pathways in the induction of HRE-dependent transcription by IH. Immunoblot analysis revealed that IH induced increased phosphorylation of ERK1, ERK2, and Jun N-terminal kinase (JNK) (Fig. 2, *A* and *B*, *top panel*) but not p38 (data not shown). However, pretreatment of cells with increasing doses of PD98059, a selective inhibitor of MAP kinase/ERK kinase (MEK), or SP600125, an inhibitor of JNK, did not inhibit HRE-dependent transcription induced by IH (Fig. 2, *A* and *B*, *bottom panel*). We then examined the effects of IH (60 cycles) on the translocation of PKC isoforms to the plasma membrane as an index of activation. Of the several PKC isoforms (α, β, γ, δ, λ, γ), IH increased PKC-α and PKC-γ levels in the membrane fraction. Similar increases in PKC-α and PKC-γ were observed after treatment with TPA (100 nM), a potent activator of PKC that served as a positive control. We examined the effects of bisindolylmaleimide 1 (Bis), which inhibits activity of the PKC α, β_1, β_{11}, γ, δ, and ϵ isoforms, on IH-induced HRE-activation. As shown in Fig. 2*C*, HRE activation was unaffected by 3 μM Bis, whereas increasing the concentration to 10 μM resulted in a modest but significant inhibition of IH-induced HRE activation (Fig. 2*C*). Because hypoxia activates PI3K in PC12 cells (33), we also tested the effects of the inhibitors LY294002 and wortmannin. Neither LY294002 nor wortmannin blocked IH-induced transcriptional activity (data not shown).

Ca^{2+} Signaling Pathways Involving CaMK II Are Required for IH-induced HIF-1 Activity—Pretreatment of cells with the intracellular Ca^{2+} chelator BAPTA-AM resulted in potent inhibition of HRE-dependent transcription induced by IH (Fig. 3*A*). These observations indicate that Ca^{2+} signaling pathways

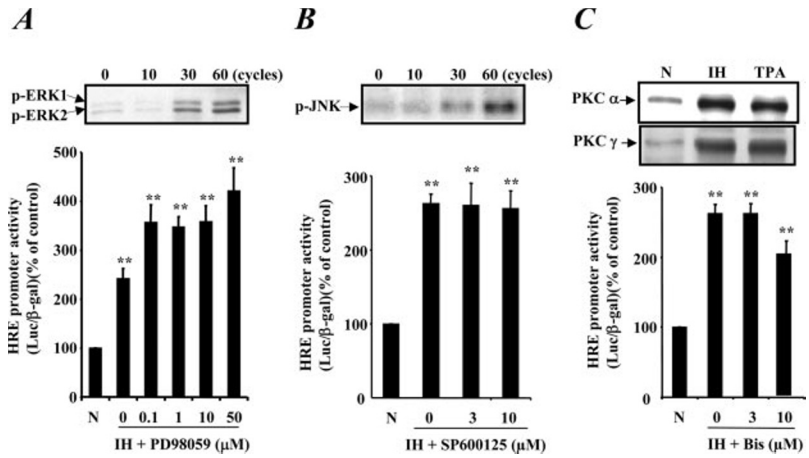
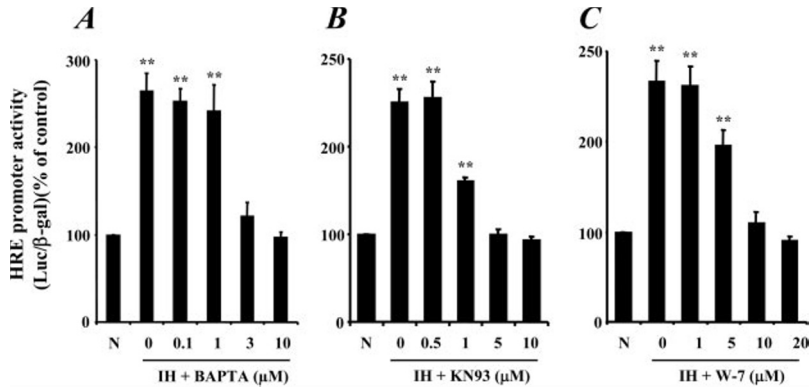


FIG. 2. **MAP kinases and PKC are not required for HRE reporter gene expression induced by IH.** *Top panel of A*, *B and C*: Activation of MAP kinases ERK and JNK, and PKC isoforms α and γ. PC12 cells were exposed to 20% O_2 (*N*) or to IH for 10–60 cycles. Immunoblot assays were performed on cell extracts using antibodies that specifically recognize the phosphorylated forms of ERK1/ERK2 (*A*) or JNK (*B*) or on membrane fractions with anti-PKC-α or anti-PKC-γ antibodies (*C*). *Bottom panel* of *A*, *B*, and *C*, MAP kinase and PKC activities are not required for IH-induced HRE activation. PC12 cells cotransfected with the p2.1 HRE reporter gene and pRSV-LacZ were pretreated with increasing concentrations of the MEK inhibitor PD98059 (*A*), SP600125, an inhibitor of JNK (*B*), or the PKC inhibitor Bis (*C*) for 30 min, exposed to 20% O_2 (*N*) or IH for 120 cycles, and harvested for analysis of luciferase and β-gal activity. Data represent mean ± S.D. from three individual experiments performed in triplicate. **, $p < 0.01$ compared with control (*N*).

FIG. 3. **Ca^{2+}-CaMK activity is required for the induction of HIF-1-dependent gene transcription in cells subjected to IH.** PC12 cells transfected with the p2.1 HRE reporter gene were untreated (*N*) or pretreated with increasing concentrations of BAPTA-AM, a Ca^{2+} chelator (*A*), KN-93, a CaMK inhibitor (*B*), or W-7, a calmodulin inhibitor (*C*), for 30 min and then exposed to 20% O_2 (*N*) or 120 cycles of IH. Data presented are mean ± S.D. ($n = 3$). **, $p < 0.01$ compared with control (*N*).



are involved in IH-induced HIF-1 transcriptional activity. CaMKs are downstream signaling molecules that participate in Ca^{2+}-mediated gene regulation. Our previous studies have shown that PC12 cells express CaMK II but not CaMK IV and that continuous hypoxia transiently increases CaMK II activity (32). We therefore examined whether CaMK II participates in IH-induced HIF-1-mediated transcription. As shown in Fig. 3*B*, IH-induced reporter gene expression was inhibited in a dose-dependent manner by pretreatment with the selective CaMK inhibitor KN93 (Fig. 3*B)* as well as with W-7, a potent inhibitor of calmodulin (Fig. 3*C*). The inhibitory effect of these agents was specific for IH-induced HRE-dependent transcription since in all cases as the expression was normalized to that of the co-transfected RSV-β-gal reporter. Further analysis of PC12 cell lysates revealed an exponential increase in CaMK II activity in response to increasing duration of IH (Fig. 4*A)* and an associated increase in the phosphorylation of CaMK II protein (Fig. 4*B*).

CaMK II Is Required for IH-induced HIF-1α Transactivation Function but Not for HIF-1α Protein Expression—We next examined whether CaMK II activity mediates changes in HIF-1α protein expression or transactivation function in cells exposed to IH. Remarkably, ectopic expression of constitutively active form CaMK II alone or in combination with HIF-1α had no significant effect on HIF-1α protein expression (Fig. 5*A*). Furthermore, KN93 did not block the induction of HIF-1α protein expression induced by IH (Fig. 5*B*). However, consistent with

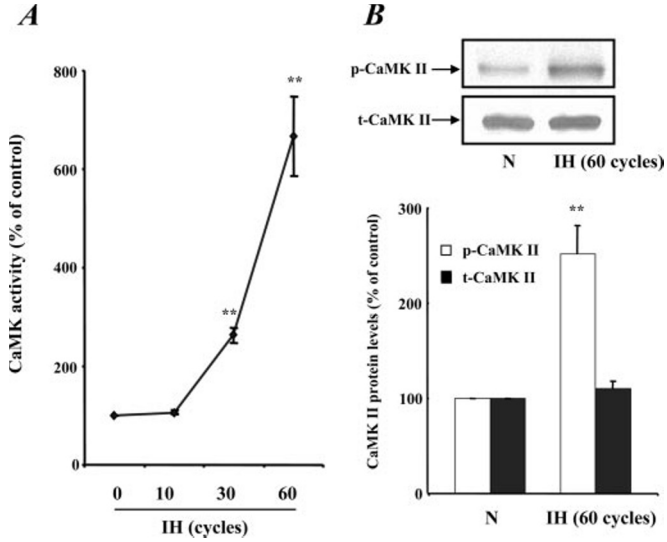


FIG. 4. **Increased CaMK II activity in PC12 cells subjected to IH.** *A*, cells were exposed to increasing cycles of IH. CaMK activity was assayed with autocamtide as a substrate in cell extracts using [γ-^{32}P] ATP. *B*, immunoblot analysis of phosphorylated (*p-CaMK II*) and total (*t-CaMK II*) CaMK II in PC12 cells exposed to 20% O_2 (*N*) or IH. *Top panel*, representative immunoblot. *Bottom panel*, densitometric analysis. Data presented are percent change from control (mean ± S.D. from three independent experiments). **, $p < 0.01$ compared with control (*N*).

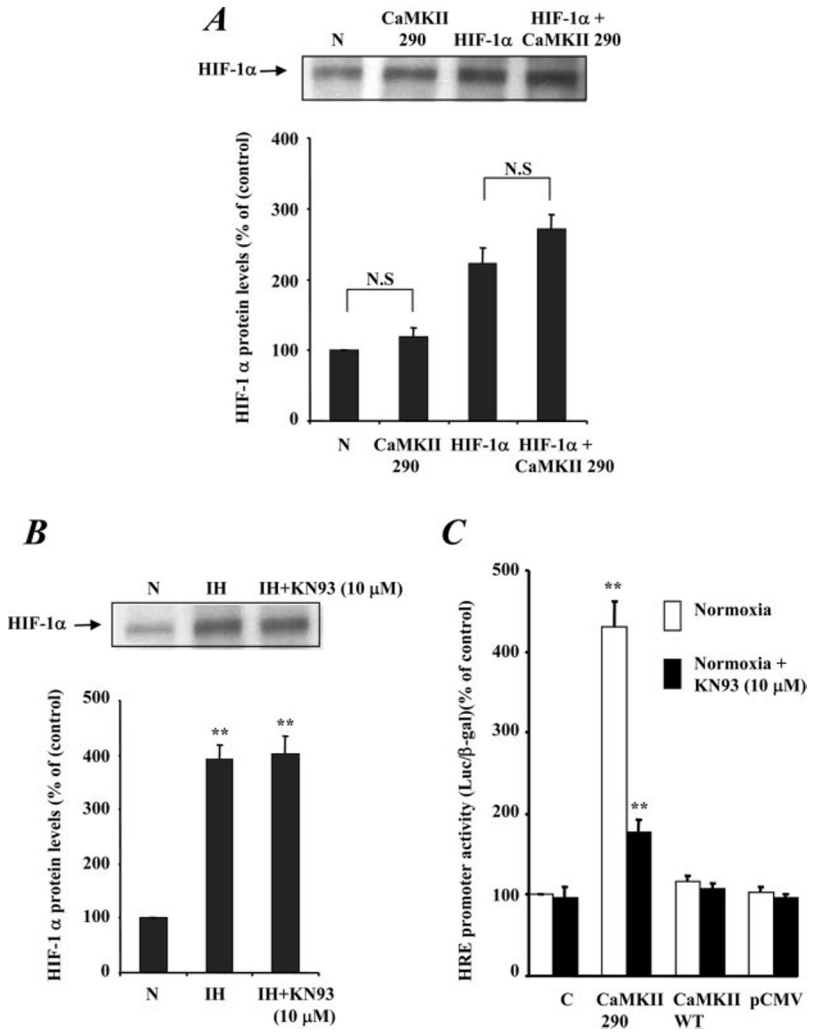


FIG. 5. **CaMK II regulates HIF-1 transcriptional activity but not HIF-1α protein expression.** *A*, PC12 cells were transfected with constitutively active form of CaMK II alone or with HIF-1α overexpression plasmid, and HIF-1α protein expression was analyzed. *Top panel*, representative immunoblot. *Bottom panel*, densitometric analysis. Overexpression of constitutively active form of CaMK II had no significant effect on HIF-1α protein expression. *B*, PC12 cells were exposed to 20% O_2 (*N*) or 60 cycles of IH in the presence of vehicle or KN-93 and HIF-1α protein expression was analyzed. *Top panel*, representative immunoblot. *Bottom panel*, densitometric analysis. *C*, expression of a constitutively active form of CaMK II induces HRE-dependent reporter gene activity in non-hypoxic cells. PC12 cells were co-transfected with pRSV-LacZ, p2.1, and either no additional plasmid (*C*) or an expression vector encoding wild type (*WT*) CaMK II, constitutively active CaMK II (290), or empty expression vector (*pCMV*). Transfected cells were cultured in the presence or absence of 10 μM KN93. Data presented are mean ± S.D. ($n = 3$). **, $p < 0.01$ compared with control (*C*).

the inhibition of IH-induced transcription by CaMK II inhibitors (see Fig. 3*B*), co-transfection of CaMK II-290, encoding a constitutively active form of CaMK II, dramatically induced HRE-dependent transcription under non-hypoxic conditions, and this induction was inhibited by KN93 (Fig. 5*C*).

To investigate the role of CaMK II in the regulation of HIF-1-dependent transcription in a greater detail, PC12 cells were cotransfected with pG5E1bLuc, a reporter plasmid in which luciferase coding sequences are present downstream of five GAL4 binding sites, and pGAL4/HIF-1α-(531–826), which encodes the DNA-binding domain of the yeast GAL4 protein fused to the transactivation domains (TADs) of HIF-1α. Increased transactivation of pG5E1bLuc by GAL4/HIF-1α-(531–826) is induced in cells exposed to continuous hypoxia (24). Transactivation mediated by GAL4/HIF-1α-(531–826) was induced in PC12 cells exposed to IH (Fig. 6*A*) or co-transfected with CaMK II-290 (Fig. 6*B*) and, in both cases, the induction was inhibited by KN93 treatment. In contrast, KN93 had no effect on reporter gene transactivation induced by 6 h of continuous hypoxia (Fig. 6*E*).

IH and CaMK II Stimulate HIF-1α Transactivation by a Mechanism That Is Independent of Asparaginyl Hydroxylation—GAL4/HIF-1α-(531–826) contains the TAD-N (N-terminal TAD; amino acids 531–575) and the TAD-C (C-terminal TAD; amino acids 786–826), which are separated by the inhibitory domain (24). FIH-1 (factor inhibiting HIF-1) binds to the inhibitory domain (34) and mediates the O_2-dependent hydroxylation of Asn-803, which prevents binding of the coactivators p300 and CBP to TAD-C (15). As a result, transactivation mediated by GAL4/HIF-1α-(531–826) is induced by continuous hypoxia, whereas GAL4/HIF-1α-(786–826), which lacks the FIH-1 binding site, is constitutively active. As shown in Fig. 6*C*, the activity of GAL4/HIF-1α-(786–826) was increased in response to IH. Co-transfection of pGAL4/HIF-1α-(786–826) with CaMK II-290 mimicked the effects of IH under non-hypoxic conditions, and KN-93 blocked the effect of IH or CaMKII-290 on GAL4/HIF-1α-(786–826) (Fig. 6*D*). However, KN-93 had no significant effect on baseline levels of transactivation mediated by either GAL4/HIF-1α-(531–826) or GAL4/HIF-1α-(786–826) under normoxia (Fig. 6, *B* and *D*). These results indicate that CaMK II stimulates TAD function via a mechanism that is independent of asparaginyl hydroxylation.

CaMK II Signaling Promotes Transactivation Mediated by p300—Several lines of evidence suggest that p300/CBP are the major coactivators for HIF-1 activation (31, 35–38). To investigate whether p300 is involved in IH- and CaMK II-mediated activation of HIF-1 transcriptional activity, PC12 cells were co-transfected with pGAL4p300 (encoding a GAL4 fusion protein containing amino acids 1–2414 of p300) and pG5E1bLuc, and exposed to 120 cycles of IH. As shown in Fig. 7*A*, IH increased p300 transcriptional activity. Co-transfection of CaMK II-290 and pGAL4p300 mimicked the effects of IH and KN-93 prevented activation of p300 by IH or CaMK II-290 (Fig. 7*A*). We next tested whether p300 was a direct substrate of CaMK II. We expressed and purified amino acids 1572–2370 of p300 as a GST fusion protein and performed an *in vitro* CaMK II assay. As can be seen from Fig. 7*B*, p300 was specifically phosphorylated by CaMK II *in vitro*.

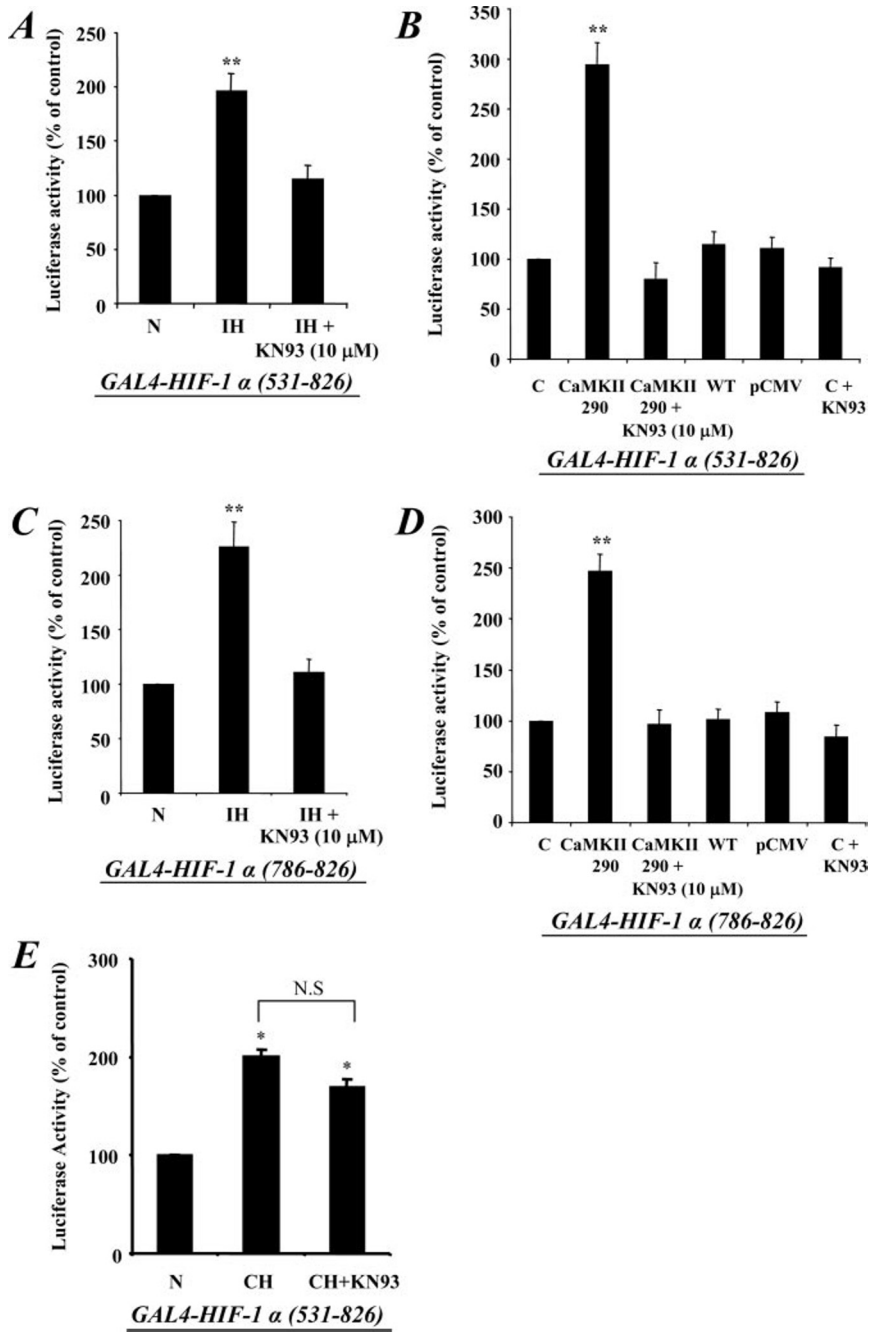


FIG. 6. **Induction of HIF-1α transactivation domain function by IH or constitutively active CaMK II.** *A*, PC12 cells were transfected with pRSV-LacZ, pGAL4/HIF-1α-(531–826) encoding a fusion protein consisting of the GAL4 DNA binding domain fused to the HIF-1α transactivation domain, and pG5E1bLuc, which contains 5 GAL4 DNA binding sites upstream of E1b promoter and luciferase coding sequences. Transfected cells were exposed to 20% O_2 (*N*) or 120 cycles of IH in the absence or presence of KN93. *B*, cells were co-transfected with GAL4-HIF-1α-(531–826) along with pCMV, or pCaMK II-WT or pCaMK II-290 in the absence or presence of KN93 and exposed to normoxia. *C*, cells were transfected with pGAL4-HIF-1α-(786–826) and pG5E1bLuc and exposed to normoxia (*N*) or 120 cycles of IH in presence and absence of KN-93. *D*, cells were co-transfected with pGAL4/HIF-1α-(786–826) along with pCMV, pCaMK II-WT or pCaMK II-290 in the absence or presence of KN93 or were untreated (*C*). *E*, cells were transfected with pRSV-LacZ, pGAL4/HIF-1α-(531–826) and exposed to 20% O_2 (*N*) or to continuous hypoxia (1% O_2 for 6 h) in presence or absence of KN-93. Data presented are percent change from control (mean ± S.D. from three independent experiments). **, $p < 0.01$ compared with control; *N.S.*, no significant difference.

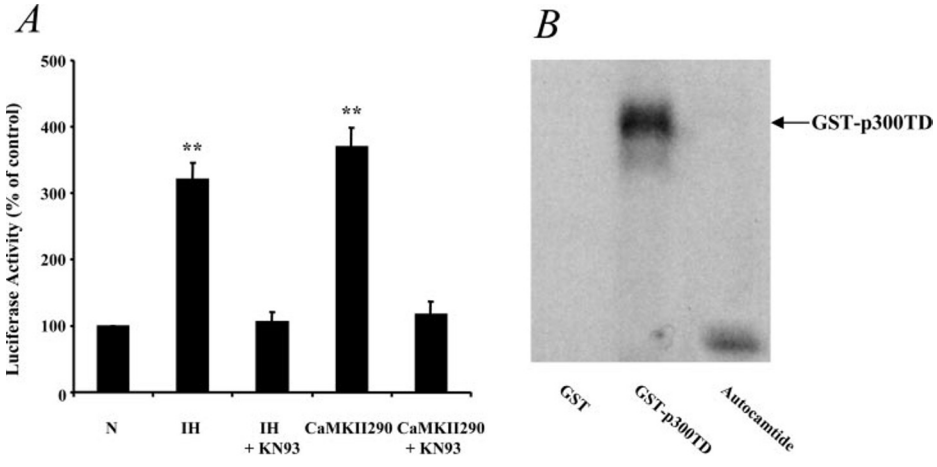


FIG. 7. **Effects of IH and CaMK II on p300.** *A*, IH and CaMK II induce transcriptional activation mediated by a GAL4/p300 fusion protein. PC12 cells were co-transfected with pRSV-LacZ, pGAL4/p300, and pG5E1bLuc with or without pCaMK II-290. Transfected cells were exposed to 20% O_2 (N) or 120 cycles of IH in the absence or presence of KN93. Data presented are mean ± S.D. ($n = 3$). **, $p < 0.01$ compared with control (N). *B*, direct phosphorylation of p300 *in vitro* by CaMK II. GST/p300TD, a fusion protein consisting of glutathione *S*-transferase and p300, was expressed in and purified from BL21 cells and used for *in vitro* CaMK II kinase assay.

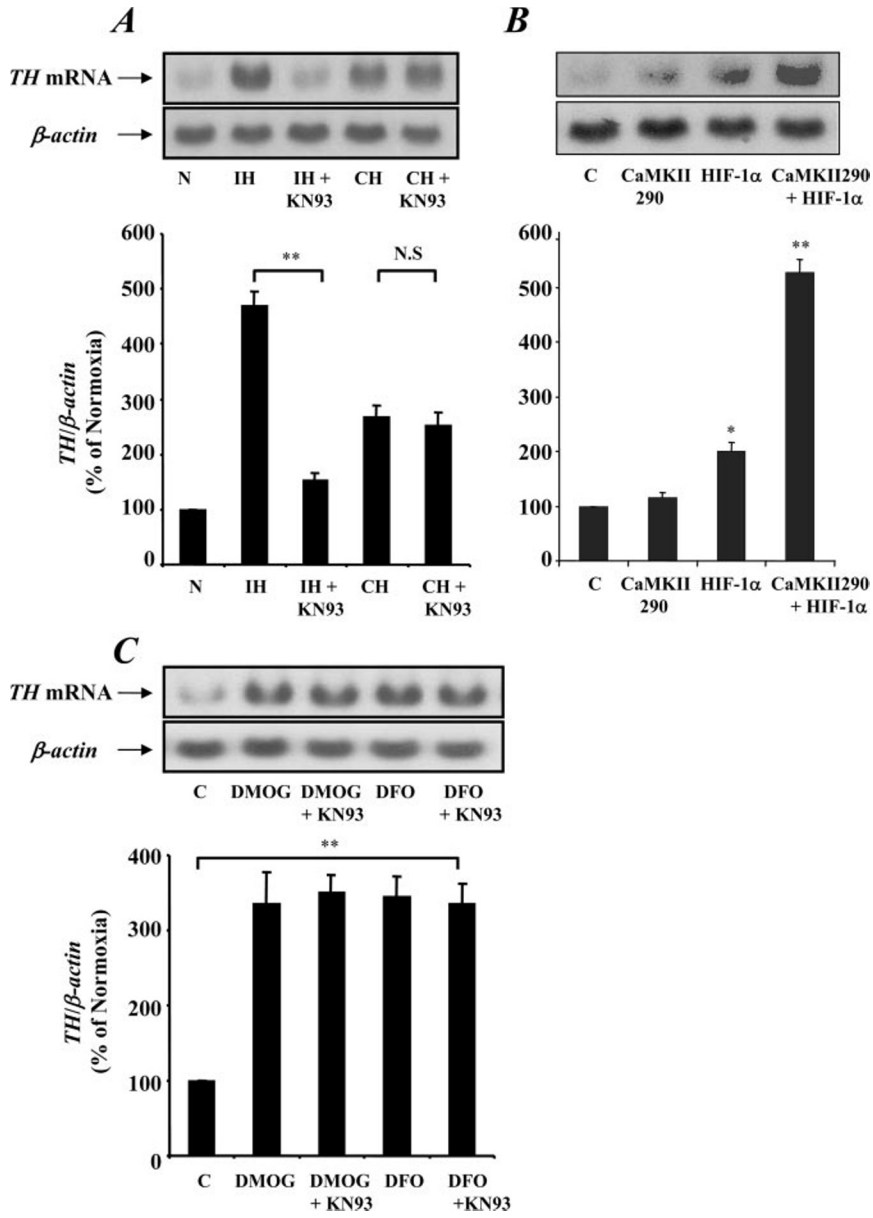


FIG. 8. **TH mRNA expression is induced by IH and continuous hypoxia via different mechanisms.** *A*, KN93 inhibits *TH* mRNA expression induced by IH but not by continuous hypoxia. PC12 cells were pretreated with KN93 (10 μM) for 30 min and then exposed to IH (120 cycles) or 6 h of continuous hypoxia (*CH*). *B*, overexpression of constitutively active form of CaMK II with HIF-1α leads to robust activation of TH mRNA. *TH* and β-actin mRNA were analyzed by Northern blot assay ($n = 3$). **, $p < 0.01$ compared with control (*N*); *N.S.*, not significant. *C*, inhibitors of HIF-1α hydroxylases increase *TH* mRNA expression in a KN93-independent manner. PC12 cells pretreated with KN93 (10 μM) for 30 min were treated with 1 mM DMOG or 1 mM desferrioxamine (DFO) for 12 h at 20% O_2.

IH Induces Expression of the HIF-1 Target Gene TH—Expression of the *TH* gene encoding tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis, is induced when PC12 cells are subjected to continuous hypoxia, an effect that is mediated by HIF-1 (39). Because catecholamine levels are elevated in recurrent apnea patients experiencing chronic IH (40, 41), we examined TH mRNA expression in PC12 cells subjected to IH. TH mRNA expression was increased 4.6-fold in PC12 cells subjected to IH and KN93 blocked this response (Fig. 8*A*). In contrast, KN93 did not attenuate the increased TH mRNA expression induced in cells subjected to continuous hypoxia for 6 h (Fig. 8*A*). Consistent with the inhibitory effects of KN-93 on IH-induced TH mRNA, over-expression of constitutively active form of CaMK II along with HIF-1α caused a robust elevation of TH mRNA under normoxia, whereas overexpression of HIF-1α had only a modest stimulatory effect on TH mRNA (Fig. 8*B*). These results are consistent with reporter gene data (Fig. 6) indicating that CaMK II potentiates transactivation mediated by HIF-1.

HIF-1 activity is induced in response to continuous hypoxia due to reduced hydroxylation of key proline and asparagine residues in HIF-1α that negatively regulate protein stability and transcriptional activity, respectively (10–15, 42). The hydroxylases contain Fe(II) in their catalytic sites and utilize O_2 and 2-oxoglutarate (α-ketoglutarate) as reaction substrates. DMOG, a competitive inhibitor of 2-oxoglutarate, and desferrioxamine (DFO), a Fe(II) chelator, induce HIF-1 activity by inhibiting the hydroxylases. Exposure of PC12 cells to DMOG or DFO enhanced the expression of TH mRNA similar to that seen with continuous hypoxia, but KN93 failed to block the response (Fig. 8*C*). Thus, IH and continuous hypoxia activate TH mRNA expression by distinct mechanisms, similar to their effects on HIF-1 activity.

DISCUSSION

HIF-1 plays a major role in coordinating physiological responses to continuous hypoxia (9–13), and recent data suggest involvement of HIF-1 in responses to IH (16–20). In this study, we have directly demonstrated for the first time the induction of HIF-1 activity in response to IH. The results presented above indicate that IH induces HIF-1α protein expression and transactivation function, two necessary prerequisites for HIF-1-dependent gene transcription. However, distinct molecular mechanisms underlie these two processes.

The induction of transactivation function is caused by increased CaMK II activity in cells subjected to IH. Our data suggest that the effects of CaMK II are mediated by phosphorylation of the co-activator p300. CaMK IV has previously been shown to phosphorylate CBP (43), which is structurally and functionally similar to p300. In several cell types, ERK signaling has been shown to induce phosphorylation of p300, which in turn increases its interaction with the transactivation domain of HIF-1α, thus stimulating HIF-1 transcriptional activity (31, 35, 44–46). Although IH increased phosphorylated ERK1 and ERK2, ERK activity appears to be dispensable for IH-induced HIF-1 transcriptional activity in PC12 cells. PI3K, JNK and PKC also do not appear to play major roles in mediating HIF-1 activation in response to IH. Taken together, our data suggest that IH induces CaMK II activity, phosphorylation of p300, and increased HIF-1α-mediated transactivation.

In contrast to IH, in cells exposed to continuous hypoxia, inhibition of CaMK II activity had no effect on the increased HIF-1 transcriptional activity, which occurs as a result of decreased O_2-dependent hydroxylation of Asn^{803} in TAD-C (15). These observations are consistent with an earlier report that continuous hypoxia induces only a modest (1.5-fold) and transient (<1 h) activation of CaMK II (32).

Whereas KN93 had striking effects on HIF-1α transactivation domain function, CaMK inhibition had no effect on the induction of HIF-1α protein expression in cells exposed to IH. The PI3K and ERK pathways, which have been shown to mediate increased HIF-1α protein expression in response to signaling via receptor tyrosine kinases and G protein-coupled receptors (47, 48) are also not required for this response, indicating the involvement of a novel signal transduction pathway. Thus, additional studies are required to delineate in greater detail the molecular mechanisms leading to increased HIF-1 activity in response to IH.

TH is the rate-limiting enzyme in catecholamine synthesis, and circulating catecholamines are increased in humans with chronic IH caused by recurrent apneas as well as animals exposed to chronic IH (40, 41, 49). A recent study has shown that IH increases TH enzymatic activity through posttranslational modification in PC12 cells (50). Our results demonstrate that IH also increases TH mRNA expression, which is the product of a known HIF-1-regulated gene (39). It is likely increased TH in turn contributes to increased catecholamine levels seen with chronic IH.

In summary, we have shown that IH is a potent stimulus for activating HIF-1, which leads to the transcription of the downstream target gene *TH*. HIF-1 activation in response to IH is mediated by signaling mechanisms that differ from those mediating HIF-1 activation in response to continuous hypoxia. The use of cell culture and animal models to further analyze the transcriptional response to IH mediated by HIF-1 may provide greater insight into the pathological consequences of sleep-disordered breathing.

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