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Oxygen-Regulated β_2-Adrenergic Receptor Hydroxylation by EGLN3 and Ubiquitylation by pVHL

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Abstract

Agonist-induced ubiquitylation and degradation of heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors (GPCRs) play an essential role in surface receptor homeostasis, thereby tuning many physiological processes. Although β-arrestin and affiliated E3 ligases mediate agonist-stimulated lysosomal degradation of the β_2-adrenergic receptor (β_2AR), a prototypic GPCR, the molecular cues that mark receptors for ubiquitylation and the regulation of receptor degradation by the proteasome remain poorly understood. We show that the von Hippel–Lindau tumor suppressor protein (pVHL)–E3 ligase complex, known for its regulation of hypoxia-inducible factor (HIF) proteins, interacts with and ubiquitylates the β_2AR, thereby decreasing receptor abundance. We further show that the interaction of pVHL with β_2AR is dependent on proline hydroxylation (proline-382 and -395) and that the dioxygenase EGLN3 interacts directly with the β_2AR to serve as an endogenous β_2AR prolyl hydroxylase. Under hypoxic conditions, receptor hydroxylation and subsequent ubiquitylation decrease dramatically, thus attenuating receptor degradation and down-regulation. Notably, in both cells and tissue, the abundance of endogenous β_2AR is shown to reflect constitutive turnover by EGLN3 and pVHL. Our findings provide insight into GPCR regulation, broaden the functional scope of prolyl hydroxylation, and expand our understanding of the cellular response to hypoxia.

Introduction

β-Adrenergic receptors (βARs) are prototypic GPCRs that play an important role in the regulation of cardiovascular and pulmonary function, and sustained βAR down-regulation (and dysfunction) is associated with diseases such as heart failure and asthma (1,2). There are three subtypes of βAR (β_1, β_2, and β_3). The β_2AR constitutes about 25 to 30% of total βARs in the human heart and is the predominant subtype present in both vascular and airway smooth muscle (1,2). In contrast, the β_1AR predominates in the heart (~70%). Whereas β_1- and β_2ARs have

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overlapping cellular actions (1–4), β_2ARs may be especially important in the failing heart where the β_1AR is down-regulated. Indeed, overexpression or pharmacological stimulation, or both, of β_2ARs increases cardiac contractility and is cardioprotective in animal models (4–8).

Continuous agonist stimulation induces β_2AR ubiquitylation, internalization, and degradation (9), thereby decreasing the total number of receptors. Agonist-stimulated removal of receptors from the cell surface involves a pathway in which the adaptor protein β-arrestin binds phosphorylated receptors (after agonist stimulation) and recruits E3 ligases (in particular NEDD4) that subserve ubiquitin-mediated lysosomal degradation (9,10). Cues other than phosphorylation may also mark receptors for ubiquitylation, internalization, or both, and events that control proteasomal (as opposed to lysosomal) degradation of receptors remain a mystery. In particular, an E3 ligase responsible for proteasomal degradation of the β_2AR has not been identified, yet proteasomal inhibitors markedly reduce receptor internalization and degradation (under both resting conditions and after agonist stimulation) (9).

The abundance and balance of βAR subtypes is thought to be regulated primarily by adrenergic state. Thus, for example, the net decreased abundance of cardiac βARs that occurs in response to hypoxia has been attributed to elevated concentrations of catecholamines (11). However, whereas β_1AR abundance decreases under severely hypoxic conditions, consistent with heightened adrenergic tone, β_2AR abundance actually increases (12), raising the possibility that the β_2AR may be regulated directly by O_2. Metallo (iron)-sensory enzymes transduce O_2-responsive signals through the modification of target proteins with which they interact. Here, we report that the iron-containing dioxygenase EGLN3 serves as an O_2-dependent β_2AR prolyl hydroxylase. After hydroxylation of the β_2AR at Pro^{382} and Pro^{395}, the von Hippel–Lindau tumor suppressor protein (pVHL)–E3 ligase complex, previously characterized in the context of hypoxia-inducible factor (HIF) regulation, is recruited to and ubiquitylates the β_2AR, promoting its down-regulation by proteasomal degradation. Accordingly, hypoxia stabilizes the β_2AR.

Results

Hypoxia limits β_2AR ubiquitylation

The effect of oxygen tension (partial pressure of oxygen, PO_2) on β_2AR stability was examined with the use of human embryonic kidney (HEK) 293 cells stably expressing human β_2ARs that contain an N-terminal FLAG tag (β_2AR-293 cells). Cells were cultured under ambient PO_2 (21% O_2) or hypoxic conditions (1% O_2) in the presence or absence of the βAR agonist, isoproterenol (10 μM, 18 hours). Receptors were immunoprecipitated from whole-cell lysates with agarose beads conjugated to an antibody against FLAG (anti-FLAG) and Western blotted with an antibody against β_2AR (anti-β_2AR) to assess the total cellular abundance of β_2AR. Hypoxia substantially increased the abundance of β_2AR in the presence or absence of agonist (Fig. 1, A, B, and D). In addition, the half-life of the β_2AR was increased significantly by 1% compared to 21% O_2, as measured in pulse-chase experiments (without agonist treatment) after metabolic labeling of cells with ^{35}S[Met] and ^{35}S[Cys] (Fig. 1C).

HIFs coordinate transcriptional adaptation to hypoxia by binding to hypoxia-response elements (HREs) within many target genes. However, it is unlikely that the increased abundance of β_2AR in the β_2AR-293 cells is regulated by HIFs because FLAG-β_2AR expression was driven by a cytomegalovirus promoter that is devoid of HREs, and overexpression of a normoxia-stabilized HIF-1α (13) had no effect on β_2AR abundance (Fig. 2E and fig. S1). Although agonist-stimulated down-regulation of overexpressed β_2AR was largely unaffected by hypoxia in HEK cells (the decrease in β_2AR abundance in response to isoproterenol was ~50% at both 21 and 1% O_2; Fig. 1A), hypoxia inhibited receptor down-regulation by agonist under more

physiologically relevant conditions. Specifically, in human umbilical venous endothelial cells (HUVECs), hypoxia not only increased the endogenous β_2AR from 10 to 22 fmol/mg under basal conditions, but also inhibited isoproterenol-stimulated down-regulation (Fig. 1B). Thus, both constitutive and ligand-induced trafficking of the β_2AR may be regulated by PO_2 in primary cell lines.

GPCR endocytosis and degradation may involve both lysosomal and proteasomal mechanisms (9). We found that under steady-state conditions, degradation of the β_2AR was blocked by proteasome inhibitors but not by lysosome inhibitors (Fig. 1D). We then examined whether β_2AR ubiquitylation was regulated by PO_2. β_2AR-293 cells were cultured overnight at 21 or 1% O_2 in the presence of the proteasome inhibitor MG132, and immunoprecipitated receptors were Western blotted with antibodies against ubiquitin (anti-ubiquitin) or against β_2AR. The β_2AR was ubiquitylated under normoxic conditions in the absence of agonist stimulation, and the degree of ubiquitylation was dramatically decreased at 1% O_2 (Fig. 1E).

pVHL complex serves as the E3 ubiquitin ligase for the β_2AR

Agonist stimulation induces rapid ubiquitylation of both the β_2AR and the regulatory protein β-arrestin (9,10). Agonist-coupled ubiquitylation of the β_2AR requires receptor phosphorylation and subsequent binding of β-arrestin 2 (9,10); thus, β-arrestin 2 acts to recruit an E3 ligase to the β_2AR. However, the β-arrestins are primarily cytosolic in unstimulated cells, in which β_2AR ubiquitylation is prominent (Fig. 1E), and constitutive trafficking of the β_2AR also occurs (14). Therefore, we considered the possibility that ubiquitylating enzymes may interact directly with the β_2AR to regulate constitutive trafficking of the receptors. The pVHL complex is an O_2-responsive E3 ligase for HIF-1α (15), and we hypothesized that it might also serve as an E3 ligase for the β_2AR. We first conducted immunofluorescence and coimmunoprecipitation experiments in both β_2AR-293 and HEK293 cells to determine whether pVHL and the β_2AR interact. Although pVHL, both overexpressed and endogenous, was broadly distributed throughout the cell, pVHL colocalized with β_2ARs associated mainly with cellular membranes (Fig. 2A and fig. S2A). Additionally, both overexpressed and endogenous pVHL coimmunoprecipitated with β_2AR both in resting cells and after treatment with isoproterenol (Fig. 2B and fig. S2B). Moreover, endogenous elongin C and cullin-2, the components of the native pVHL complex (16), were also present in β_2AR immunoprecipitates (Fig. 2B), and the direct interaction between the β_2AR and pVHL complex was confirmed by far-Western blot (fig. S2C). Collectively, these findings suggest that the pVHL–E3 ligase complex associates with the β_2AR in cultured cells.

We performed an in vitro ubiquitylation assay to determine whether the pVHL–E3 ligase complex directly ubiquitylated β_2AR. FLAG-pVHL was transiently overexpressed in HEK293 cells, immunoprecipitated, and mixed with purified recombinant β_2AR in a cell-free ubiquitylation system. As revealed by Western blotting with anti-β_2AR antibody, native receptor migrated at ~55 kD (monomer) or 110 kD (dimer) (Fig. 2C). Multiple smeared bands of higher molecular weight, representing polyubiquitylated β_2ARs, appeared only when immunoprecipitate from cells expressing pVHL was added to the in vitro system (Fig. 2C). In vitro ubiquitylation of the β_2AR was verified by Western blotting with an antibody against ubiquitin (anti-ubiquitin) (Fig. 2D) and by control experiments in which omitting the E1 ubiquitin-activating enzyme from the in vitro assay completely eliminated the ubiquitin signal (Fig. 2D). Silencing small interfering RNA (siRNA) studies provided further evidence that the pVHL complex serves as the endogenous E3 ligase, because knockdown of endogenous pVHL in HUVECs resulted in a significant increase in the abundance of endogenous β_2AR (Fig. 2E and fig. S2E).

The role of pVHL in O_2-dependent ubiquitylation of the β_2AR was further corroborated by studies in pVHL-deficient 786-O cells in which the stabilizing effect of hypoxia on the β_2AR

was absent (Fig. 2F). Introduction of pVHL restored O_2 responsivity and thereby decreased β_2AR abundance in normoxia relative to 1% O_2 (Fig. 2F). Additionally, β_2AR ubiquitylation was greatly increased at 21% O_2 and inhibited at 1% O_2, and this effect was dependent on pVHL (Fig. 2F). These findings suggest that, as is the case for HIF-1α, the pVHL complex is an E3 ligase for the β_2AR.

Hydroxylation of the β_2AR at proline residues P^{382} and P^{395}

HIF is a dimer of α and β subunits, with the α subunit regulated by oxygen and the β subunit constitutively present (17). Oxygen dependence is mediated by a family of enzymes (EGLN 1 to 3) that catalyze hydroxylation of specific prolyl residues within the α subunits of HIF (18,19). The hydroxylated α subunit is then targeted by pVHL for ubiquitylation and proteasomal degradation under normoxic conditions. Hypoxia decreases HIF-1α prolyl hydroxylation, which is required for the interaction between pVHL and HIF-1α (20,21), and thus stabilizes HIF. We noted that hypoxia similarly decreases the interaction between pVHL and the β_2AR (Fig. 3, A and B, and fig. S2D), raising the possibility that proline hydroxylation might also mediate the interaction of β_2AR with pVHL. Proline hydroxylation of HIF-1α occurs at a conserved motif L[XX]LAP (18,22). Although a substitution of flanking Leu or Ala residues has little effect on HIF-1α hydroxylation (23), an acidic amino acid at the +5 position (relative to Pro) seems to be important (24). There are three Pro residues within intracellular domains of the β_2AR and acidic amino acids are found downstream of two of them (Pro^{382} and Pro^{395}). Mutation of these two proline residues ($\beta_2AR^{P382A/P395A}$) decreased the interaction between receptor and pVHL relative to that of wild-type β_2AR (Fig. 3B). In addition, exposing cells expressing the proline mutant receptor to hypoxia or treatment with dimethyloxalyglycine (DMOG), a broad-spectrum prolyl hydroxylase inhibitor, failed to increase the abundance of $\beta_2AR^{P382A/P395A}$ (Fig. 3C).

To verify directly hydroxylation of the β_2AR, the receptor was purified from β_2AR-293 cells (cultured at 21 or 1% O_2) with alprenolol-Sepharose affinity resin, digested with trypsin, and then analyzed by liquid chromatography–mass spectrometry (LC-MS). A +16-dalton mass shift, indicative of hydroxylation, was identified for a peptide corresponding to β_2AR(376–404), which contains P^{382} and P^{395}, but does not contain any free Cys thiol or Met, thus excluding *S*-oxidation (Fig. 3D). MS/MS analysis showed that both proline residues could be hydroxylated in normoxia (Fig. 3E and fig. S3); hydroxylation was not detected under hypoxic conditions. Collectively, these data suggest that either or both prolines are hydroxylated.

EGLN3 interacts with the β_2AR and promotes receptor hydroxylation

Considering the similarities between the O_2-dependent regulation of HIF-1α and the β_2AR, we examined whether the HIF prolyl hydroxylase family called EGLN might also serve as a hydroxylase for the β_2AR. EGLN2 is a nuclear protein and thus not likely to hydroxylate β_2AR, whereas both EGLN1 and EGLN3 are cytosolic (25). In HEK293 cells, EGLN3, both overexpressed (Fig. 4A) and endogenous (Fig. 4F), but not EGLN1 or 2, specifically coimmunoprecipitated with the β_2AR; agonist stimulation did not affect this interaction (fig. S4A). The C-terminal double-stranded β-helix (DSBH) fold is the defining structure of the 2OG dioxygenase superfamily (26) and this structure is highly conserved among the EGLN proteins, which are 86% identical and share about 98% similarity. Therefore, it would not be expected that the β_2AR would interact with this DSBH domain of EGLN3 without similarly binding EGLN1 and 2. To further delineate the region of EGLN3 responsible for β_2AR binding, we made a set of truncated EGLN3 glutathione *S*-transferase (GST) fusion proteins and found that only GST-$EGLN3^{(73\text{-}116)}$ could pull down recombinant β_2AR (fig. S4, B and D). Additional deletion experiments confirmed that residues 73 to 116 of EGLN3 are required for interaction with the β_2AR (fig. S4C). Further deletion experiments showed that EGLN3 residues from 88 to 104 were sufficient and required for its interaction with the β_2AR (Fig. 4,

B and C). The two hydroxylated prolines are located in the C-terminal tail of the β_2AR, and the GST-β_2AR C-terminal tail [GST-β_2AR(330–413)] pulled down EGLN3 from cell lysates (Fig. 4D). We then developed a peptide based on human EGLN3(89–104) and a mutant peptide EGLN(4R) with four residues changed to Arg, which served as a control (see Materials and Methods for details). Only peptide EGLN3(89–104), but not EGLN(4R), competed with recombinant EGLN3 for binding to GST-β_2AR(330–413) (Fig. 4E). Both peptides were efficiently taken up by β_2AR-293 cells (fig. S4E). However, only peptide EGLN3(89–104), but not EGLN3(4R), significantly increased the cellular abundance of the β_2AR (Fig. 4G). Moreover, EGLN3(89–104) did not affect HIF-1α abundance (fig. S4F). Coimmunoprecipitation experiments further confirmed that endogenous EGLN3 associated with β_2AR and their interaction was eliminated by peptide EGLN3(89–104) (Fig. 4F).

Finally, we assessed the functional role of EGLN3 as a β_2AR hydroxylase in cells and animals. Depletion of endogenous EGLN3 (but not EGLN1) with siRNA markedly increased the abundance of β_2AR in β_2AR-293 cells under normoxic conditions (Fig. 5A). Thus, the O_2-dependent turnover of the β_2AR can be associated specifically with EGLN3 activity. Capture of pVHL has been used as a surrogate assay for HIF-1α hydroxylation (18); we developed a similar strategy to assay for hydroxylation of the β_2AR. Immobilized GST-β_2AR(330–413) was incubated with [^{35}S]pVHL produced by in vitro transcription and translation either directly or after incubation with lysates from HEK293 cells. The amount of pVHL trapped by the C terminus of the β_2AR was increased by exposure to lysates (Fig. 5B), consistent with the interpretation that cellular extracts exhibit β_2AR hydroxylase activity. To identify this activity directly with EGLN3, we examined with matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry a GST-β_2AR(330–413) that had been exposed either to native HEK293 cell lysates or to lysates depleted of EGLN3 with siRNA. Notably, the β_2AR(376–404) peptide exposed to native lysate exhibited a +16-dalton shift in mass indicative of hydroxylation, and this modification was largely eliminated by knockdown of EGLN3 with siRNA (Fig. 5C). Thus, β_2AR is a substrate for EGLN3.

To support a role for hydroxylation in regulation of β_2AR abundance in vivo, we compared βAR abundance in wild-type and EGLN3-knockout mice. The abundance of β_2AR in hearts from EGLN3-knockout mice was significantly increased compared to that from wild-type littermates. In contrast, we observed a minor decrease in β_1AR abundance (Fig. 5D). Thus, the classic predominance of β_1AR over β_2AR in the heart reflects the constitutive turnover of the β_2AR by endogenous EGLN3. Together with the demonstration that endogenous β_2AR is regulated by endogenous pVHL (Figs. 1B and 2E), these studies make a compelling case for physiological relevance of the regulation of βAR abundance by oxygen-responsive EGLN3 and pVHL.

Discussion

The cellular response to physiological concentrations of O_2 is characterized by sequential actions of EGLN and pVHL–E3 ligase complex that destabilize HIF protein, a master regulator of hypoxic genes. Hydroxylation of proline residues in HIF-1α promotes binding of the pVHL ubiquitylation complex, which then targets HIF-1α for proteasomal degradation. Thus, under resting O_2 concentrations, HIF is negatively regulated by O_2-dependent hydroxylation (mediated by EGLN) and ubiquitylation (mediated by pVHL). Interestingly, RNA polymerase II and collagen IV were recently shown to interact with pVHL in a prolyl hydroxylase–dependent manner (27,28), raising the possibility that pVHL may have additional substrates (29). However, physiological roles for these interactions have not been well demonstrated. Thus, whether the EGLN and pVHL system might serve more broadly in hypoxic signaling has remained an open question. The present findings suggest that both constitutive and agonist-stimulated GPCR abundance can be controlled by the O_2-regulated EGLN and pVHL system

and, in particular, that the β_2AR is a physiological substrate of EGLN3 and the pVHL–E3 ligase complex. Our data highlight a transcription-independent role for the EGLN and pVHL system, which mediates the influence of O_2 on receptor-mediated cellular responsiveness.

The type 2 form of von Hippel–Lindau disease (a hereditary cancer syndrome caused by inactivating mutations of von Hippel–Lindau tumor suppressor gene) is characterized by the presence of pheochromocytomas, a neuroendocrine tumor of the adrenal glands, and that accordingly, type 2 patients often show excessive secretion of catecholamines (endogenous β_2AR ligands) and increased sympathetic nervous system activity (16). It is of interest that mice deficient in EGLN3 also display an adrenergic phenotype characterized by alterations in blood pressure and cardiac contractility (30). β_2AR dysregulation resulting from type 2 mutations might contribute to the symptomology and etiology of von Hippel–Lindau disease, as well as to the EGLN3$^{-/-}$ phenotype.

All mammalian EGLN isoforms can hydroxylate HIF in vitro, but only EGLN1 regulates HIF-1α abundance in vivo (31,32). Whether EGLN proteins exhibit functional redundancy or play distinct roles is largely unknown (25,33). Our finding that only EGLN3 binds and regulates the β_2AR and that the interaction is mediated through the N-terminal region, which is highly variable among EGLN isoforms, suggests that each isoform may have unique substrates.

EGLN3 is most abundant in cardiac and smooth muscle (33,34), where the β_2AR is highly abundant in vivo. βAR activation increases cardiac contractility and relaxes vascular and airway smooth muscle, and βAR dysregulation may be broadly involved in the pathogenesis of cardiovascular and airway diseases, including heart failure, hypertension, and asthma (2, 35,36). Notably, the β_2AR, in particular, enhances bronchodilation and alveolar fluid clearance (which increase O_2 uptake), enhances cardiac output and peripheral vasodilation (which increase O_2 delivery), and enhances cardioprotection and angiogenesis under ischemic conditions (35–39), thereby effectively recapitulating the integrated physiological response to hypoxia. Thus, our results showing up-regulation of the β_2AR in response to hypoxia puts the function of the receptor in new light. The ability of the EGLN-pVHL hydroxylation and ubiquitylation pathway to regulate the β_2AR and the implications of that regulation for the response to ischemia and hypoxia suggest previously unidentified targets in the treatment of cardiovascular and respiratory diseases. In addition, our findings may implicate age-dependent increases in *EGLN3* expression, observed in both humans and mice (40), in multiple degenerative diseases.

Materials and Methods

Cell culture and mouse tissue

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). 786-O cells were maintained in RPMI 1640 with 10% FBS. HUVECs were cultured in EBM-2 medium with 10% FBS supplemented with EGM-2 SingleQuots Kit. PO_2 was controlled by incubating cells at 37°C in humidified, O_2-CO_2–regulated incubators (Sanyo) adjusted to 5% CO_2 and the indicated PO_2 (balanced by N_2), or in a standard CO_2-controlled incubator maintained at 5% CO_2–95% air. EGLN3 knockout mice were described previously (41). The hearts of knockout mice and their wild-type littermates at 6 weeks old were removed and perfused with cold 0.9% saline. Hearts were then flash-frozen in liquid nitrogen and stored at −80°C.

siRNA and EGLN peptides

ON-TARGET plus siRNAs for suppressing human VHL (J-003936-09-0020, J-003936-10-0020) were purchased from Thermo Scientific. siRNA duplexes designed to

suppress the expression of human EGLN1 (sense, 5′-CAAGGUAAGUGGAGGUAUAUU-3′; antisense, 5′-UAUACCUCCACUUACCUUGUU-3′; GenBank accession number, EGLN1 NM_022051) and EGLN3 (sense, 5′-GCAAAUACUACGU-CAAGGAUU-3′; antisense, 5′-UCCUUGACGUAGUAUUUGCUU-3′ and sense, 5′-UUCAGGAAUUUAACUAGGAUU-3′; antisense, 5′-UCCUAGUUAAAUUCCUGAAUU-3′; GenBank accession number, EGLN3 NM_022073) and scrambled siRNA control were purchased from Dharmacon. HEK293 cells at 40 to 50% confluency were transfected with siRNAs (50 nM) by exposure to lipofectamine 2000 (Invitrogen) for 48 hours. HUVECs were electrotransfected by a Bio-Rad GenePulser Xcell2 for 72 hours. The effects of suppression were validated by Western blotting with antibodies that recognized pVHL (BD Biosciences), EGLN1, or EGLN3 (Novus-Biologicals). Fluorescein isothiocyanate (FITC)– labeled peptide derived from human EGLN3(89–104) (EAISFLLSLID-RLVLY) or mutant peptide EGLN3(4R) (EARSFLRSLIRRLRLY) with four amino acids in EGLN3(89–104) mutated to Arg were purchased from EZBiolab.

Pulse-chase analysis

β_2AR-293 cells were radiolabeled in Met-Cys–free medium supplemented with [^{35}S]Met] and [^{35}S]Cys (100 μCi/ml) for 12 hours at 21 or 1% O_2. Cells were washed with DMEM containing excess cold Met and Cys (3 mM), then cells were chased in complete DMEM medium plus excess cold Met and Cys (3 mM) at 21 or 1% O_2. FLAG-β_2AR was immunoprecipitated from the cell lysates and resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography.

In vitro ubiquitylation assay

Recombinant β_2AR (2 μg) reconstituted in phospholipid vesicles (9) was used as the substrate. pVHL–E3 ubiquitin ligase complex was immunoprecipitated with anti-FLAG beads (Sigma) from HEK293 cells transfected with FLAG-pVHL (cells transfected with empty vector as the control) and eluted with FLAG peptide (Sigma). The reactions were carried out at 30°C for 60 min in buffer containing 50 mM tris (pH 7.4), 2 mM adenosine triphosphate–Mg^{2+}, 2 mM dithiothreitol (DTT), and 2.5 mM $MgCl_2$ supplemented with 250 ng of human recombinant E1 (BostonBiochem), 500 ng of human recombinant UbcH5b (Boston-Biochem), and 10 μg of ubiquitin (BostonBiochem).

In vitro hydroxylation and pVHL capture assay

To assay hydroxylation in vitro, 5 μg of GST-β_2AR(330–413) captured on glutathione (GSH)-Sepharose beads (Sigma) were incubated with cell lysates from HEK293 cells (transfected with siRNA for EGLN3 or scrambled siRNA) in hypotonic extraction buffer [HEB: 20 mM tris (pH 7.5), 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT] supplemented with 2 mM ascorbate, 0.2 mM 2-oxoglutarate, and 0.1 mM Fe^{2+} at 30°C for 60 min; this was repeated twice more with fresh cell lysate. The beads were then washed three times with 20 mM ammonium bicarbonate (pH 8.0) and digested with trypsin (Trypsin Gold; Promega) at 37°C for 16 hours. Proline hydroxylation was confirmed by MALDI-TOF (Applied Biosystems, Model 4700).

To assay pVHL capture, GST-β_2AR(330–413) was mixed with [^{35}S]pVHL produced by in vitro transcription and translation, either directly or after incubation with HEK293 cell extract as above. The mixture was incubated with rotation in binding buffer [50 mM tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and 10% glycerol] for 60 min at 4°C. Bound [^{35}S]pVHL was eluted and analyzed by SDS-PAGE and autoradiography.

EGLN3 binding to β_2AR: GST pull-down assay and coimmunoprecipitation

Complementary DNA (cDNA) corresponding to EGLN3 residues 1 to 36, 37 to 72, 73 to 116, 89 to 104, or 222 to 239 or β_2AR(330–413) residues were amplified by polymerase chain reaction and cloned into pGEX-2T vector. GST or GST fusion proteins were purified from *Escherichia coli* with GSH-Sepharose (Sigma). GST or GST fusion proteins (5 μg) were incubated overnight with equal amounts of β_2AR (from β_2AR-293) or EGLN3 (derived from transiently transfected HEK293 cells or recombinant protein from SF9 cells) at 4°C in 0.5 ml of binding buffer [50 mM tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, and 10% glycerol]. Thirty microliters of 50% GSH-Sepharose slurry was added to the mixture followed by rotation for another hour at 4°C. Beads were then washed five times with 1 ml of binding buffer. Proteins were separated by SDS-PAGE and analyzed by Western blot. For coimmunoprecipitations of β_2AR and/or EGLNs, cells were lysed [50 mM tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 10% glycerol, and protease inhibitor cocktail] and lysates were clarified by centrifugation at 14,000*g*. After addition of 1 to 2 μg of antibody, lysates were incubated with rotation overnight at 4°C and for an additional hour after the addition of 25 μl of 50% protein A or G agarose. Beads were washed five times with lysis buffer and proteins were analyzed by Western blotting.

Far-Western analysis

One microgram of recombinant β_2AR reconstituted in lipid vesicles or empty vesicles was separated by nonreducing SDS-PAGE and either immunoblotted with anti-β_2AR or incubated with 100 nM recombinant pVHL–elongin C–elongin B complex (VCB) (42) in phosphate-buffered saline (PBS), 0.1% Tween-20, and 5% milk. Bound VCB was detected by anti-pVHL.

Immunofluorescence and confocal microscopy

Immediately after removal from the incubator, cells were washed with PBS and fixed and permeabilized in phosphate-buffered 2% paraformaldehyde and 0.2% Triton X-100 for 30 min at 4°C. Immunofluorescence labeling was performed with mouse anti-FLAG M2 (1:500 dilution; Sigma) or anti-pVHL (BD Biosciences) followed by tetramethyl rhodamine isothiocyanate (TRITC)–conjugated goat secondary antibody against mouse (Jackson ImmunoResearch Laboratories). Images were acquired by confocal laser-scanning microscopy (LSM5 Pascal; Carl Zeiss, Inc.).

Radioligand binding

Membranes of HUVECs cultured under 21 or 1% O_2 and membrane preparations from hearts of wild-type or EGLN3-knockout mice were prepared, and ^{125}I-labeled cyanopindolol binding was performed in triplicate in the presence or absence of the antagonist propranolol or cGP-20712A as described previously (9,43).

Liquid chromatography–tandem mass spectrometry analysis

Human β_2AR was purified from stably overexpressing HEK293 cells with alprenolol-Sepharose affinity resin, as described previously (9). After reduction with DTT (10 mM, 37°C, 30 min) and alkylation with iodoacetamide (20 mM for 30 min at room temperature in the dark), purified receptors were digested with trypsin in solution at a working concentration of 5 ng/μl. Digested peptides were purified by Stage tip chromatography, lyophilized, and reconstituted in 5% acetonitrile–5% formic acid. Liquid chromatography–tandem mass spectrometry (LC– MS/MS) experiments were performed on a hybrid linear quadrapole ion trap/FT-ICR (LTQ FT) mass spectrometer (Thermo Electron, San Jose, CA) equipped with a Finnigan Nanospray II electrospray ionization source (Thermo Electron), an Agilent 1100 Series binary high-performance liquid chromatography (HPLC) pump (Agilent Technologies, Palo Alto, CA), and a Famos autosampler (LC Packings, San Francisco, CA). Peptide mixtures

were loaded onto a 125-μm inside diameter fused-silica microcapillary column packed in-house with C18 reversed-phase resin (Magic C18AQ; particle size, 5 μm; pore size, 200 Å; Microsom Bioresources, Auburn, CA), and separation was achieved with a 75-min gradient at a flow rate of 300 nl/min provided across a flow splitter by HPLC pumps. The LTQ-FT mass spectrometer was operated in the data-dependent mode with the FT10 strategy (44). In brief, a scan cycle was initiated with a full survey scan of high mass accuracy [mass/charge ratio (*m/z*), 350 to 1700) in the FT-ICR mass spectrometer. This was followed by MS/MS scans in the linear ion trap of the 10 most abundant ions in the survey scan, with dynamic exclusion of previously selected ions. Singly charged ions were excluded from MS/MS analysis.

Database search for MS/MS

Instrument control and primary data processing were carried out with the Xcalibur software package, Version 1.4 SR1 (Thermo Electron). Raw data were converted to the mzXML format and in-house Perl scripts were used to identify the charge state and the monoisotopic *m/z* for peptide ions chosen for MS/MS analysis. This information was used to generate .dta files that were subjected to SEQUEST search analysis against a small database containing the human β_2AR. Searches were performed with the following parameters: full-trypsin specificity, a mass tolerance of 1.1 Da, static modifications of oxidized Met (+15.9949), carboxyamidomethylated Cys (+57.0215), and dynamic modifications of hydroxylated Pro (+15.9949). All peptide matches were filtered by XCorr, mass accuracy, and dCn′ (defined as the normalized difference between XCorr values of the top-ranked candidate peptide and the next candidate with a different amino acid sequence).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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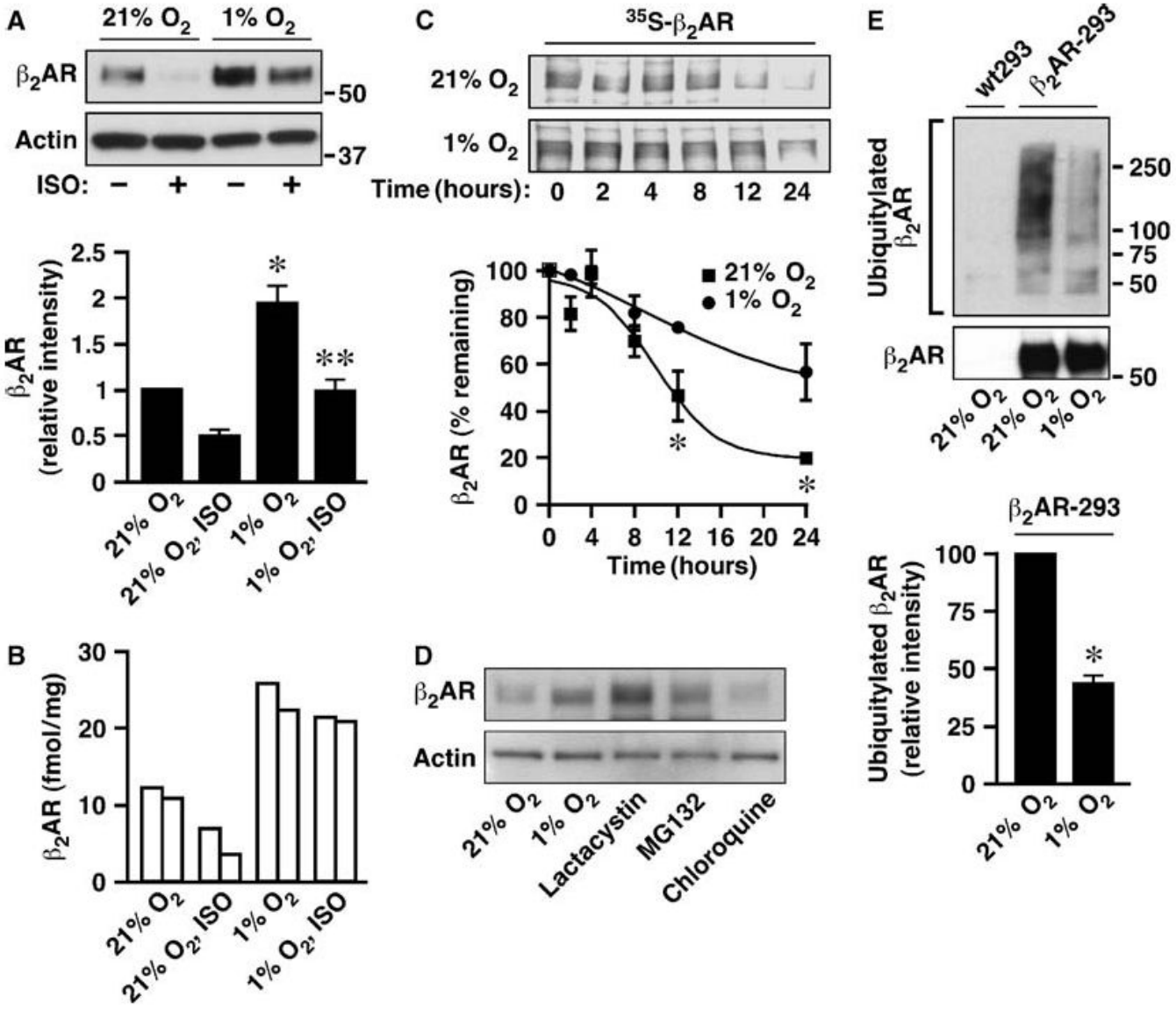

Fig. 1.

Hypoxia stabilizes the β_2AR by inhibiting its ubiquitylation. (**A**) FLAG-tagged β_2AR was isolated with anti-FLAG beads from β_2AR-293 cells cultured under 21 or 1% O_2 with or without 10 μM isoproterenol (ISO) for 18 hours, and Western blotted with anti-β_2AR antibody. Actin was used as the loading control. $n = 3$ (n = the number of independent experiments, here and elsewhere), $^*P < 0.01$ versus 21%, $^{**}P < 0.05$ versus 21% + ISO (Student's t test). **(B)** HUVECs were cultured under 21 or 1% O_2 with or without 10 μM ISO for 18 hours. Cell membranes were collected and β_2AR density was measured by radioligand binding ($n = 2$; each bar represents the result of an individual experiment). (**C**) β_2AR-293 cells were metabolically labeled with ^{35}S[Met] and ^{35}S[Cys] for 12 hours followed by a chase period of the indicated durations carried out at 21 or 1% O_2. FLAG-β_2AR was immunoprecipitated from the cell lysates and signals were detected by SDS-PAGE followed by autoradiography. $n = 3$, $^*P < 0.05$ versus 1% by analysis of variance (ANOVA). (**D**) β_2AR-293 cells were treated with MG132 (10 μM), lactacystin (20 μM), or chloroquine (20 μM) for 18 hours. Receptor was collected with anti-FLAG beads and Western blotted with anti-β_2AR. **(E)** β_2AR was collected

with anti-FLAG beads from β_2AR-293 cells cultured at 21 or 1% O_2 with MG132 (10 μM) for 18 hours and Western blotted with anti-ubiquitin or anti-β_2AR. $n = 3$, $^*P < 0.01$ versus 21% (Student's *t* test). In (A), (C), and (E), densitometric analysis (mean ± SEM) was performed.

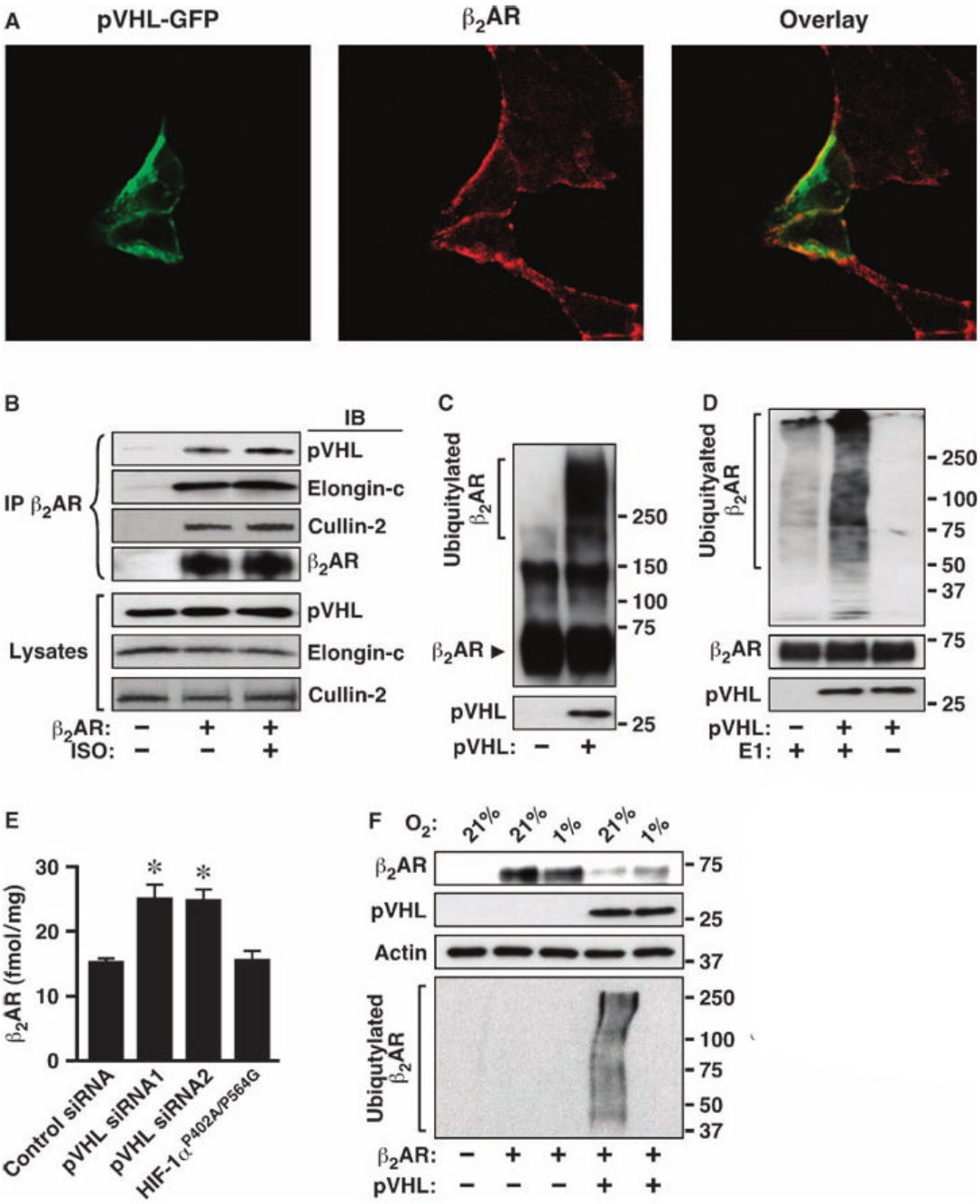

Fig. 2.

The pVHL complex associates with the β_2AR and serves as an E3 ubiquitin ligase. (**A**) β_2AR-293 cells were transiently transfected with pVHL-GFP, fixed, and stained with anti–FLAG M2 (β_2AR) and TRITC-conjugated secondary antibody (red). **(B)** FLAG-pVHL and β_2AR or pcDNA3 were cotransfected into HEK293 cells. ISO stimulation was for 15 min. Cell lysates were immunoprecipitated (IP) with anti-β_2AR and Western blotted (IB) with anti-β_2AR, anti-FLAG (for pVHL), anti–cullin-2, or anti–elongin-C as indicated. (**C** and **D**) In vitro ubiquitylation assays were performed for recombinant β_2AR (150-kD dimer in C is reflective of high recombinant protein concentration) in the presence or absence of pVHL complex or E1 ubiquitin-conjugating enzyme. Samples were Western blotted with anti-β_2AR (C) or anti-

ubiquitin (D). Bottom panels show the pVHL input. (**E**) HUVECs were electrotransfected with scrambled siRNA, siRNAs for human VHL, or plasmid expressing HIF-1$\alpha^{P402A/P564G}$ for 72 hours. Cell membranes were collected and β_2AR density was measured by radioligand binding. $n = 3$, $^*P < 0.01$ versus scrambled siRNA (Student's *t* test). (**F**) β_2AR was transfected into 786-O or 786-O–pVHL cells; after 24 hours, cells were incubated for an additional 18 hours at 21 or 1% O_2. Proteins were Western blotted with specific antibodies as indicated. The illustrated pVHL-dependent effects of PO_2 are representative of three experiments, but cotransfection with pVHL had variable effects on the overall abundance of transfected β_2AR in 786-O cells.

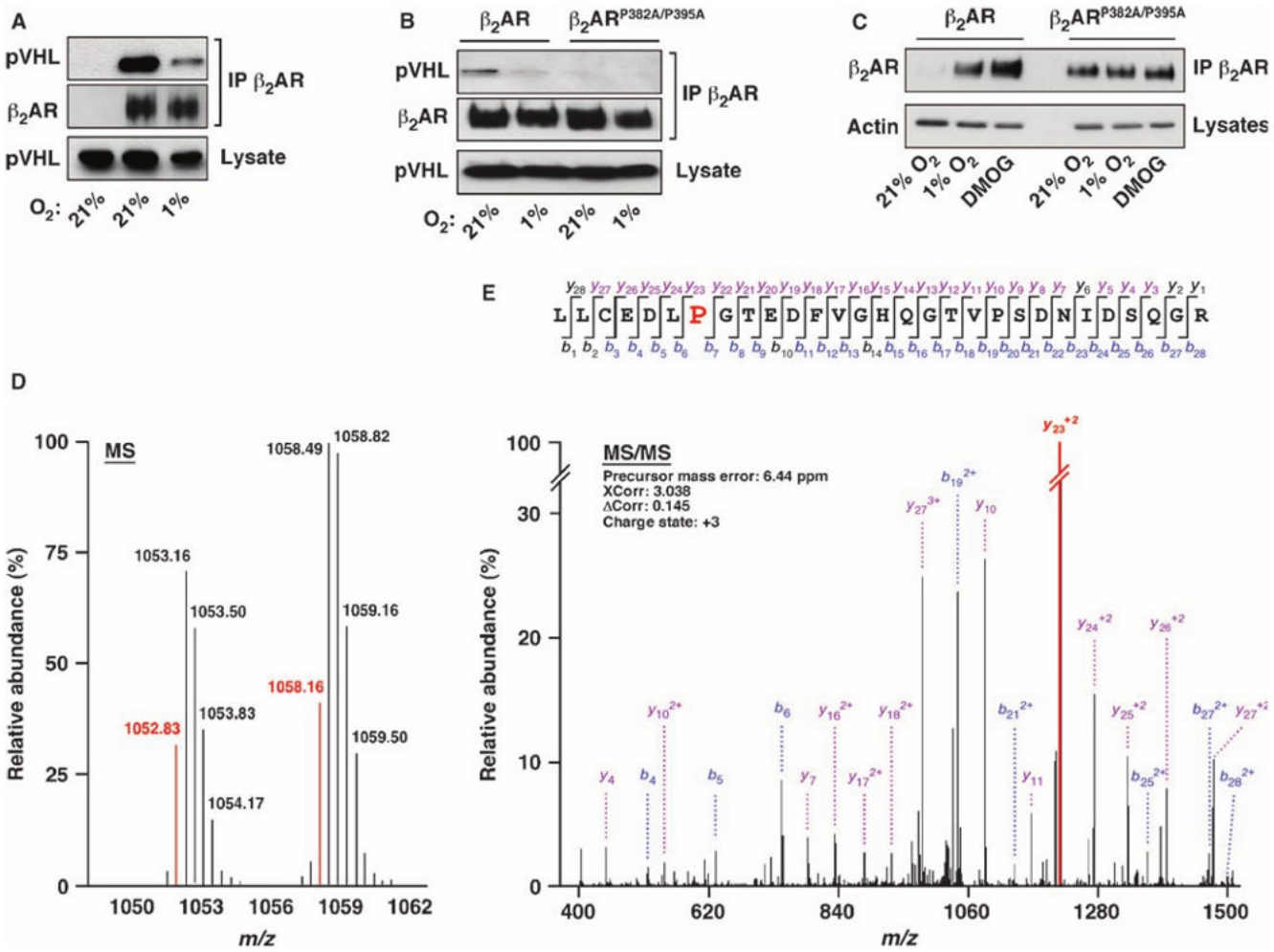

Fig. 3.

The β_2AR is hydroxylated at proline residues P^{382} and P^{395}. (**A** and **B**) FLAG-pVHL and β_2AR [(A) and (B)] or $\beta_2AR^{P382A/P395A}$ (B) were cotransfected into HEK293 cells; in (A), the leftmost lane shows the result of control transfection with pcDNA3 rather than pcDNA3-β_2AR. After 24 hours, cells were incubated for an additional 18 hours at either 21 or 1% O_2 in the presence of 10 μM MG132. Cell lysates were immunoprecipitated with anti-β_2AR and Western blotted with anti-FLAG (for pVHL) or anti-β_2ARs. (**C**) HEK293 cells stably expressing FLAG-β_2AR or FLAG-$\beta_2AR^{P382A/P395A}$ were incubated at 21 or 1% O_2 or treated with 1 mM DMOG for 18 hours at 21% O_2. β_2AR or $\beta_2AR^{P382A/P395A}$ was isolated with anti-FLAG beads and Western blotted with anti-β_2AR antibody. Actin was used as loading control. (**D**) FLAG-β_2AR purified from β_2AR-293 cells cultured at 21% O_2 was digested with trypsin and analyzed by LC-MS. Monoisotopic precursor ions (shown in red) at *m/z* = 1052.83 and 1058.16 correspond to $[M+3H]^{+3}$ of the unhydroxylated and hydroxylated LLCEDLPGTEDFVGHQGTVPSDNIDSQGR peptides, respectively. (**E**) Tandem mass spectrum of the precursor ion at *m/z* = 1058.16. Large red "P" indicates a hydroxylated proline residue. The peak heights are the relative abundances of the corresponding fragment ions, with the annotation of the identified matched N terminus–containing ions (b ions) in blue and C terminus–containing ions (y ions) in magenta. For clarity, only the major identified peaks are labeled (a complete table of fragment ions for this spectrum is presented in fig. S3B).

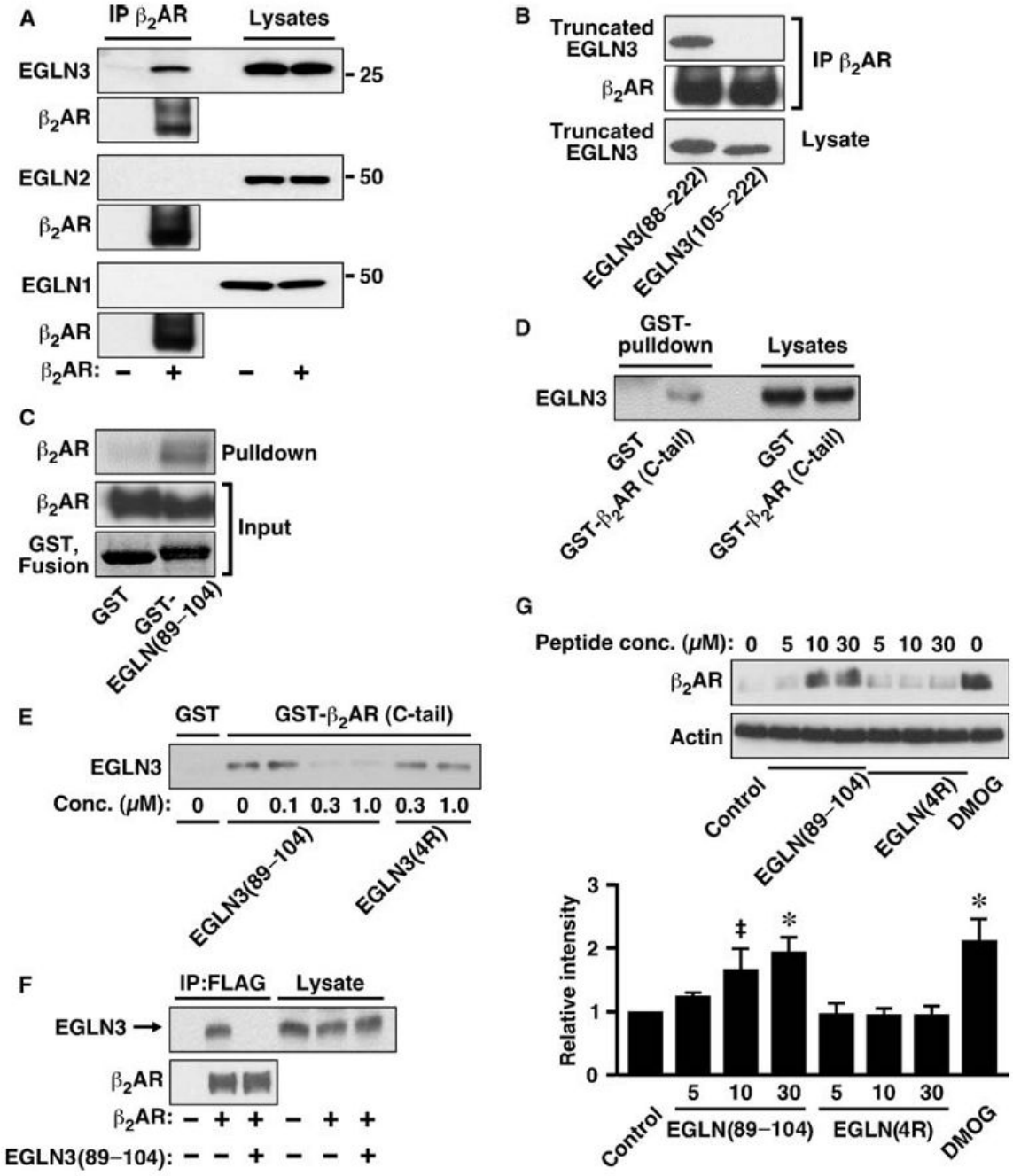

Fig. 4.

EGLN3 interacts specifically with the β_2AR. (**A**) HEK293 cells were cotransfected with FLAG-EGLN1, 2, or 3 and with β_2AR or pcDNA3 control. Cell lysates were immunoprecipitated with anti-β_2AR and Western blotted with anti-β_2AR or anti-FLAG (for EGLNs). (**B**) β_2AR-293 cells were transfected with truncated FLAG-EGLN3 constructs. Cell lysates were immunoprecipitated with anti-β_2AR and Western blotted with anti-FLAG or anti-β_2AR. (**C**) GST or truncated GST-EGLN3(89–104) fusion protein (5 μg) were incubated with an equal amount of β_2AR before GST pulldown. Pulldowns and inputs were separated by SDS-PAGE and Western blotted with anti-β_2AR or stained with Coomassie blue (for GST or the fusion protein). (**D**) GST or GST-β_2AR(330–413) (GST-β_2AR-c-tail) (5 μg) were used to pull

down EGLN3. Pulldowns were Western blotted with anti-EGLN3 antibody. (**E**) 100 nM recombinant EGLN3 and 100 nM GST or GST-β_2AR-c-tail were incubated in the presence of different concentrations of FITC-labeled peptide derived from human EGLN3(89–104) or mutant peptide EGLN3(4R). GST or GST fusion were then pulled down and Western blotted with anti-EGLN3. (**F**) HEK293 cells were transfected with pcDNA3 or FLAG-β_2AR and treated with 30 μM EGLN3(89–104) peptide as indicated for 4 hours. Cell lysates were immunoprecipitated with anti-FLAG beads and Western blotted with anti-EGLN3 or anti-β_2AR as indicated. (**G**) β_2AR-293 cells were treated with different concentrations of EGLN3 (89–104) and EGLN3(4R) mutant peptides or 1 mM DMOG as indicated for 18 hours. β_2ARs were collected by anti-FLAG beads and Western blotted with anti-β_2AR. Actin was the loading control. Densitometric analysis (mean ± SEM) was performed; $n = 3$, $^{*}P < 0.01$ versus control, $^{+}P < 0.05$ versus control (Student's *t* test).

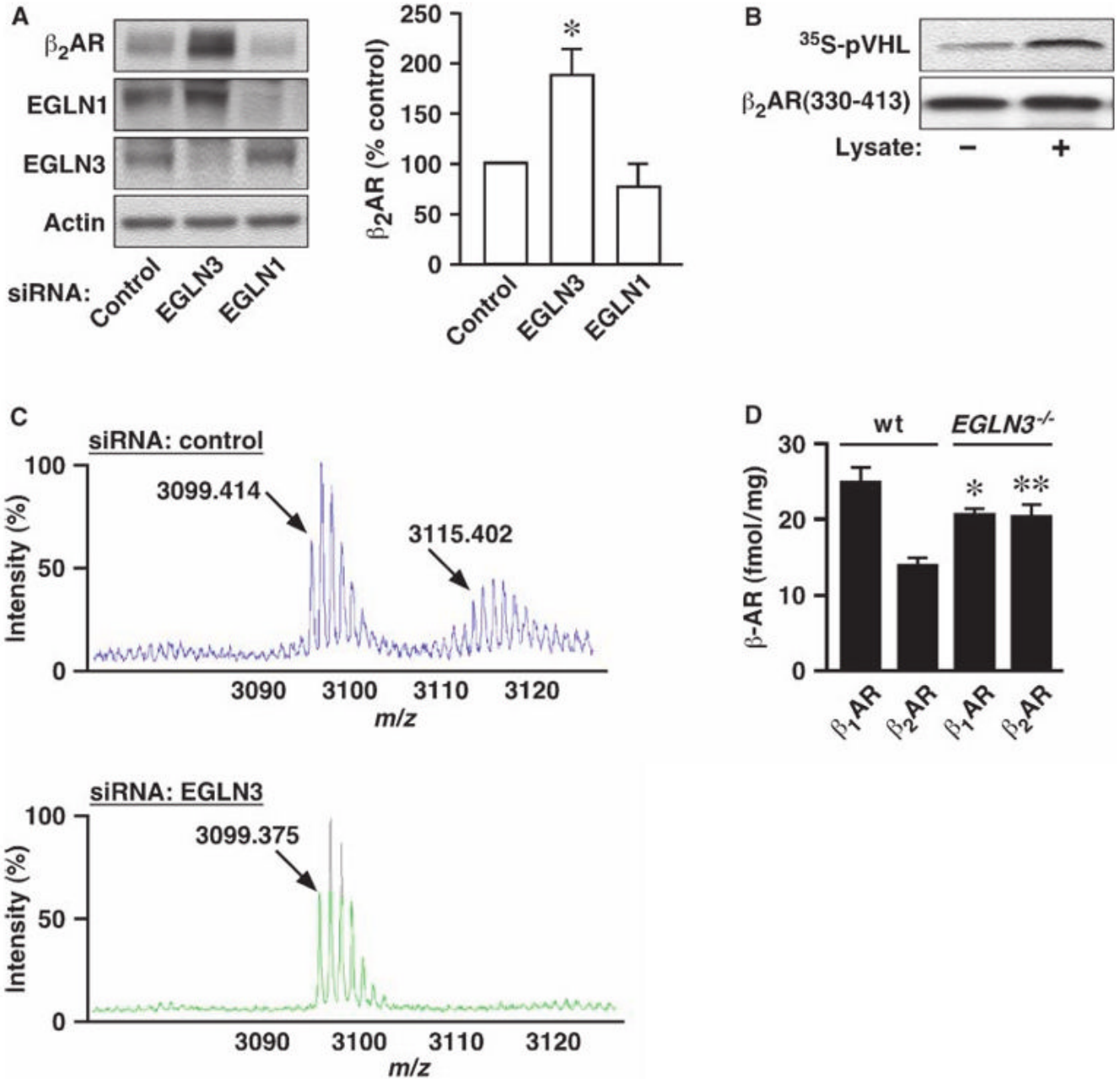

Fig. 5.

EGLN3 serves as an endogenous hydroxylase for the β_2AR. (**A**) β_2AR-293 cells (expressing FLAG-β_2AR) were transfected with EGLN1 or EGLN3 siRNA for 48 hours, and cell lysates were Western blotted with anti-β_2AR, anti-EGLN1, anti-EGLN3, and anti-actin. Densitometric analysis (mean ± SEM) was performed. $n = 3$, $^*P < 0.01$ versus siRNA-scramble (control). (**B**) GST-β_2AR(330–413) immobilized on GSH-Sepharose was incubated with [^{35}S]pVHL (60 min, 4°C) either directly or after three 60-min incubations with fresh HEK293 cell lysate. The beads were eluted and the eluants were analyzed by SDS-PAGE and autoradiography. The bottom panel shows the inputs of GST-β_2AR(330–413). (**C**) Immobilized GST-β_2AR(330–413) was incubated with lysates from HEK293 cells transfected with siRNA to EGLN3 or control (scrambled) siRNA and digested with trypsin (37°C, 16 hours). Digests were analyzed

by MALDI-TOF. Peaks at *m/z* = 3099.414 or 3099.375 and 3115.402 correspond to unhydroxylated and hydroxylated LLCEDLPGTEDFVGHQGTVPSDNIDSQGR peptides, respectively. **(D)** βARs densities on membranes derived from hearts of wild-type (wt) and EGLN3 knockout mice were measured by radioligand binding. $n = 7$, $^{*}P < 0.02$ versus wild type and $^{**}P < 0.01$ versus wild type (Student's *t* test).