

Prolyl hydroxylase 2: a novel regulator of β_2 -adrenoceptor internalization

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Abstract

Adrenergic receptor (AR)-mediated signalling is modulated by oxygen levels. Prolyl hydroxylases (PHDs) are crucial for intracellular oxygen sensing and organism survival. However, it remains to be clarified whether or how PHDs are involved in the regulation of β_2 -adrenoceptor (β_2 -AR) signalling. Here we show that PHD2 can modulate the rate of β_2 -AR internalization through interactions with β -arrestin 2. PHD2 hydroxylates β -arrestin 2 at the proline (Pro)¹⁷⁶, Pro¹⁷⁹ and Pro¹⁸¹ sites, which retards the recruitment of β -arrestin 2 to the plasma membrane and inhibits subsequent co-internalization with β_2 -AR into the cytosol. β_2 -AR internalization is critical to control the temporal and spatial aspects of β_2 -AR signalling. Identifying novel regulators of β_2 -AR internalization will enable us to develop new strategies to manipulate receptor signalling and provide potential targets for drug development in the prevention and treatment of diseases associated with β_2 -AR signalling dysregulation.

Keywords: prolyl hydroxylase • β_2 -adrenoceptor • internalization • β -arrestin 2 • hydroxylation

Introduction

The β_2 -adrenoceptor (β_2 -AR), a member of the seven-transmembrane receptor family, is encoded by a gene on chromosome 5 and widely distributed in the respiratory and cardiovascular systems [1]. Because of their involvement in a large number of physiolog-

ical and pathological processes, β_2 -AR has been subject to intensive investigation and represents an important target for current pharmacological intervention. Prolonged stimulation of β_2 -AR often results in its internalization into endosomes to prevent β_2 -AR overstimulation [2]. Furthermore, β_2 -AR internalization is also involved in the regulation of receptor signalling, including signal termination and propagation and receptor re-sensitization [3, 4].

Prolyl hydroxylases (PHDs) belong to the superfamily of 2-oxoglutarate-Fe (II)-dependent dioxygenases. They act as important oxygen sensors *in vivo* and respond to many intracellular signals, including reactive oxygen species, nitric oxide and certain metabolites [5]. To date, three PHD isoforms have been identified, which have distinct tissue distributions and roles in mammalian

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cells. Previous studies reveal that PHD2 inactivation severely disrupts normal murine embryonic development, resulting in embryonic lethality, whereas PHD1^{-/-} and PHD3^{-/-} mice are still viable [6]. PHD1^{-/-} or PHD3^{-/-} mice do not display apparent erythropoiesis defects; however, conditional somatic inactivation of PHD2 causes polycythemia in mice, similar to hypomorphic PHD2 mutations in human beings [7, 8]. These evidence implicate that PHD2 as playing a crucial role in basal physiological conditions.

β_2 -AR abundance, sympathetic nerve activity and catecholamine release have been reported to be modulated by oxygen state [9, 10]. Because PHDs are crucial oxygen sensors that transmit O₂-responsive signals through the modification of target proteins [11], we postulated that PHDs may play an important role in the regulation of β_2 -AR signalling. In the present study, we mainly investigated the role of PHDs in β_2 -AR internalization.

Materials and methods

Plasmids and plasmid construction

The β -arrestin 1 and β -arrestin 2 plasmids were generously provided by Dr. Robert J. Lefkowitz (Duke University, Durham, NC, USA). The PHD1, PHD2 and PHD3 plasmids were provided by Dr. Felix Oehme (Bayer Schering Pharma AG, Berlin, Germany). The GRK2 (G protein-coupled receptor kinases), GRK5 and GRK6 plasmids were from Dr. Jose R. Naranjo (Spanish National Research Council, Madrid, Spain). The enhanced green fluorescent protein (pEGFP-C1) plasmid was obtained from Clontech (Mountain View, CA, USA). The pcDNA 3.0 and pcDNA 3.0-EGFP plasmids were purchased from Invitrogen (Carlsbad, CA, USA). The PHD2 cDNA was generated by PCR and cloned into pET-32a (Novagen, Madison, WI, USA) for bacterial expression with an N-terminal thioredoxin-6 histidine tag. The β -arrestin 2 cDNA was cloned into pGEX 4T3 vector (Amersham, Piscataway, NJ, USA) for bacterial expression with an N-terminal glutathione *S*-transferase (GST) fusion tag. The β -arrestin 2-GFP, PHD2 Δ MYND domain (myeloid, Nervy, and DEAF-1) and PHD2 Δ CD plasmids were constructed using basic molecular cloning techniques as described in the Supporting Information.

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells stably expressing β_2 -AR (β_2 -AR-293 cells) were provided by Dr. Gang Pei (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China). The cells were maintained in DMEM supplemented with 10% foetal bovine serum (Gibco, Grand Island, NY, USA). Transient transfection was performed with Lipofectamine 2000 (Invitrogen). Cardiomyocytes were obtained through primary isolation from one-day-old Sprague-Dawley rats, and the transfection was conducted through lentivirus-mediated gene delivery. The lentiviral vectors were constructed shown in the Supporting Information.

Receptor internalization assay

The β_2 -AR internalization assay was performed as previously described [12]. Briefly, β_2 -AR-293 cells or cardiomyocytes were stimulated with

freshly prepared isoproterenol (ISO, 10 μ M; Sigma-Aldrich, St. Louis, MO, USA) for the indicated time. After washing with ice-cold phosphate-buffered saline, the cells were incubated with anti- β_2 -AR antibody (1:1000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 hr, washed, and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Invitrogen) for 1 hr. Receptor internalization was defined as the fraction of total cell surface receptors removed from the plasma membrane after agonist treatment.

Small interfering RNA (siRNA) preparation and transfection

To screen for the effective siRNAs, candidate siRNA sequences for each PHD enzyme were selected as previously described [13, 14]. Effective siRNA sequences were chemically synthesized with two deoxythymidine nucleotide 3' overhangs and PAGE-purified (Ambion, Austin, TX, USA). siRNAs targeting PHD1 mRNA (GenBankTM accession number NM_053046) correspond to nucleotides 835–855 and 1471–1489; those targeting PHD2 (GenBankTM accession number NM_022051) correspond to nucleotides 3901–3921 and 4077–4097 and those targeting PHD3 (GenBankTM accession number NM_022073) correspond to nucleotides 671–691 and 715–735. The control used in these experiments was SilencerTM negative control siRNA (Ambion), a sequence provided by the manufacturers that targets nucleotides 2558–2578 of the *D. melanogaster* hypoxia-inducible factor-1 α (HIF-1 α) homolog, Sigma (GenBankTM accession number U 43090), which has no significant homology to any mammalian gene. Cells were seeded at 30% confluence 24 hrs prior to transfection. Lipofectamine 2000 (Invitrogen) was used to transfect β_2 -AR-293 cells with 20 nM siRNA duplex twice at 24 hr intervals unless otherwise stated. The efficacy of the siRNA transfection in each experiment was ascertained by Western blots.

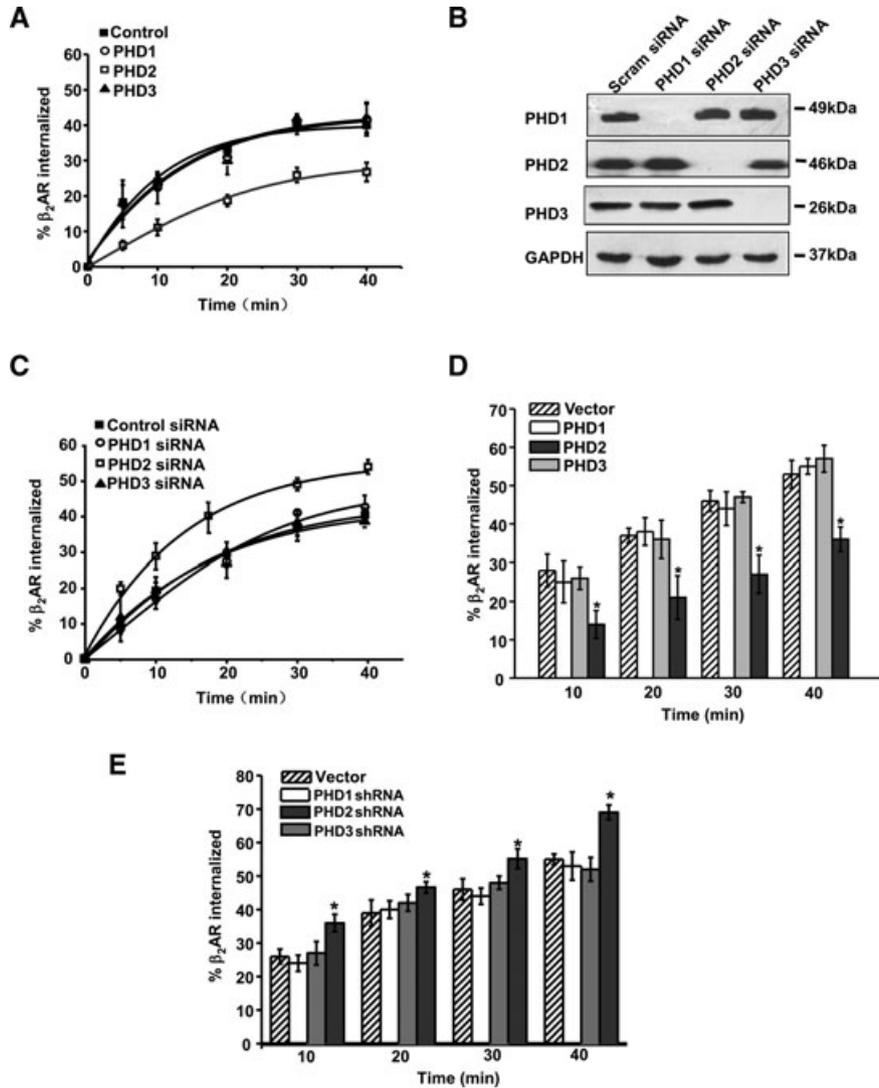
Coimmunoprecipitation

β_2 -AR-293 cells were cotransfected with polyhistidine tagged (His-tagged) PHD2 with FLAG (peptide sequence N-DYKDDDDK-C)-tagged β -arrestin 1, β -arrestin 2, GRK2, GRK5 or GRK6 for 48 hrs. For coimmunoprecipitations, these cells were resuspended in the lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 0.5% NP-40, 10% glycerol and protease inhibitor cocktail) for 30 min. For pre-clearing, the supernatant was supplemented with 5 μ g IgG and 25 μ l of protein G-Sepharose 4 Fast Flow (GE Healthcare, Piscataway, NJ, USA) and incubated at 4°C for 1 hr on a rotary incubator. The supernatant was recovered by centrifugation at 6000 \times *g* for 5 sec. at 4°C. Then 10 μ g antibodies were added to the supernatant, and the mixture was incubated for 6 hrs at 4°C on a rotary incubator. Next, 25 μ l of protein G-Sepharose 4 Fast Flow was added, and the mixture was incubated for an additional 3 hrs. The beads were washed five times by resuspension in the lysis buffer. Finally, 40 μ l 2 \times SDS sample buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 40% [vol/vol] glycerol, 0.01% bromophenol blue, 100 mM dithiothreitol) was added to elute the protein on the beads during incubation at 95°C for 3 min. The eluted proteins were analysed by SDS-PAGE and Western blots.

β -arrestin 2-GFP recruitment assay

β_2 -AR-293 cells were transfected with β -arrestin 2-GFP with or without the PHD2 plasmid. After 48 hrs of expression, the cells were stimulated with

Fig. 1 PHD2 regulates the rate of β_2 -AR internalization. **(A)** Overexpression of PHD2, but not PHD1 or PHD3, inhibits β_2 -AR internalization. β_2 -AR-293 cells were transfected with PHD1, PHD2, PHD3 or the vector (pcDNA3.0) plasmid. Forty-eight hours after transfection, these cells were stimulated with ISO (10 μ M) for the indicated time to determine β_2 -AR internalization as described in 'Materials and methods'. Internalized receptors are considered to be those removed from the plasma membrane after agonist treatment. **(B)** β_2 -AR-293 cells were transfected with PHD1, PHD2, PHD3 or scramble (Scram) siRNA for 48 hrs. The expression of each PHD isoform was determined by Western blots. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control. **(C)** PHD2 silencing promotes β_2 -AR internalization. Endogenous PHD expression in β_2 -AR-293 cells was silenced by specific siRNA. Forty-eight hours after transfection, β_2 -AR was stimulated with ISO (10 μ M) for the indicated time to conduct a β_2 -AR internalization assay. **(D)** Neonatal rat cardiomyocytes were infected with PHD1, PHD2, PHD3 or vector virus (control) for 48 hrs, and then these cells were stimulated with ISO for the indicated time to determine β_2 -AR internalization. **(E)** Neonatal rat cardiomyocytes were infected with PHD1, PHD2, PHD3 shRNA-lentiviral constructs or vector virus (control), and then these cells were stimulated with ISO for the indicated time for β_2 -AR internalization assay. The data are shown as the means \pm S.E.M. of representative experiments performed at least three times in triplicate. Asterisk (*) denotes a value that is significantly different from controls ($P < 0.05$).



the receptor agonist (ISO, 10 μ M) for various times. Finally, these cells were fixed, permeabilized and stained for β_2 -AR. A confocal microscope (A1R, Nikon, Tokyo, Japan) was used to observe the relative locations between β -arrestin 2 and β_2 -AR.

Mass spectrometry analysis of β -arrestin 2 hydroxylation

β -arrestin 2 was purified from β_2 -AR-293 cells and digested with trypsin at a working concentration of 5 ng/ml. Digested peptides were purified by stage tip chromatography, lyophilized and reconstituted in 5% acetonitrile-5% formic acid for liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Thermo-Finnigan LTQ-XL, San Jose, CA, USA). Raw data were subjected to search analysis using proteomics software SEQUEST against a small database containing human β -arrestin 2.

Statistical analysis

Each experiment was repeated at least three times. Statistical significance was determined using a paired Student's t-test. Differences were considered statistically significant at $P < 0.05$.

Results

PHD2, but not PHD1 or PHD3, regulates β_2 -AR internalization

To determine the role for PHDs in the regulation of β_2 -AR internalization, PHD1, PHD2, PHD3 or the vector plasmid was

transfected into β_2 -AR-293 cells, respectively. After 48 hrs of expression, these cells were treated with ISO (10 μ M) to activate β_2 -AR. In PHD1- or PHD3-overexpressing cells, ISO stimulation resulted in the loss of surface receptors with a $t_{1/2}$ of approximately 8 min., similar to the cells expressing β_2 -AR only (Control). In contrast, PHD2 overexpression resulted in a dramatic decrease in β_2 -AR internalization rate ($t_{1/2}$ of approximately 12 min.; Fig. 1A) in response to receptor activation, suggesting a substantial role of PHD2 in the regulation of β_2 -AR internalization.

To further confirm this finding, we evaluated the effect of endogenous PHDs on β_2 -AR internalization by silencing PHD1, PHD2 or PHD3 expression. Figure 1B shows that PHD1, PHD2 and PHD3 expression were substantially reduced in the cells transfected with siRNAs specific to each isoform but not with the control siRNA. PHD2 silencing accelerated β_2 -AR internalization in response to agonist stimulation, whereas PHD1 or PHD3 silencing had no influence on β_2 -AR internalization (Fig. 1C). In Fig. S1, the confocal images clearly reveal that the rate of β_2 -AR internalization was greater in PHD2-silenced cells than untreated cells or PHD2-overexpressing cells after 40 min. of receptor activation.

To assess whether the observation could be extended to other cell lines, we also performed similar studies in neonatal rat cardiomyocytes that constitutively express β_2 -ARs. The result revealed that PHD2, but not PHD1 or PHD3, overexpression resulted in an obvious decrease in ligand-induced β_2 -AR internalization (Fig. 1D). In contrast, PHD2 down-regulation could obviously accelerate β_2 -AR internalization, whereas PHD1 or PHD3 down-regulation did not affect the rate of β_2 -AR internalization (Fig. 1E). Taken together, all of the results suggest that PHD2 plays an inhibitory role in the regulation of β_2 -AR internalization.

The CD domain of the PHD2 protein is required for the regulation of β_2 -AR internalization

PHD2 contains an N-terminal MYND-type Zn²⁺ finger domain from residues 21 to 58 and a catalytic domain from residues 205 to 391 [15]. To identify the region responsible for β_2 -AR internalization, we generated a series of GFP-tagged PHD2 plasmids, including full-length PHD2, a MYND domain deletion-mutant (PHD2 Δ MYND) and a catalytic domain deletion mutant (PHD2 Δ CD) as shown in Fig. 2A. The PHD2 Δ MYND and PHD2 Δ CD mutant proteins were mainly distributed in the cytosol (Fig. 2B), similar to the cellular distribution of full-length PHD2 [16]. PHD2 Δ MYND overexpression retarded β_2 -AR internalization as efficiently as full-length PHD2 overexpression. In contrast, PHD2 Δ CD overexpression had no effect on β_2 -AR internalization upon receptor stimulation (Fig. 2C), suggesting that the CD domain is required for the regulation of β_2 -AR internalization.

β_2 -AR internalization is regulated through PHD2- β -arrestin 2 interaction

Subsequently, we began to explore the mechanism of PHD2 regulation of β_2 -AR internalization. Previous studies revealed that decreased oxygen availability contributes to the stability of β_2 -AR in response to agonist stimulation. β_2 -AR stability is tightly associated with β_2 -AR responsiveness and also affects the rate of β_2 -AR internalization [17]. Thus, we first estimated the levels of β_2 -AR expression during our experiments. Western blots analysis revealed that PHD2 expression did not result in any change in β_2 -AR expression (Fig. S2). Moreover, no treatment caused HIF-1 α induction (Fig. S3), a hypoxia indicator, implicating that all of the experiments were performed in the normoxic state and that changes in β_2 -AR internalization are not mediated by the actions of HIF-1 α but are conferred by PHD2. In addition, consistent with previous study [11], β_2 -AR-PHD2 association was not detected in β_2 -AR-293 cells through coimmunoprecipitation experiments (Fig. 3A). Collectively, these results suggest that PHD2 may affect the downstream effectors of β_2 -AR signalling.

β_2 -AR internalization is mediated by receptor phosphorylation and β -arrestin translocation [17]. β_2 -ARs are phosphorylated by GRKs. Then, β -arrestin 1 and 2 bind to the phosphorylated β_2 -AR on the plasma membrane and co-internalize with β_2 -AR into the cytosol [18]. The GRK subtypes, GRK2, 5 and 6, have been reported to participate in the regulation of β_2 -AR internalization [19]. However, we did not detect PHD2-GRKs association irrespective of agonist addition (Fig. S4). When PHD2 was co-expressed with β -arrestin 1 or β -arrestin 2, PHD2 was found to be immunoprecipitated by β -arrestin 2 but not β -arrestin 1 (Fig. 3B). β -arrestin 2 specifically pulled down PHD2 Δ MYND but not PHD2 Δ CD (Fig. 3C). Moreover, we detected an endogenous association between PHD2 and β -arrestin 2 in β_2 -AR-293 cells (Fig. 3D) and neonatal rat cardiomyocytes (Fig. 3E).

The endogenous PHD2- β -arrestin 2 association prompted us to determine whether it was a direct interaction. For this purpose, we generated and purified recombinant PHD2 and β -arrestin 2 proteins. GST pull-down assays indicated that PHD2 could interact with β -arrestin 2 under cell-free conditions (Fig. 3F), suggesting that PHD2 directly interacts with β -arrestin 2. Taken together, these results reveal that PHD2 interacts with β -arrestin 2 via the CD domain.

PHD2 hydroxylates β -arrestin 2, thereby affecting β_2 -AR internalization

PHD2 is an important oxygen sensor that modifies the corresponding target proteins through its hydroxylase activity [5]. Hypoxia condition or dimethylxalylglycine (DMOG) treatment resulted in the inhibition of PHD activity, and in turn obviously accelerated β_2 -AR internalization compared with the cells cultured under normoxic conditions, suggesting that PHD activity plays a

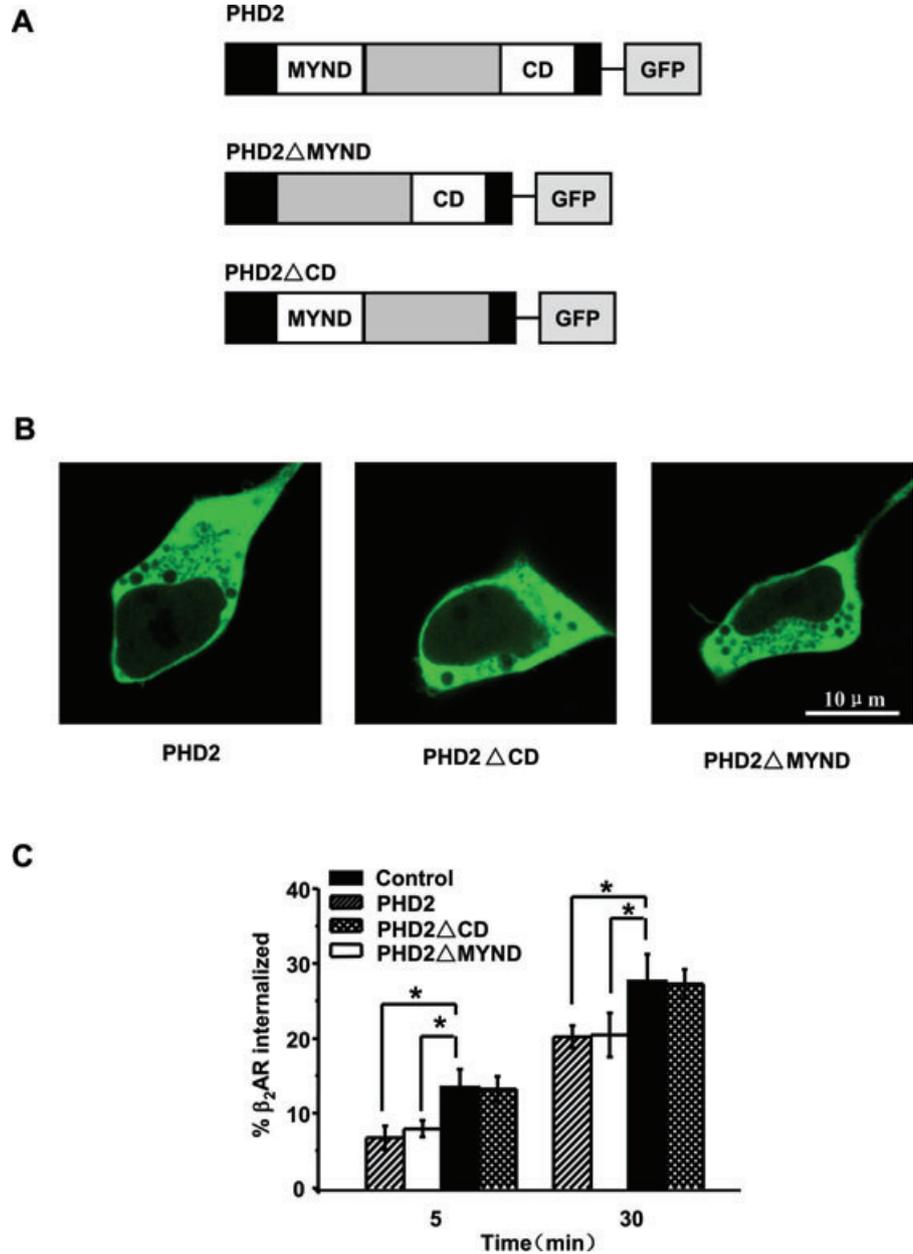


Fig. 2 The CD domain of PHD2 is required for β_2 -AR internalization. **(A)** Schematic representation of PHD2 and the truncated mutants. Full-length PHD2 and PHD2 Δ MYND and PHD2 Δ CD truncated mutants were generated as GFP-tagged fusion proteins. **(B)** Each GFP-tagged PHD2 mutant was transfected into β_2 -AR-293 cells for 48 hrs. The localization was analysed by confocal microscopy. A representative image is shown. **(C)** β_2 -AR-293 cells were transfected with GFP (control), PHD2-GFP, PHD2 Δ MYND-GFP or PHD2 Δ CD-GFP for 48 hrs. Receptor internalization was quantified at 5 and 30 min. after β_2 -AR stimulation. Experiments were performed in triplicate, and the graph represents the mean \pm S.E.M. ($n = 4$). Asterisk (*) denotes the value that is significantly different from controls ($P < 0.05$).

negative role in the regulation of β_2 -AR internalization (Fig. 4A). However, hypoxia or DMOG is a broad-spectrum PHD inhibitor, and these treatments failed to distinguish which PHD isoform activity participated in the regulation of β_2 -AR internalization. Next, we adopted double gene knockdown technique combined with DMOG treatment to solve this problem. As shown in Fig 4B, compared with the untreated cells (Ctrl), PHD1/PHD3 double knockdown (PHD1 $^-$ /PHD3 $^-$) did not affect the rate of β_2 -AR internalization upon β_2 -AR activation, whereas subsequent DMOG treatment could inhibit PHD2 hydroxylase activity, and

accelerate β_2 -AR internalization in response to agonist stimulation. In contrast, compared with the untreated cells, PHD1/PHD2 (PHD1 $^-$ /PHD2 $^-$) or PHD2/PHD3 (PHD2 $^-$ /PHD3 $^-$) double knockdown obviously accelerated β_2 -AR internalization, but the effect was mainly mediated by PHD2 depletion. Although subsequent DMOG treatment resulted in the inhibition of PHD1 or PHD3 hydroxylase activity, the rate of β_2 -AR internalization did not further change. Collectively, these results suggest that the hydroxylase activity of PHD2, but not PHD1 or PHD3, is involved in the regulation of β_2 -AR internalization.

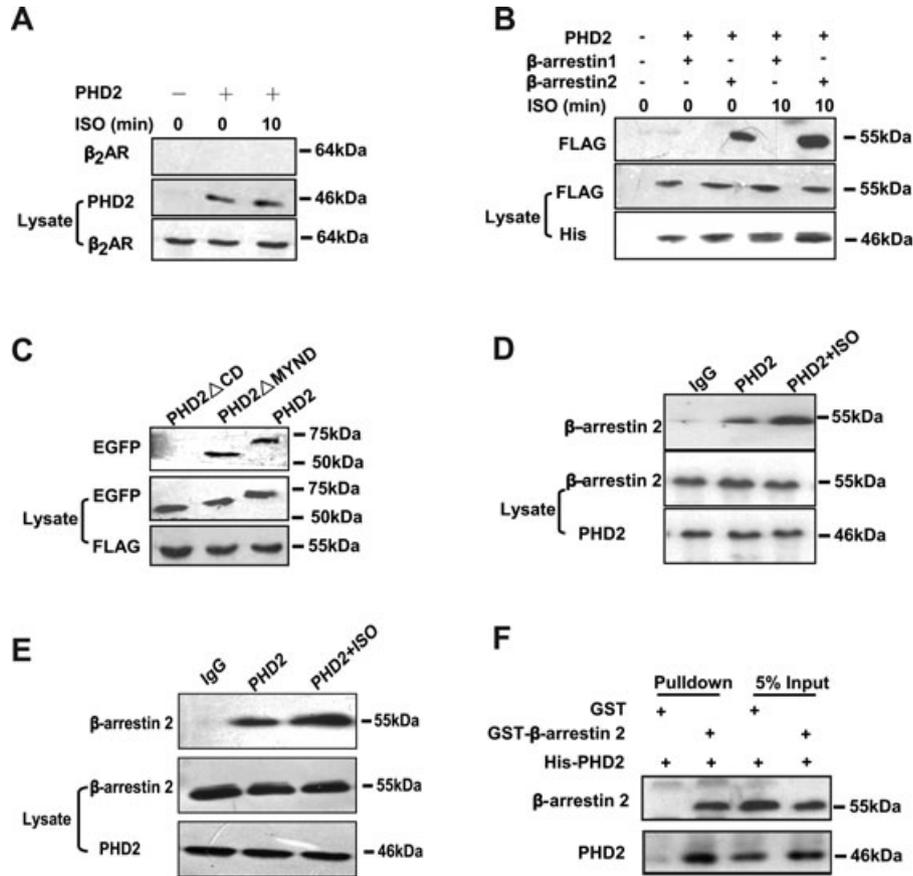


Fig. 3 PHD2 interacts specifically with β -arrestin 2. (A) β_2 -AR-293 cells were transfected with His-tagged PHD2 for 48 hrs and then treated with ISO (10 μ M) for the indicated time. The total cell lysates were immunoprecipitated with anti-His antibody (for PHD2). Western blots were performed with antibodies against β_2 -AR or His (for PHD2). (B) β_2 -AR-293 cells were cotransfected with His-tagged PHD2 and FLAG-tagged β -arrestin 1 or 2. Forty-eight hours after transfection, these cells were treated as shown in the figure, and then the cell lysates were immunoprecipitated with anti-His antibody (for PHD2). The immunoprecipitates and cell lysates were blotted with the indicated antibodies. (C) β_2 -AR-293 cells were transiently cotransfected with FLAG-tagged β -arrestin 2 along with the full length PHD2, PHD2 Δ MYND or PHD2 Δ CD plasmids. Cell lysates were immunoprecipitated with the anti-FLAG antibody for β -arrestin 2. Western blots were performed with the antibody against FLAG (for β -arrestin 2) or GFP (for PHD2 and truncated mutants). (D, E) The cell lysate of β_2 -AR-293 cells or neonatal rat cardiomyocytes was subjected to immunoprecipitation with anti-IgG or anti-PHD2 antibody, respectively. The immunoprecipitates were then blotted with the indicated antibodies. (F) Purified PHD2 protein was incubated with an equal amount of GST or GST- β -arrestin 2 coupled to GSH-Sepharose. Proteins retained on Sepharose were then detected with the indicated antibodies. A representative image is shown.

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Because β -arrestin 2 directly interacts with PHD2, it is essential to test whether it is directly hydroxylated by PHD2. β -arrestin 2 was purified from agonist-simulated β_2 -AR-293 cells (cultured at 21% or 1% O_2) using an alprenolol-Sepharose affinity resin, digested with trypsin and analysed by LC-MS. As shown by a +16-dalton mass shift, which is indicative of hydroxylation, three hydroxylated proline (Pro) residues (Pro¹⁷⁶, Pro¹⁷⁹ and Pro¹⁸¹) were identified on the peptide corresponding to β -arrestin 2 (172–189) (Fig. 4C and D). However, β -arrestin 2 hydroxylation was not detected once the hydroxylase activity of PHD2 was inhibited by hypoxic treatment.

PHD2 retards β -arrestin 2 recruitment to the plasma membrane and subsequent internalization of β_2 -AR

The process of β -arrestin 2-mediated β_2 -AR internalization involves the initial translocation of β -arrestin 2 to the plasma membrane, the subsequent formation of the membrane cluster, and β -arrestin 2/ β_2 -AR co-internalization into the cytosol [19]. To

determine how PHD2 affected β -arrestin 2 movement, we monitored the agonist-dependent trafficking of β -arrestin 2-GFP in β_2 -AR-293 cells in response to receptor activation (Fig. 5A). Membrane clusters were detected as early as 1 min. after the addition of ISO. Compared to the control group, PHD2 overexpression exhibited delayed formation of membrane clusters (34.44% \pm 1.92% versus 25.56 \pm 3.85%, Fig. 5B). The differences in internal clusters were first observed at 10 min., with a greater internalization percentage in control cells than in PHD2-overexpressing cells (28.89 \pm 3.85% versus 20 \pm 3.33%). The greatest internalization percentages (46.67 \pm 3.33% versus 33.33 \pm 3.33%) were observed at 40 min. for both the control and PHD2 overexpressing cells, but the value is significantly higher in control cells. These results support and strengthen the findings observed in Fig. 1A, namely that PHD2 retards β_2 -AR internalization. During the experiment, Western blots revealed that PHD2 expression level had no effect on the endogenous β -arrestin 2 expression (Fig. 5C). Taken together, these results suggest that PHD2 delays β_2 -AR internalization by retarding the recruitment of β -arrestin 2 to the plasma membrane and its subsequent co-internalization with β_2 -AR.

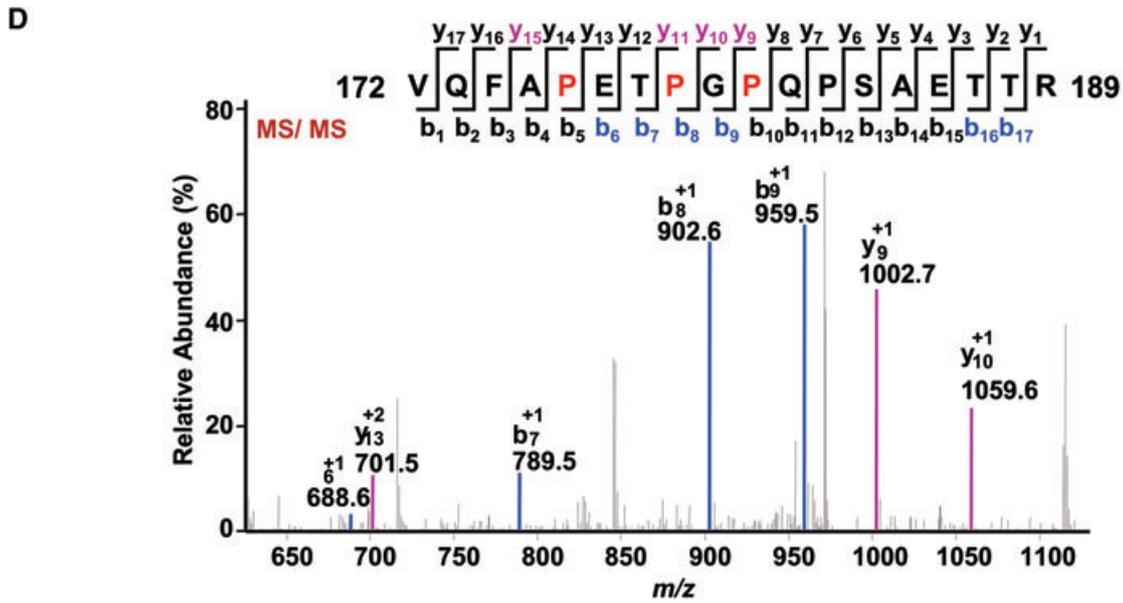
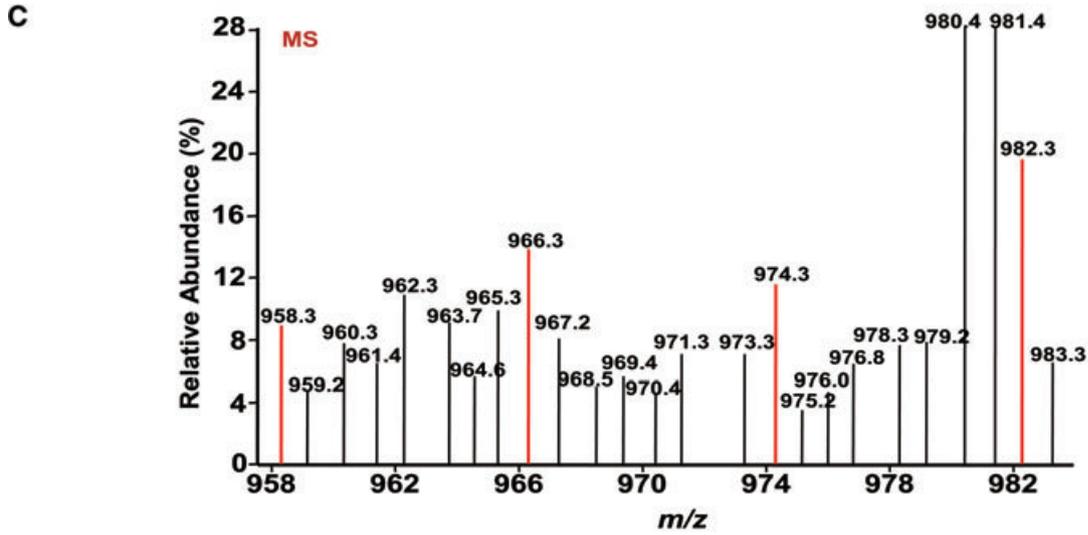
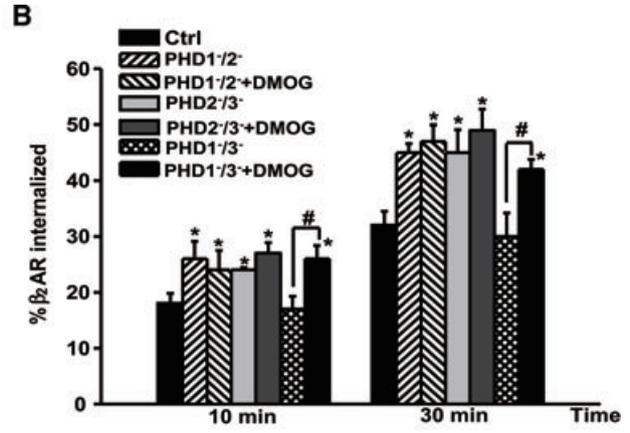
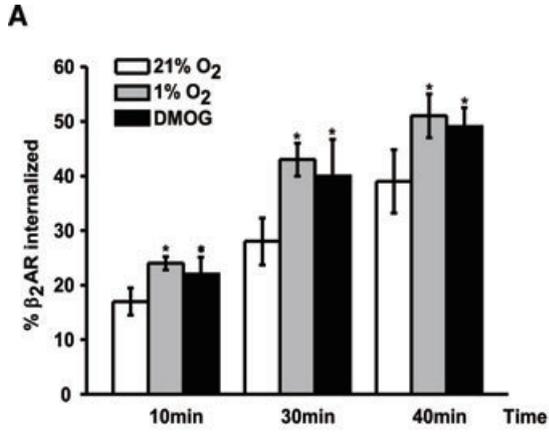




Fig. 4 PHD2 regulates β_2 -AR internalization depending on the hydroxylase activity. **(A)** β_2 -AR-293 cells were incubated in 21% or 1% O_2 or treated with DMOG (1 mM) for 12 hrs, and then stimulated with ISO (10 μ M) for 10, 30 or 40 min. Receptor internalization was determined as described in 'Materials and methods'. The graph shows values as the means \pm S.E.M. for four independent experiments. Asterisk (*) denotes a significant difference compared with cells incubated in 21% O_2 ($P < 0.05$). **(B)** β_2 -AR-293 cells were transfected with PHD1/PHD2 siRNA, PHD2/PHD3 siRNA, PHD1/PHD3 siRNA or scramble siRNA (Ctrl) for 36 hrs, and then a portion of these cells were treated with DMOG (1 mM) for an additional 12 hrs. Receptor internalization was determined as described in 'Materials and methods'. The graph shows values as the means \pm S.E.M. for four independent experiments. Asterisk (*) denotes a significant difference compared with the cells transfected with scramble siRNA (Ctrl). '#' denotes a significant difference between cells treated with or without DMOG in the same siRNA transfection group. **(C)** FLAG- β -arrestin 2 was purified from β_2 -AR-293 cells cultured in 21% or 1% O_2 , digested with trypsin and analysed by LC-MS. Monoisotopic precursor ions (shown in red), at $m/z = 958.3, 966.3, 974.3$ and 982.3 , correspond to $[M+2H]^+$ for the unhydroxylated, monoproline hydroxylated, diproline hydroxylated and triproline hydroxylated VQFAPETPGQPQSAETTR peptides, respectively. **(D)** A tandem mass spectrum of the precursor ion at $m/z = 971.5$. A large red 'P' indicates hydroxylated proline residues. The peak heights show the relative abundances of the corresponding fragment ions with the annotations identifying the matching N terminus-containing ions (b ions) in blue and C terminus-containing ions (y ions) in magenta. For clarity, only the major peaks are labelled.

Discussion

PHDs are a group of enzymes that includes collagen hydroxylases and members of the α -ketoglutarate-dependent dioxygenase [5]. They use oxygen, α -ketoglutarate and a prolyl residue as substrates and act as important oxygen sensors *in vivo*. Most PHD studies are related to HIF proteins, which are tightly controlled by the hydroxylase activity of PHDs [20–22]. In normoxic conditions, PHD activation results in rapid degradation of HIF-1 α or HIF-2 α protein [23]. Besides their differential involvement in the HIF signal transduction pathway, there is increasing evidence for PHD-dependent functions that are unrelated to HIF. For PHD1, PHD2 and PHD3, a regulation of the I κ B kinase, the large subunit of RNA polymerase II and activating transcription factor 4 (ATF4) has been suggested, respectively [24, 25]. A recently described isobaric tags for relative and absolute quantitation (iTRAQ) proteome approach has additionally led to the suggestion that proteins related to the cytoskeleton are regulated as a function of PHD2 [26].

Xie *et al.* also extended the role of PHDs to arenas beyond HIF biology and demonstrated that the increase in β_2 -AR abundance during hypoxia is not mediated by HIF transcriptional effects but instead is conferred by post-translational modifications induced by PHD3 [11]. PHD3 interacts directly with β_2 -AR to serve as an endogenous β_2 -AR PHD. Under hypoxic conditions, receptor hydroxylation and subsequent ubiquitylation decrease dramatically, thus attenuating receptor degradation and down-regulation. The finding first elucidated the relationship between PHD3 and β_2 -AR and provided considerable molecular and biochemical data to demonstrate a potential role of PHD3 in the regulation of β_2 -AR signalling.

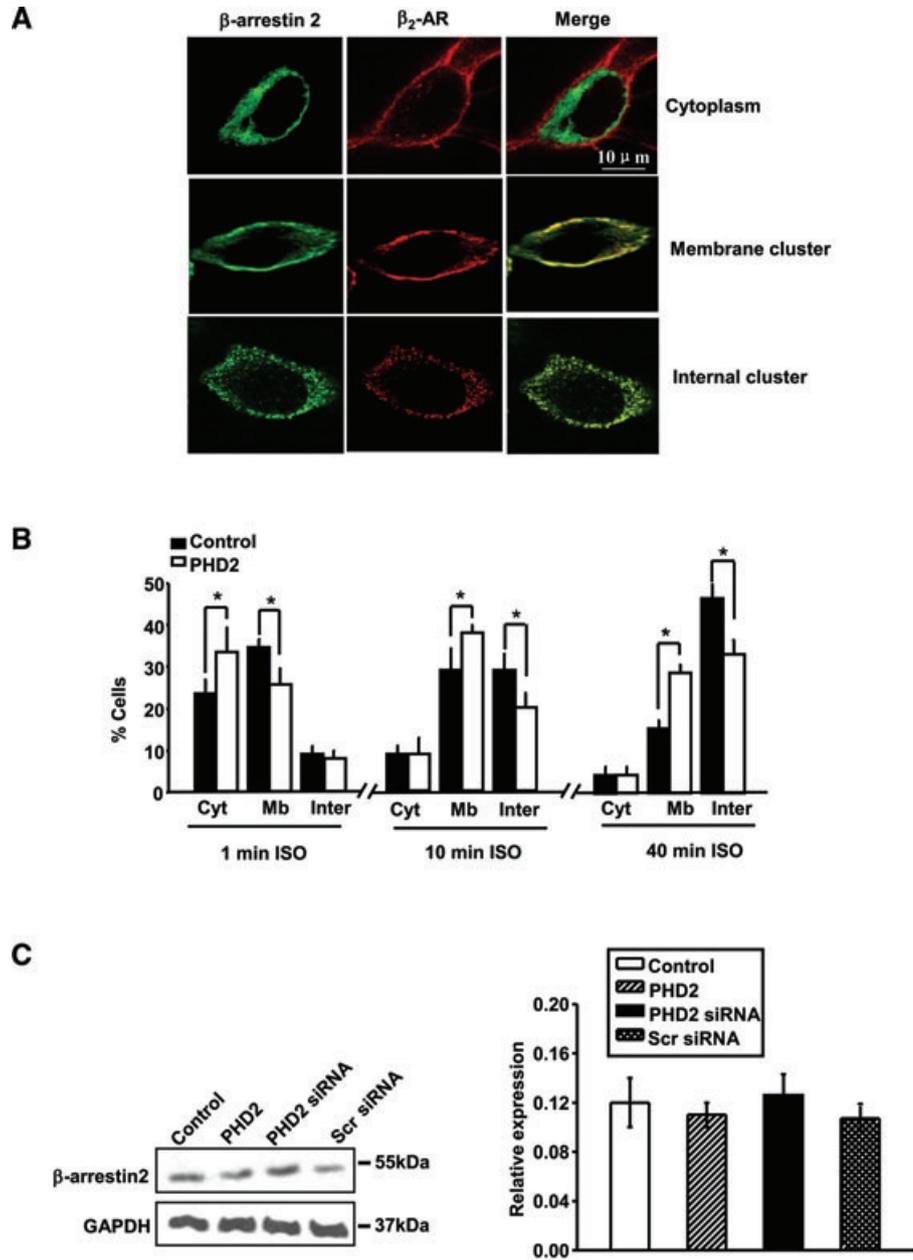
PHD3 is only expressed in cardiac and neural tissue under non-stressed conditions, whereas PHD2 is constitutively expressed in cells and tissue [5]. The cardiovascular phenotypes of postnatal PHD2 knockout mice include dilated cardiomyopathy and vascular abnormalities [6, 7], but cardiac structural abnormalities were not reported in PHD3 knockout mice, implying that PHD2 may play a greater role especially in normal physiological conditions. In the current study, we identified a novel role for PHD2 in modulating the internalization of β_2 -AR. β -arrestin 2 functions as an endocytic

adaptor to promote receptor concentration in clathrin-coated pits. It is, thus, an important component of β_2 -AR internalization machinery. PHD2 directly interacts with β -arrestin 2 protein and affects β -arrestin 2 trafficking which in turn changes the rate of β_2 -AR internalization. To our knowledge, the present results are the first to identify that β -arrestin 2 is a non-HIF hydroxylation target of PHD2. β_2 -AR abundance is tightly associated with β_2 -AR signalling responsiveness. PHD2 or PHD3 knockdown did not affect β_2 -AR abundance (Fig. S2 and S5), suggesting that the change of β_2 -AR internalization is caused by the modification of β -arrestin 2 but not of β_2 -AR itself. Previous study revealed that PHD3 knockdown leads to a significant increase in β_2 -AR expression [11]. The following reason is likely to explain the discrepancy between Xie's results and our result. β_2 -AR expression was detected in the same cell line, however, these cell lines were constructed by different labs. Thus, the level of endogenous β_2 -AR expression may vary greatly. β_2 -AR expression is regulated by multiple genetic factors, thus PHD3 knockdown alone may not be enough to change β_2 -AR expression.

β -arrestin interacts with a number of proteins, such as clathrin, adaptor protein 2 (AP2), phosphoinositides, GTPase-activating proteins and guanine nucleotide exchange factors, to form a complex that mediates β_2 -AR internalization. Phosphoinositide 3-kinase (PI3K) has been reported to alter the rate of β_2 -AR internalization by affecting the formation of these complexes [19]. Our study reveals a novel mechanism that regulates β_2 -AR internalization (*i.e.* the hydroxylation of β -arrestin 2 by PHD2). Interestingly, PI3K activity increases in a load-dependent manner in failing human hearts, and inhibition of the interaction between GRK2 and PI3K restores contractility in human beings and ameliorates the development of murine heart failure [27]. The mechanism for the restoration of function is likely due to the redistribution of down-regulated receptors in the plasma membrane [28]. It is possible that interference of PHD2 function may have an effect similar to PI3K in the treatment of heart failure, which must be verified in future research.

Post-transcriptional hydroxylation plays a great role in protein stability and protein-ligand interactions [29]. The idea is that it is far easier to create a spectrum of slightly different proteins by taking one basic protein scaffold and then fine-tuning or entirely switching its properties than to constantly transcribe new

Fig. 5 PHD2 overexpression retards the recruitment of β -arrestin 2 to the plasma membrane and its co-internalization with β_2 -AR into the cytosol. **(A)** β_2 -AR-293 cells were transfected with β -arrestin 2 with or without PHD2. β -arrestin 2 dynamics upon receptor stimulation were imaged by confocal microscopy and categorized after imaging into three groups: I, cytosolic β -arrestin 2 (resting state); II, β -arrestin 2 translocated to the plasma membrane and clustered with β_2 -AR at the plasma membrane; and III, β -arrestin 2 co-internalized with β_2 -AR into the cytosol. Representative images of cells in each state are shown. **(B)** For quantitative analysis, the percentage of cells conforming to the phenotypes outlined in Fig. 5A was determined 1, 10 or 40 min. after agonist stimulation. The graph represents the mean \pm S.E.M. ($n = 4$) with statistically significant differences marked with * ($P < 0.05$). **(C)** β_2 -AR-293 cells were transfected with PHD2, PHD2 siRNA, or scramble siRNA (Scr siRNA) or left untreated (control) for 48 hrs, and then these cells were lysed. Western blots were conducted to determine the level of β -arrestin 2 expression. GAPDH expression was used as the loading control. β -arrestin 2 expression was determined as the ratio of densitometric value compared to GAPDH expression. Representative immunoblots are shown along with quantitative data showing the mean \pm S.E.M. from four separate blots.



proteins. β -arrestin 2 was found to be hydroxylated at the Pro¹⁷⁶, Pro¹⁷⁹ and Pro¹⁸¹ sites by PHD2. These modifications endue β -arrestin 2 dynamic protein functions that can rapidly respond to systemic stimuli, such as oxygen levels or hormone concentrations. Post-translational hydroxylation of proteins is relatively uncommon, but it has been intensively studied for decades. The most abundant eukaryotic protein, collagen, has been reported to hydroxylate at lysine and proline residues [30]. Asn and Asp residues are hydroxylated in various secreted proteins, including epidermal growth factor-like domains in blood coagulation factors (VII, IX and X as well as in thrombomodulin and in low-density

lipoprotein receptors [31, 32]. Our research provides a significant contribution to the research on protein hydroxylation.

Identification of a negative regulator of β_2 -AR internalization reveals a new function of PHD2 and highlights a cellular requirement for exquisite regulation of receptor dynamics. PHD2 directly interacts with β -arrestin 2 and results in the hydroxylation of β -arrestin 2 at the Pro¹⁷⁶, Pro¹⁷⁹ and Pro¹⁸¹ sites, which in turn lead to the retardation of β -arrestin 2 recruitment to the plasma membrane and subsequent co-internalization with β_2 -AR into the cytosol. This discovery provides new insight into the regulation of β_2 -AR signalling and may open a new avenue for the prevention

and treatment of diseases associated with β_2 -AR signalling [33]. β -arrestin 2 and PHD2 is expressed ubiquitously in all cells and tissues. β -arrestin 2 is not only involved in the regulation of β_2 -AR internalization but also participates in the regulation of the internalization processing of other receptors such as V2R and C-X-C chemokine receptor type 4 (CXCR-4) [34–36]. Thus, in the future, it is necessary to verify whether other β -arrestin 2-associated receptors are modulated in a fashion similar to how PHD2 regulates β_2 -AR internalization.

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Conflict of interest

None declared.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Intracellular localization of β_2 -AR after 40 min receptor stimulation. β_2 -AR-293 cells were transfected with PHD2 or PHD2 siRNA or left untreated. 48 hrs after transfection, these cells were stimulated with freshly prepared isoproterenol (ISO, 10 μ M, Sigma-Aldrich) for 40 min. After washing with ice-cold PBS, the cells were incubated with anti-HA antibody (for β_2 -AR, Santa Cruz) for 1 hr, washed, and incubated with FITC-conjugated

secondary antibody (Invitrogen) for 1 hr. Then the cells were analyzed by confocal microscopy. A representative image is shown.

Fig. S2 β_2 -AR expression in β_2 -AR-293 cells after PHD2 interference. β_2 -AR-293 cells were transfected with PHD2 or PHD2 siRNA or left untreated for 48 hrs. β_2 -AR expression was detected by western blots. GAPDH was used as the loading control. A representative image is shown.

Fig. S3 HIF-1 α expression in β_2 -AR-293 cells after different treatment. β_2 -AR-293 cells were transfected with PHD2 or PHD2 siRNA for 48 hrs, then stimulated with ISO (10 μ M) for the indicated time. MG132 (10 μ M) was added to prevent the degradation of HIF-1 α . Cells cultured in 1% O₂ for 48 hrs were taken as a positive control. After treatment, the expression of HIF-1 α was determined by western blot. GAPDH was used as a loading control. Scores of 0, 1 or 2 indicate PHD2 knockdown, normal PHD2 expression, or PHD2 overexpression, respectively. A representative image is shown.

Fig. S4 Interaction of PHD2 with GRK protein. β_2 -AR-293 cells were co-transfected with His-tagged PHD2 and FLAG-tagged GRK 2, 5 or 6 plasmids for 48 hrs. The whole cell lysates were immunoprecipitated with the His antibody (for PHD2). The presence of GRKs in the immunoprecipitates was detected by western blots. A: Cells without ISO stimulation; B: Cells stimulated with ISO (10 μ M) for 10 min.

Fig. S5 Estimation the expression of β_2 -AR protein after PHD3 interference. β_2 -AR-293 cells were transfected with PHD3 siRNA, scramble siRNA (Scr) or left untreated (Ctrl) for 48 hrs, and then these cells were lysed. Western blots were conducted to determine the level of β_2 -AR expression. GAPDH expression was used as the loading control. β_2 -AR expression was determined as the ratio of densitometric value compared to GAPDH expression. Representative immunoblots are shown along with quantitative data showing the mean \pm S.E.M. from four separate blots.

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