

Nur77 Activated by Hypoxia-Inducible Factor-1 α Overproduces Proopiomelanocortin in von Hippel-Lindau-Mutated Renal Cell Carcinoma

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ABSTRACT

Mutation in the von Hippel-Lindau (VHL) protein associated with renal cell carcinoma causes hypoxia-inducible factor (HIF) to stabilize and consequently to induce various HIF-targeting proteins. In this study, we found that proopiomelanocortin (POMC), an adrenocorticotrophic hormone precursor, is up-regulated constitutively in VHL-mutated renal cell carcinoma. A critical transcription factor responsible for POMC overproduction was identified as Nur77, a member of the orphan steroid receptor superfamily. Little is known about how VHL mutation leads to activation of Nur77. We report that Nur77 is directly regulated by HIF. We show that HIF-1 α , but not HIF-2 α , binds to a putative HIF responsive element in the Nur77 promoter, activating the expression of Nur77. Mutation or deletion of the HIF binding site in the Nur77 promoter abrogates activation of a luciferase reporter gene under the control of Nur77 promoter by HIF-1 α . The treatment of Nur77 antisense oligonucleotide reduces POMC transcription under hypoxic conditions. We confirmed that Nur77 and POMC are up-regulated in VHL-mutated renal cell carcinoma. In this study, we provide the first molecular evidence that Nur77 activated by HIF under hypoxic conditions regulates production of the peptide hormone precursor POMC.

INTRODUCTION

Hypoxia-inducible factor (HIF) -1 α is a member of the basic helix-loop helix/PER-ARNT-SIM superfamily of transcription factors that form a heterodimer with HIF-1 β (ARNT). It plays key roles in the regulation of glucose metabolism, angiogenesis, and erythropoiesis under oxygen-limited conditions (1, 2). HIF-2 α (EPAS/HLF/HRF/MOP2) also binds to HIF-1 β as heterodimer (3–6). HIF-2 α is known to have different biological functions from those of HIF-1 α , although they share a high degree of sequence homology and similar domain structures (7). The tumor suppressor von Hippel-Lindau protein (pVHL) is a HIF repressor through direct binding to HIF- α subunits, and it targets them to the ubiquitin-dependent degradation pathway (8) and/or by recruiting histone deacetylase complexes to HIF- α -containing complex (9). It was found recently that pVHL specifically recognizes 4-hydroxyproline residues within the oxygen-dependent domain of HIF- α subunits catalyzed by hypoxia prolyl-4-hydroxylases (10–13). Mutations of VHL have been shown to result in constitutive stabilization of HIF- α subunit, leading to malignancy (14).

Clear type of renal cell carcinoma (RCC) belongs to a von Hippel-Lindau (VHL) disease characterized by the development of highly vascular tumors caused by overproduction of vascular endothelial growth factor (VEGF) as a consequence of constitutive activation of

HIF. In addition, RCC shows various neoplastic phenomena. Among these, Cushing's syndrome may occur in patients with RCC, although it is rare (15). It is known that Cushing's syndrome is caused by the overproduction of adrenocorticotrophic hormone (ACTH), mainly in the pituitary gland, and by lung cancer (16, 17).

ACTH biosynthesis is controlled by several transcription factors through the promoter of proopiomelanocortin (POMC), the precursor to ACTH, in pituitary cells (18, 19). The main transcription factor for ACTH production by corticotrophin-releasing hormone was identified as Nur77, an orphan nuclear receptor, which binds to the Nur response element site in the POMC promoter (18, 20). Nur77 is known to serve as a transcription factor to produce POMC in small cell lung cancer (21). Retinoic acid has been shown to prevent Cushing's syndrome by inhibiting Nur77 transcription activity (22). These results prompted us to investigate whether Nur77 also mediates the overproduction of POMC in RCC and, if so, how VHL mutation activates Nur77.

MATERIALS AND METHODS

Cell Culture and RCC Tissue. All of the cells in this study were purchased from American Type Culture Collection (Manassas, VA). Human embryonic kidney 293 cells, human neuroblastoma SK-N-SH cells, and human renal carcinoma cells (786-O) were maintained and grown with DMEM containing 10% fetal bovine serum, and 50 unit/ml streptomycin and penicillin. VHL-expressing RCC (786-O/VHL) was obtained by transfection of 786-O with mammalian expression vector for HA-tagged VHL, and selected with 1 mg/ml of G418 (Life Technologies, Inc., Rockville, MD). Normal and tumor tissues from kidneys of RCC patients were obtained at Seoul National University Hospital with consent from each patient.

DNA Constructs and Transfection. Cells were transiently transfected with various combinations of plasmids using Lipofectamine Plus reagent (Life Technologies, Inc.). Expression vectors for c-myc-tagged Nur77 (FL) (pMT-Nur77 [FL]) and Nur77 (267–601) (pMT-Nur77 [267–601]) were constructed by subcloning the corresponding Nur77 fragments into pCS2 + MT vector. pNur77-Luciferase reporter genes were constructed by subcloning appropriate PCR fragments into pGL2-basic vector (Promega, Madison, WI). pCMV-HA-HIF-1 α was obtained from Dr. W. G. An (Kyongpook National University). cDNA of HIF-2 α was obtained by PCR from Marathon-ready human liver cDNA library (Clontech Laboratories, Inc., Palo Alto, CA) and subcloned into pcDNA3-flag mammalian expression vector (Invitrogen, Carlsbad, CA). Antisense and sense oligonucleotides (1 μ M) were transfected with Lipofectamine reagent (Life Technologies, Inc.).

RNA Analysis and Reverse Transcription-PCR. Total RNAs were extracted from cells grown under 1% hypoxic conditions or from tissues from RCC patients with TRIzol reagent (Life Technologies, Inc.). cDNAs were synthesized using reverse transcriptase cDNA synthesis kit (Takara, Japan) with total RNAs as template. PCR primers used in this study are as follows: Nur77 (sense 5'-TCTGCTCAGGCCTGGTGTCTAC, antisense 5'-GGCACCAAGTCTCCAGCTTG-3'), VHL (sense 5'-GCGTCGTGCTGCCCGTATG-3', antisense 5'-TTCTGCACATTTGGGTGGTCTTC-3'), β -actin (sense 5'-GGCATCCACGAAACTACCTT-3', antisense 5'-CTGTGTGGACTTGGGAGAGG-3'), HIF-1 α (sense 5'-CTCAAAGTCGGACGCCTCA-3', antisense 5'-CCCTGCAGTAGGTTTCTGCT-3'), HIF-2 α (sense 5'-CTTCTCAATCTACATCAGGACG-3', antisense 5'-CTGCCCTCCTCACAATAGTC-3'), VEGF (sense 5'-CCTCCGAAACCATGAACCTTT, antisense 5'-AGAGATCTGGTTCCCGAAAC-3'),

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and POMC (sense 5'-AGAAGCGCGCAGGACGTCTCA-3', antisense 5'-CCTTCTGTAGGCGTCTTG-3').

Electrophoretic Mobility Shift Assay and Biotin-Streptavidin Pull-Down Assay. Nuclear extracts (15 μ g) from SK-N-SH cells transfected with mammalian expression vectors for HIF-1 α and HIF-1 β were incubated with ³²P-labeled probe in the presence of either cold wild-type probe or mutant probe for 1 h. For the supershift assay, anti-HIF-1 α antibody (Transduction Laboratories, Lexington, KY) was preincubated with nuclear extracts before addition of ³²P-labeled probe. Reaction mixtures were loaded into 6% polyacrylamide gel and analyzed by autoradiography. Nuclear extracts (200 μ g) were incubated with biotin-labeled probe and streptavidin-agarose (Oncogene, Darmstadt, Germany) for 4 h, followed by washing with washing buffer [25 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 25 mg/ml poly(dI/dC), and 1 mM phenylmethylsulfonyl fluoride]. Samples were subjected to 8% SDS-PAGE, transferred to nitrocellulose membrane, and underwent Western blot analysis with anti-HIF-1 α antibody.

Cell Lysis, Immunoprecipitation, and Western Blot Analysis. Cells were lysed with a lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP40, and 1 mM phenylmethylsulfonyl fluoride]. Whole cell lysates were incubated with suitable antibodies along with protein-(A/G) beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 h. The beads were washed with the lysis buffer three times. The bound proteins were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and underwent Western blot analysis with appropriate antibodies. Tissues were broken with a homogenizer (PowerGen 700; Fisher Scientific, Pittsburgh, PA), and nuclear fractions were separated from cytosolic fractions. One hundred μ g of cytosolic fraction were used for detection of VHL, and 50 μ g of nuclear protein were used for HIF-1 α and HIF-2 α expression.

Genomic DNA and Sequencing. Genomic DNAs were extracted from tissues from RCC patients with TRIzol reagent (Life Technologies, Inc.). VHL genomic DNAs were synthesized using PCR, purified by PCR purification kit (Qiagen, Valencia, CA), and sequenced by automatic DNA sequencer.

ACTH Hormone Assay. ACTH was measured by ELISA method (Research Diagnostics, Inc., Flanders, NJ).

RESULTS

Nur77 Is a Hypoxia-inducible Protein. To examine whether Nur77 is induced under hypoxic conditions, VHL^(+/+) cells were treated with increasing amounts of cobalt chloride, which acts as a hypoxia mimic to stabilize HIF- α . Nur77, along with HIF-1 α , was dramatically induced by cobalt chloride in a dose-dependent manner (Fig. 1A). We next investigated whether hypoxia induces Nur77 mRNA and protein under hypoxic (1% O₂) conditions. VHL^(+/+) types of cells grown under normoxic or hypoxic conditions were harvested, and Nur77 expression was detected using reverse transcription-PCR and Western blot analysis. Although Nur77 mRNA or protein is undetected under normoxic conditions, it is induced under hypoxic conditions, similar to VEGF (Fig. 1, B and C). These results demonstrate that Nur77 is a hypoxia-inducible protein.

HIF-1 α Specifically Binds to the Nur77 Promoter. Next, we searched for potential HIF binding sites within the promoter region of Nur77. HIF binds to a core sequence 5'-RCGTG-3' within a hypoxia-response element (HRE; Ref. 1). We identified two putative HREs in the mouse Nur77 promoter, which is localized between -214 and -203 (5'-CGTGCGGTACG-3'). It is noteworthy that these two sites are adjacent to each other as a reverse repeats. These HRE sites are partially conserved in human and rat Nur77 promoters (Fig. 2A). We then determined whether the putative HIF binding site is capable of binding HIF using electrophoretic mobility shift assay (Fig. 2B). On incubation of nuclear extracts with ³²P-labeled probe (5'-AACCGCGTGCGGTACG-3'), the protein-DNA complex is formed. This binding is specific to the putative HIF binding site in the Nur77 promoter, because the labeled probe can be competed with wild-type oligonucleotide but not with a mutant. Preincubation of nuclear extracts with anti-HIF-1 α antibody caused supershift of protein-DNA

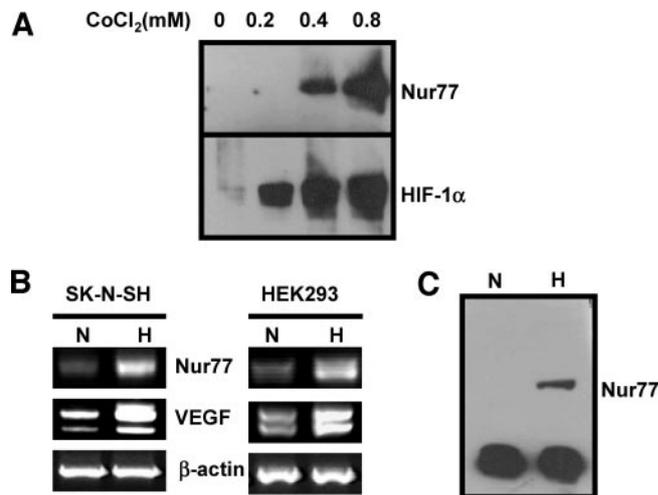


Fig. 1. Hypoxia induces Nur77 expression. A, cobalt chloride induces Nur77 and stabilizes hypoxia-inducible factor (HIF) -1 α . B, hypoxia activates Nur77 transcription. SK-N-SH cells and human embryonic kidney 293 cells were incubated under low-oxygen (1%) conditions overnight. Reverse transcription-PCR was performed using extracted total RNAs. C, hypoxia induces Nur77 protein expression. SK-N-SH cells were incubated under hypoxic conditions (1% oxygen) for 16 h. The cells were harvested, lysed, and immunoprecipitated with anti-Nur77 polyclonal antibody. Samples were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-Nur77 antibodies.

complex band, confirming that the protein bound to the probe contains HIF-1 α (Fig. 2B). We additionally confirmed that HIF-1 α and HIF-1 β bind to Nur77 promoter by incubation of the probe conjugated to biotin with nuclear extracts from cells that are transiently transfected with HIF-1 α and HIF-1 β (Fig. 2C). To determine whether the putative HIF binding site is essential for mediating Nur77 activation by HIF, we generated several mutants of the Nur77 promoter in front of the luciferase reporter gene and determined their response to HIF-1 α coexpression. Deletion (-206 or -100) or mutation [-250 (mut)], in which the consensus CGTG is converted to CATA in the HRE (5'-AACCGCGCATACGTACG-3') of the putative HIF-binding site, completely abrogates the response of the reporter gene to HIF-1 α overexpression (Fig. 2D). These results indicate that the putative HIF binding site binds to HIF and is capable of mediating the activation of Nur77 expression in RCC.

HIF-1 α , Not HIF-2 α , Is Responsible for the Induction of Nur77. To distinguish between the functional roles of HIF isoforms in Nur77 induction, we transiently transfected either HIF-1 α or HIF-2 α in SK-N-SH cells and detected transcription of Nur77 by reverse transcription-PCR. We had assumed that both of them could activate endogenous Nur77 expression. Surprisingly, only HIF-1 α increased Nur77 mRNA, although both of them increased VEGF mRNA (Fig. 3A). To additionally assess the specificity of HIF-1 α for Nur77 induction, we chose 786-O [HIF-1 α ^(-/-), HIF-2 α ^(+/+), and VHL^(-/-)] RCC, which constitutively expresses HIF-2 α alone, not HIF-1 α , to determine the level of Nur77 and other proteins. In 786-O RCC, we failed to detect Nur77 even after exposing cells to low oxygen stress, whereas we found high expression of VEGF (Fig. 3B) under normoxic and hypoxic conditions, implicating that HIF-2 α is not essential for induction of Nur77. We then generated stable cells (786-O/VHL), in which VHL expression is restored and HIF-2 α is degraded under normoxic conditions. Although VHL and HIF-2 α are expressed in 786-O/VHL, Nur77 cannot be induced under hypoxic stress. Expression of HIF-1 α restored the induction of Nur77 by hypoxic stress. Thus, HIF-1 α , rather than HIF-2 α , is specific for the HIF binding site in the Nur77 promoter.

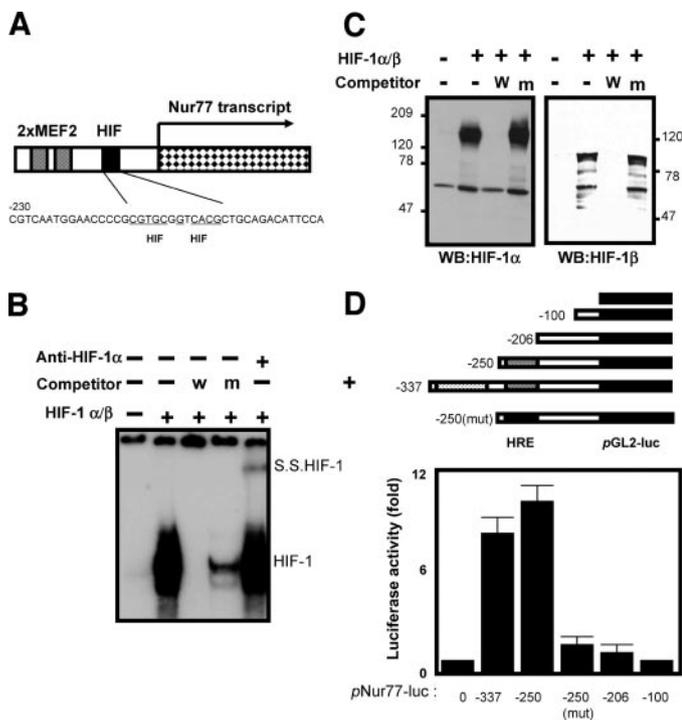


Fig. 2. Hypoxia-inducible factor (HIF) binds to the Nur77 promoter. **A**, the putative HIF-response element (HRE) in the Nur77 promoter. **B**, electrophoretic mobility shift assay for binding of HIF to the putative HRE in the Nur77 promoter. Nuclear extracts from cells that are transiently transfected with HIF-1α/β (pCMV-HIF-1α and pcDNA-HIF-1β) were incubated with ³²P-labeled HRE primers and subjected to 6% polyacrylamide gel; mobility was analyzed by autoradiography (w, wild-type cold probe; m, mutant cold probe). **C**, biotin-streptavidin pull-down assay. Biotinylated HRE probe was incubated with nuclear extracts from cells that are transiently transfected with HIF-1α/β (pCMV-HIF-1α and pcDNA-HIF-1β). Samples precipitated with streptavidin-agarose were subjected to SDS-PAGE and probed with either anti-HIF-1α or anti-HIF-1β antibody. **D**, the putative HRE in the Nur77 promoter is required for HIF-dependent reporter gene activation. SK-N-SH cells were transiently transfected with different reporter constructs under the control of the wild-type and deletion mutants of Nur77 promoter along with HIF-1α expression vector (pCMV-HA-HIF-1α).

Hypoxia-Inducible Nur77 Is Critical for Production of POMC in RCC. Because Nur77 is known to be a transcription factor involved in POMC expression, we investigated whether hypoxia also induces POMC in VHL^(+/+) cells. When we incubated cells overnight under 1% O₂, we could detect an increase in VEGF mRNA as expected. Under the same conditions, we also observed a significant increase in POMC and Nur77 mRNA (Fig. 4A). To determine whether Nur77 may be involved in the induction of POMC in RCC, we expressed transiently either c-myc-tagged full-length Nur77 or transactivation-deficient mutant of Nur77 and analyzed POMC mRNA. Expression of full-length Nur77 triggers a dramatic increase in POMC in 786-O RCC, whereas transactivation-deficient mutant did not induce POMC (Fig. 4B). To determine whether Nur77 directly mediates POMC transcription under hypoxic conditions, we transfected cells transiently with either antisense-Nur77 or sense-Nur77 oligonucleotide, and cultured them for 24 h before subjecting the cells to hypoxic conditions. Treatment with antisense-Nur77 results in significant decrease in POMC even under hypoxic conditions (Fig. 4C). Taken together, these results strongly suggest that Nur77 is necessary and sufficient for hypoxia-dependent transcription of POMC.

We next obtained normal and tumor tissues of kidney from a RCC patient, and detected POMC and Nur77 mRNA and other control markers using reverse transcription-PCR and Western blot analysis. The genomic analysis of VHL gene in this patient revealed that asparagine-90 is converted to isoleucine, nearer the NH₂ terminus, in the evolutionary conserved region of exon 1 (Fig. 5A). It has been

reported that this mutation (N90I) of pVHL is associated with tumors by losing the binding ability of VHL to HIF-1α, ultimately leading to tumor generation (23). We ascertained that this patient suffered from RCC because he has constitutive stabilization of HIF-1α and HIF-2α by Western blot analysis (Fig. 5B). In contrast to its normal tissue control, the tumor tissues produce abnormally high amounts of Nur77 and POMC in this patient (Fig. 5A).

DISCUSSION

In the present study, we identify a novel transcriptional regulatory mechanism that HIF-1α induces Nur77 mRNA and protein during hypoxic conditions. We found that HIF-1α directly binds to Nur77 promoter region between -214 and -203 where two HREs are adjacent to each other as reverse repeats, similar to HRE in mouse lactate dehydrogenase promoter (24, 25). Deletion or mutation of putative HRE site within Nur77 promoter dramatically decreases reporter gene activity, supporting the belief that HIF binds directly to the HRE within the Nur77 promoter.

HIF-1α is an important transcription factor for regulation of various gene expressions of angiogenesis, glucose metabolic enzymes, and oxygen-carrying proteins, all of which are critical for oxygen usage in

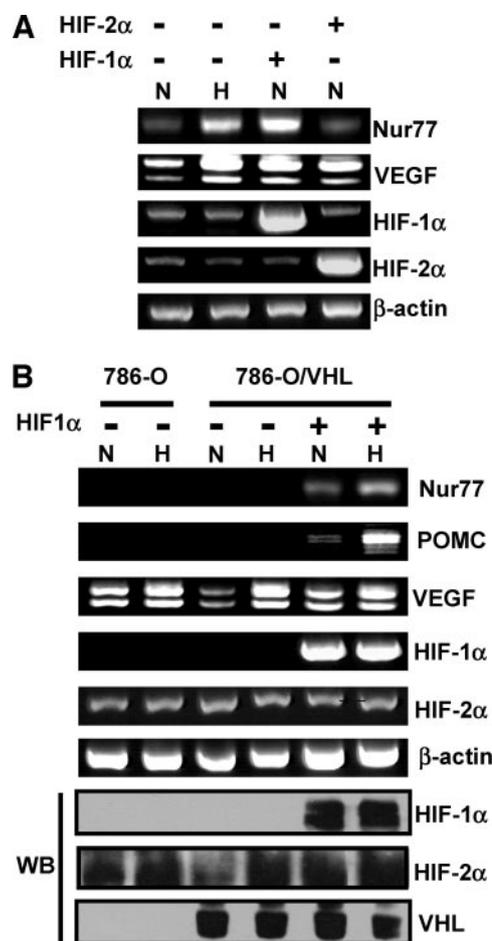


Fig. 3. Hypoxia-inducible factor (HIF) -1α, not HIF-2α, specifically induces the Nur77 expression. **A**, SK-N-SH cells were transiently transfected with either HIF-1α (pCMV-HIF-1α) or HIF-2α (pcDNA3-HIF-2α) expression vector. mRNA level of Nur77 and VEGF is analyzed by reverse transcription-PCR. **B**, 786-O RCC [VHL^(-/-), HIF-1α^(-/-)] or 786-O/HA-VHL [HIF-1α^(-/-)] stable transfectants were transiently transfected with HA-HIF-1α. mRNAs of proopiomelanocortin and Nur77 were detected by reverse transcription-PCR with the corresponding primers. The expression of VHL, HIF-1α, and HIF-2α was assessed by Western blot analysis with corresponding antibodies.

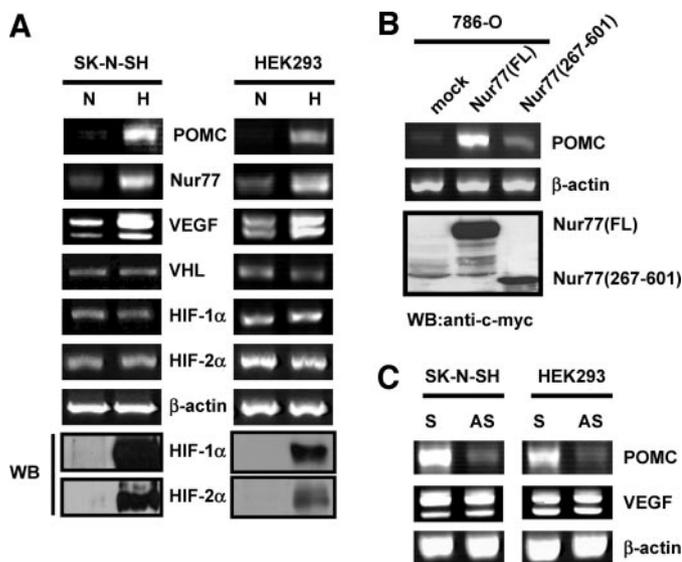


Fig. 4. Nur77 is necessary and sufficient for hypoxia-induced proopiomelanocortin (POMC) transcription. **A**, human embryonic kidney 293 cells and SK-N-SH cells were incubated under hypoxic conditions, and various mRNAs, including POMC, were detected using reverse transcription-PCR. **B**, 786-O renal cell carcinoma cells were transfected with expression vector for either c-myc-tagged full-length Nur77 (pMT-Nur77 [FL]) or Nur77 (267–601) (pMT-Nur77 [267–601]), and mRNA level of POMC was measured using reverse transcription-PCR. The expression of c-myc-tagged Nur77 (FL) and c-myc-Nur77 (267–601) was probed with anti-c-myc monoclonal antibody. **C**, antisense-Nur77 reduces the expression of POMC under hypoxic conditions. Human embryonic kidney 293 cells were transfected with sense or antisense-Nur77 oligonucleotides using Lipofectamine reagent (Life Technologies, Inc.). After a 24-h incubation, cells were grown under low-oxygen conditions. Levels of mRNA for POMC, vascular endothelial growth factor, and β -actin were determined by reverse transcription-PCR.

the human body. VHL mutation causes the constitutive stabilization of HIF-1 α , leading to malignant tumors in certain organs.

It was known previously that hypoxia activates hypothalamic-pituitary-adrenal axis, resulting in increase of ACTH and ACTH receptor (26, 27), suggesting that oxygen fluctuation may control the production of cellular hormone. However, little was known about the effect of oxygen tension on hormone homeostasis, despite the possibility that the hormone regulates metabolic homeostasis, proliferation, and differentiation in an oxygen concentration-dependent manner.

Nur77 is involved in regulation of various genes in the hypothalamic-pituitary-adrenal axis related to production of POMC, a precursor of ACTH, and enzymes related to steroid hormone synthesis (18, 28, 29). Nur77 serves as a main transcription factor to produce POMC in response to a physiologic stimulus, corticotrophin-releasing hormone (18). Mutation of Nur response element within the POMC promoter decreases markedly the POMC transcription in the pituitary tumor cell line (30).

Thus far, it has been reported that two types of signals are mainly involved in Nur77 induction. First, Nur77 induction in T cells is mainly mediated by calcium-activated myocyte enhancer factor-2D transcription factor during apoptosis of immature T cells (31). The mechanism by which calcium regulates myocyte enhancer factor-2D is through dissociation of calcium-sensitive myocyte enhancer factor-2 corepressors (Cabin1/HDACs and HDAC4/5), and the association with calcineurin-activated transcription factor nuclear factor of activated T cells and the coactivator p300 (32, 33). Second, activator protein superfamily of JunD is known to activate Nur77 transcription. Nerve growth factor and membrane depolarization activate Nur77 through binding of JunD in pheochromocytoma-derived cell line PC12 (34), and a calcium/calmodulin-dependent activation of extracellular signal-activated protein kinases 1/2 mediates JunD phosphorylation and induction of Nur77 by prostaglandin F $_2\alpha$ in ovarian cells (28). Moreover, the Tax viral transactivator induces Nur77 by activation of JunD through two AP1-like response elements (35).

In addition to these kinds of mechanisms known previously for induction of Nur77, we provide a third mechanism by which HIF regulates Nur77 transcription in this report. We demonstrate that HIF-1 α specifically induces Nur77 transcription. HIF- α subunit exists in isoforms HIF-1 α , HIF-2 α , and HIF-3 α . Among the three isoforms, HIF-1 α and HIF-2 α are subject to oxygen-dependent proteosomal degradation mediated by the pVHL (8). Interestingly, only HIF-1 α , not HIF-2 α , specifically induces Nur77 production. Neither Nur77 nor POMC was induced in 786-O RCC deficient in HIF-1 α under hypoxic conditions; on the contrary, overexpression of HIF-1 α in 786-O restored the induction of Nur77 and POMC. Despite the similarities between HIF-1 α and HIF-2 α , they differ in biological functions, because HIF-2 α has lower transactivation activity than HIF-1 α under hypoxic conditions, and its target genes are more restricted than those of HIF-1 α (36). Unlike HIF-1 α , DNA binding of HIF-2 α is regulated by a redox mechanism through unique cysteine residue within DNA binding domain (37). It has been reported that redundancy is limited between HIF-1 α and HIF-2 α because genetic ablation of either HIF- α isoform is lethal embryonically (38–41). Therefore, we suggest that HIF-1 α is necessary for the activation of Nur77 in VHL-mutated RCC.

We demonstrate that HIF-1 α -targeting Nur77 induces POMC transcription in VHL-mutated RCC by showing that treatment of Nur77 antisense oligonucleotide reduces the POMC transcription even under hypoxic conditions. HIF-1 α is constitutively active in RCC as a result of mutation in pVHL. If it can activate Nur77 transcription, which in turn can induce POMC, we predict that Nur77 and POMC should be expressed in RCC tissues but not in their normal counterpart in humans. We verify this prediction by confirming the expression of Nur77 and POMC from kidney tumor tissues from RCC patients.

In the present study, we provide for the first time molecular

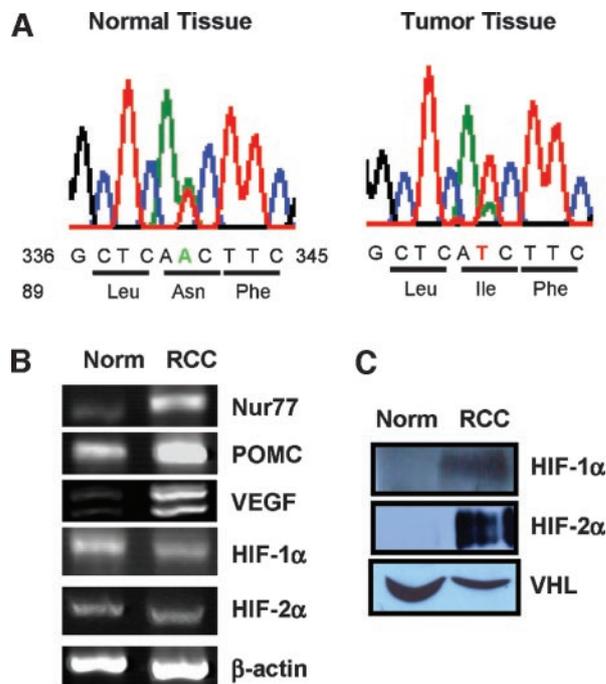


Fig. 5. Overproduction of proopiomelanocortin (POMC) and Nur77 in patients with renal cell carcinoma (RCC). **A**, mutation of von Hippel-Lindau (VHL) protein in a RCC patient. **B**, reverse transcription-PCR analysis for POMC and Nur77 expression. Normal and tumor tissues were obtained from kidney of a patient with RCC. Tissues were homogenized with TRIzol reagent (Life Technologies, Inc.), and total RNAs were extracted and used in reverse transcription-PCR. **C**, expression levels of hypoxia-inducible factor (HIF)-1 α , HIF-2 α , and VHL by Western blot analysis. Tissue lysates (100 μ g) or nuclear proteins (50 μ g) were loaded onto SDS-PAGE and transferred into nitrocellulose, and each of the proteins was detected with anti-VHL, anti-HIF-1 α , and anti-HIF-2 α antibodies.

evidence that HIF-1 α can control production of POMC, a peptide hormone precursor, whereas both of the HIF- α isoforms induce an angiogenic factor, VEGF, in VHL-mutated RCC patients.

However, it is still unclear whether POMC overproduction in VHL-mutated RCC is directly involved in Cushing's syndrome. Actually, we could not detect ACTH increase from either patient tumor tissues or hypoxia-incubated 786-O cell lines (data not shown), probably because RCC has less active proteases for cleavage of POMC into ACTH than pituitary gland cells because proteolytic processing is known to be inevitable to make active ACTH. Therefore, it is suggested that Cushing's syndrome only occurs by the continuous processing by overproduction, followed by cleavage of POMC, which also may be the reason why Cushing's syndrome occurs rarely in patients with RCC.

Furthermore, we will need to investigate whether VHL mutation cooperates with POMC proteases for increase of ACTH in RCC at the molecular level, and to find patients who have mutations of VHL and POMC protease for understanding the rarity of Cushing's syndrome in patients with RCC.

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