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Prolyl hydroxylase domain protein 2 regulates the intracellular cyclic AMP level in cardiomyocytes through its interaction with phosphodiesterase 4D

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ABSTRACT

Cyclic adenosine 3',5'-monophosphate (cAMP), which is synthesized by adenylyl cyclase (AC) and degraded by phosphodiesterase (PDE), plays crucial roles in the regulation of multiple cellular functions and physiological processes. Prolyl hydroxylase domain (PHD) proteins, which belong to a family of dioxygenases that function as oxygen sensors through their hydroxylation activity, have been implicated in multiple signaling pathways. Here, we aimed to determine whether PHD played a role in regulating intracellular cAMP level in cardiomyocytes. Through the overexpression/knockdown of the PHD gene and the measurement of the cAMP content, we found that PHD2, but not PHD1 or PHD3, acts as a regulator of intracellular cAMP. In neonatal rat cardiomyocytes and H9c2 cells, the overexpression of PHD2 increased the intracellular cAMP level, whereas the PHD2 knockdown reduced it. There was no alteration in the AC expression or activity in cells that overexpressed or downregulated PHD2. The overexpression of PHD2 decreased both the protein expression and the activity of phosphodiesterase 4D (PDE4D), whereas the PHD2 knockdown increased the PDE4D expression and activity. Co-immunoprecipitation experiments revealed a direct binding between PHD2 and PDE4D and liquid chromatography–tandem mass spectrometry analyses identified the specific hydroxylation sites on PDE4D. In conclusion, PHD2 may directly interact with PDE4D to function as a novel regulator of the intracellular cAMP levels in cardiomyocytes.

1. Introduction

The second messenger system is an important component of the intracellular signaling network and plays a vital role in the delivery and amplification of transmembrane signals. Cyclic adenosine 3',5'-monophosphate (cAMP) is one of the most well-known second messengers that regulates various cellular functions and biological processes, including gene transcription, cell proliferation, metabolism, as well as more specialized functions depending on the specific cell type [1,2]. In cardiomyocytes, cAMP is a key regulator of sarcomere contraction, relaxation and automaticity and also mediates the positive inotropic and lusitropic effects of β-adrenergic activation [2,3]. It is now well established that the cAMP levels are functionally associated with many cardiovascular disorders, such as cardiac hypertrophy, arrhythmia and vascular stenosis [3].

cAMP is synthesized by adenylyl cyclase (AC) through the stimulation of G protein-coupled receptors (GPCRs) and degraded via phosphodiesterase (PDE)-catalyzed hydrolysis. In general, the intracellular cAMP level is the result of a balance between its synthesis and degradation [4]. Nevertheless, the regulation of cAMP signaling is complicated as many levels of regulation by various modulators have been discovered [5]. Novel regulatory mechanisms of the intracellular cAMP content therefore need to be explored.

Prolyl hydroxylase domain (PHD) proteins, also known as EGL nine homologs (EGLNs), belong to the superfamily of dioxygenases that use oxygen and 2-oxoglutarate as substrates, and iron and ascorbate as cofactors [6]. In mammalian cells, three isoforms of PHD (PHD1–3) have been identified [7]. Acting as oxygen sensors, PHDs are able to modify the stability of the hypoxia-inducible factor transcription factor (HIF) in response to the oxygen availability. This process is achieved through the catalysis of the oxygen-dependent hydroxylation of certain proline residues on HIF [8]. Re-

Abbreviations: AC, adenylyl cyclase; PDE, phosphodiesterase; PHD, prolyl hydroxylase domain protein; H9c2 cells, a cell line derived from embryonic rat heart; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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cent studies have demonstrated that PHDs also participate in some HIF-independent substrate modifications [9], which suggest that PHDs have diverse roles in intracellular signaling. In fact, the β2-adrenergic receptor (β2-AR), which is a major adrenergic GPCR in the heart, has been identified as a substrate for PHD3 [10–11]. The current study was designed to investigate whether PHDs play a role in the regulation of the intracellular cAMP level in cardiomyocytes and to explore the underlying mechanism of this regulation.

2. Materials and methods

2.1. Chemicals and reagents

Forskolin, SQ22536, isobutylmethylxanthine (IBMX), α-ketoglutarate (α-KG), dimethyloxalylglycine (DMOG), MG132 and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich. The anti-PHD1 and anti-PHD3 antibodies were obtained from Abcam. The anti-PHD2 and anti-GAPDH antibodies were purchased from Cell Signaling, and the anti-PDE4D, anti-AC5/6 and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology.

2.2. Plasmids and lentiviral vector construction

The rat PHD1 and PHD2 plasmids were provided by Prof. Christian Frelin (National Center for Scientific Research, France). The rat PHD3 plasmid was provided by Prof. Robert S. Freeman (University of Rochester Medical Center, USA). The human PHD2 plasmid was provided by Dr. Felix Oehme (Bayer Schering Pharma AG, Germany). The rat PDE4D plasmid was obtained from Prof. Miles D. Houslay (University of Glasgow, UK). The cDNAs of rat PHD1–3 were prepared through the PCR amplification of the corresponding plasmids and then cloned into pLenti6 V5-D (Invitrogen) to generate the pLenti-PHD expression vectors. For the siRNA duplex targeting each PHD isoform, a previously validated sequence was used to generate the pLenti-PHD-siRNA vector [12]. The lentivirus particles were produced using the ViraPower Lentiviral Expression System (Invitrogen) according to the manufacturer's protocol.

2.3. Cell culture and transfection

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the policy of the Animal Care and Use Committee at the Tongji University School of Medicine. Neonatal rat cardiomyocytes (NRCM) were isolated from the hearts of 1–2-day-old Sprague–Dawley rat pups, as previously described [13]. The NRCM, H9c2 and human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (Gibco). The NRCM and H9c2 cells were transfected with the lentiviral vectors and the transfection in the HEK293 cells was performed using the Attractene Transfection Reagent (Qiagen) following the manufacturer's instructions.

2.4. cAMP measurement

The NRCM and H9c2 cells were cultured in 24-well plates and either transfected with the corresponding vectors or treated for 30 min with forskolin (30 μM) or SQ22536 (1 mM). The cells were washed twice with phosphate-buffered saline (PBS) and harvested by trypsinization. The cell pellets were resuspended in buffer containing 50 mM Tris–HCl (pH 7.5), 4 mM EDTA and 50 μM IBMX. After a brief sonication, the cells were boiled for 4 min and centrifuged at 12,000*g* and 4 °C for 10 min to remove any cellular debris. The intracellular cAMP content was determined using the commercially available cAMP-Glo™ Assay Kit (Promega) according to the manufacturer's instructions. The cAMP values were normalized to the assayed proteins.

2.5. Adenylyl cyclase assay

The cells were harvested in lysis buffer containing 20 mM HEPES (pH 7.4), 0.1% TritonX-100, 0.5 mM dithiothreitol (DTT), 5 mg/ml leupeptin, 5 mg/ml aprotinin, 10 mg/ml pepstatin A and 1 mM phenylmethylsulfonyl fluoride (PMSF). An appropriate volume of the obtained homogenate was added to an ATP regenerating system, which was comprised of 20 mM HEPES (pH 7.4), 0.8 mM $MgCl_2$, 0.3 mM KCl, 100 mM NaCl, 0.5 mM ATP, 5 mM creatine phosphate, 1 mM EGTA, 70 U creatine phosphokinase, 0.5 mM DTT and 0.2 mM IBMX, and incubated for 20 min at 30 °C. At the end of the incubation period, the medium was extracted to determine the cAMP level using the method described above.

2.6. Phosphodiesterase assay

The cells were lysed and sonicated in KHEM buffer containing 50 mM KCl, 50 mM HEPES (pH 7.4), 1.94 mM $MgCl_2$, 10 mM EGTA, 1 mM DTT, 5 mg/ml leupeptin, 5 mg/ml aprotinin, 10 mg/ml pepstatin A and 1 mM PMSF. The PDE4D from the lysate was precipitated using PDE4D antibody and Protein G Sepharose Beads (GE Healthcare). The PDE4D activity was assayed using a PDE-Glo™ Phosphodiesterase Assay Kit (Promega) according to the manufacturer's instructions. The activity of PDE4D was presented as the relative luminescence units (RLU) and normalized to the assayed proteins.

2.7. Western blot

The cells were lysed with RIPA lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 1% sodium deoxycholate, 1% NP-40, 1 mM PMSF and 1 mM EDTA. After centrifugation at 20,000*g* and 4 °C for 10 min, 2 × SDS gel sample buffer was added into the cleared lysate. The proteins were size-fractionated by SDS–PAGE, transferred onto a polyvinylidene fluoride (PVDF) membrane and immunoblotted using the corresponding antibodies.

2.8. Immunoprecipitation

After pre-clearing with Protein G Sepharose for 1 h at 4 °C, the cell lysates were incubated with the primary antibody for 4 h at 4 °C. The immune complexes were then precipitated with Protein G Sepharose for 2 h at 4 °C with continuous mixing. After three washes in PBS, the adsorbed proteins were eluted with boiling SDS sample buffer and used for immunoblotting.

2.9. Liquid chromatography–tandem mass spectrometry analysis (LC–MS) for PDE4D hydroxylation

The PDE4D proteins were purified from the H9c2 and HEK293 cells (cultured at 21% or 1% O_2), reduced with DTT (10 mM at 37 °C for 30 min) and alkylated with iodoacetamide (20 mM for 30 min at room temperature in the dark). The purified proteins were digested with a trypsin solution at a working concentration of 5 ng/ml. The mass spectrometry analyses were performed as previously described [12]. The raw data were subjected to the TurboSEQUEST program in the BioWorks™ 3.0 software (Thermo Finnigan, San Jose, CA, USA). A database containing rat or human PDE4D was used in the search analysis.

2.10. Statistical analysis

Each experiment was repeated at least three times. The comparison of the means between different groups was performed using Student's *t*-test or One-Way ANOVA (with post-hoc). The data are shown as the means ± S.E.M. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Manipulation of the PHD2 expression altered the intracellular cAMP level

To determine whether PHDs affect the intracellular cAMP level, NRCM and H9c2 cells were transfected with one of the three isoforms of PHD (PHD1–3) or with PHD siRNA vectors. The efficiency of the PHD overexpression or knockdown in the NRCM was tested by Western blot (Fig. S1). The intracellular cAMP concentration was measured to assess the changes in the cAMP level. Similarly to the AC agonist forskolin, the overexpression of PHD2 significantly increased the intracellular cAMP concentration in both NRCM and H9c2 cells compared with the control, whereas the overexpression of PHD1 or PHD3 did not have such effect (Fig. 1A). However, the siRNA-mediated knockdown of PHD2 significantly decreased the cAMP level compared with the control, which was similar to the effect of the AC inhibitor SQ22536 (Fig. 1B). The knockdown of PHD1 or PHD3 did not change the intracellular cAMP concentration. These results suggest that PHD2 plays an important role in the regulation of the intracellular cAMP level. This effect was not observed with PHD1 or PHD3.

3.2. PDE but not AC mediated the increase in the cAMP level induced by PHD2 overexpression

Previous studies have identified that AC5/6 are the dominant isoforms of AC in the heart [14]. We found that neither the overexpression nor the knockdown of PHD2 had any effect on the expression of AC5/6 in NRCM (Fig. 2A). Similar results were observed in H9c2 cells (data not shown). Subsequently, the AC5/6 activity was assayed using the AC agonist forskolin and the antagonist SQ22536 as the positive controls. While forskolin and SQ22536 significantly altered the AC5/6 activity in both NRCM and H9c2 cells, the PHD2 overexpression/knockdown did not have an effect (Fig. 2B), which suggests that PHD2 may not regulate the intracellular cAMP level through the AC pathway.

We then investigated whether PHD2 affects the cAMP level through the regulation of PDE. In rat cardiomyocytes, PDE4D is the dominant isoform of PDE that hydrolyzes cAMP [4,15]. Therefore, PDE4D was chosen as a potential target of PHD2. As shown in Fig. 2C, PHD2 overexpression led to a significant decrease in the PDE4D expression in NRCM, whereas the knockdown of PHD2 increased the PDE4D expression level. The experiments were also performed in H9c2 cells and similar results were obtained (data not shown). The co-immunoprecipitation experiments showed that both recombinant and endogenous PHD2 could pull down PDE4D in NRCM and vice versa (Fig. 2D). Moreover, the PHD2 overexpression had a similar inhibitory effect on the PDE4D activity as the PDE antagonist IBMX, whereas the knockdown of PHD2 resulted in a marked increase in the PDE4D activity (Fig. 2E). These data suggested that PHD2 directly interacts with PDE4D to regulate both its expression and activity.

3.3. PHD2 regulated the PDE activity via hydroxylation modification

We used the PHD2 agonist α-KG and the PHD2 antagonist DMOG to determine whether PDE4D is regulated by the hydroxylase activity of PHD2. In NRCM, α-KG attenuated the PDE4D activity, whereas DMOG enhanced it (Fig. S2). These results suggest that the hydroxylation activity of PHD2 may be involved in its regulation of the PDE4D activity. Protein mass spectrum (MS) analyses revealed that two proline (P) sites (P^{238} and P^{260}) of PDE4D in H9c2 cells were hydroxylated by both endogenous and recombinant PHD2, and three proline sites (P^{29}, P^{382}, and P^{419}) in HEK293 cells were hydroxylated only by recombinant PHD2 (the MS and peptide analyses of the P^{260} site in H9c2 cells and the P^{382} site in HEK293 cells are shown in Fig. 3 and Fig. S3, respectively; the data on the other sites are not shown). Interestingly, the P^{382} site in human PDE4D and the P^{260} site in H9c2 cells are homogeneous (both sites are present within the same sequence of amino acids, KLMHSSSLTNSSIPR). However, the hydroxylation of PDE4D was not detected under hypoxic conditions, in which the hydroxylase activity of PHD2 was inhibited. Thus, PDE4D is likely a target of PHD2.

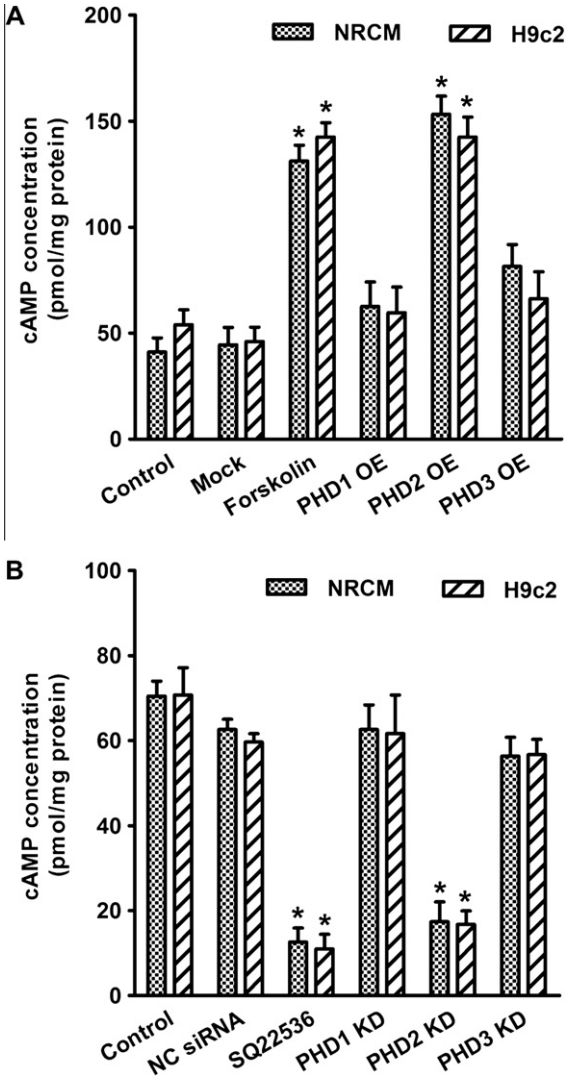

Fig. 1. The effect of PHD overexpression or knockdown on the intracellular cAMP level. (A) Changes in the cAMP concentration in PHD-overexpressing NRCM and H9c2 cells. The cells were either transfected with pcDNA3.1 (mock), PHD1, PHD2 or PHD3, or treated with DMSO (control) or forskolin for 30 min before the cAMP level was measured. The cAMP values were normalized to the assayed proteins. (B) The effects of the knockdown of PHD on the cAMP concentration in NRCM and H9c2 cells. The cells were either transfected with a negative control siRNA (NC siRNA), PHD1 siRNA, PHD2 siRNA or PHD3 siRNA vector, or treated with DMSO (control) or SQ22536 for 30 min. $^{*}p < 0.05$ vs. control. OE: overexpression. KD: knockdown.

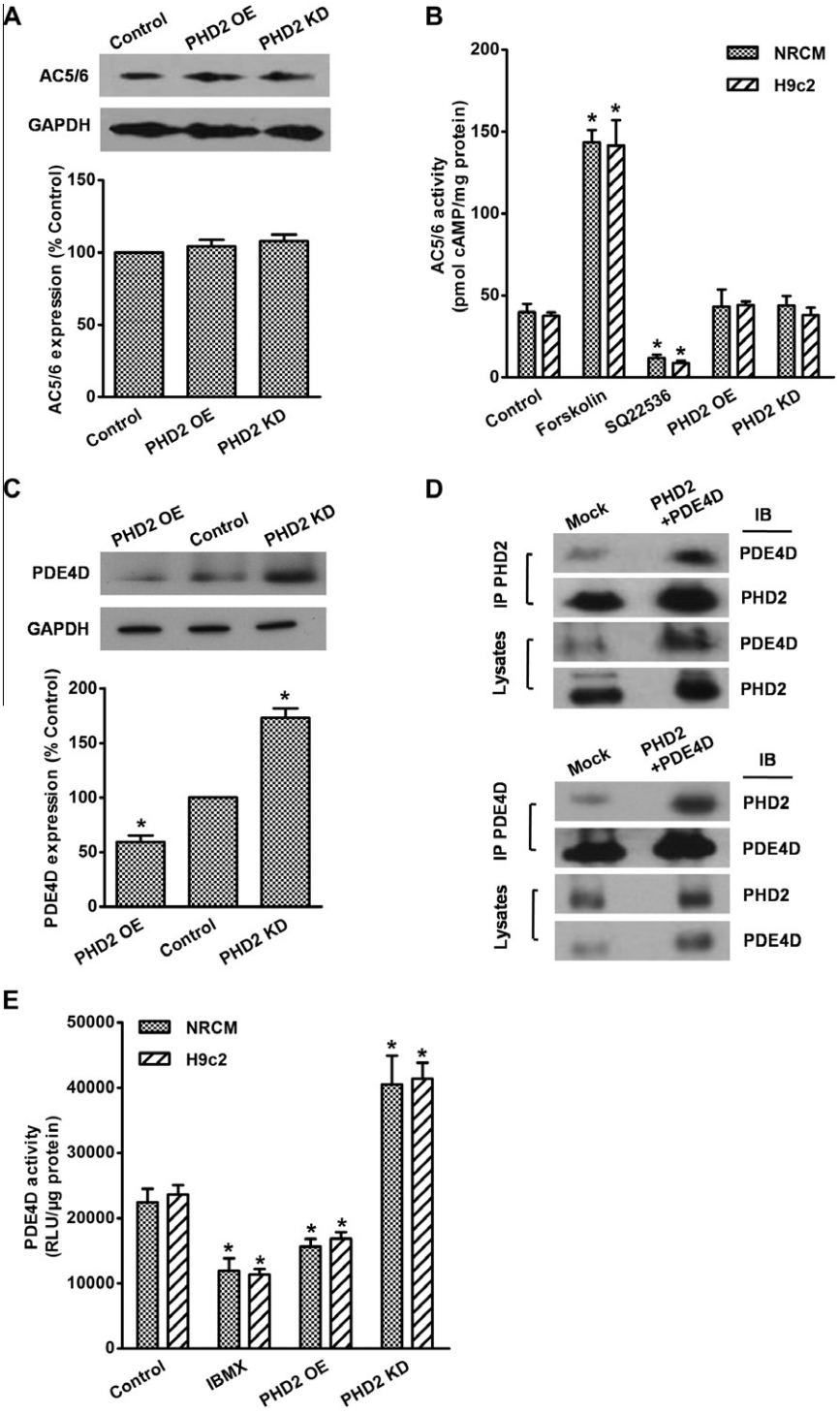

Fig. 2. PHD2 regulates the intracellular cAMP level through PDE4D but not through AC5/6. (A) The effect of PHD2 overexpression or knockdown on the AC5/6 expression. The NRCM were transfected with PHD2 or PHD2 siRNA. Untreated cells were used as a control. A typical Western blot of AC5/6 (upper panel) and the pooled data (lower panel) are shown, with GAPDH as a loading control. (B) PHD2 did not affect the AC5/6 activity. The NRCM and H9c2 cells were either treated with forskolin or SQ22536 for 30 min, or transfected with PHD2 or PHD2 siRNA. The AC5/6 activity was measured according to the protocol described in Section 2 (C) The effect of PHD2 overexpression or knockdown on the PDE4D expression in NRCM. (D) PHD2 interacts with PDE4D. NRCM were transfected with pcDNA3.1 (Mock) or cotransfected with PHD2 and PDE4D. The cell lysates were immunoprecipitated with anti-PHD2 and blotted with anti-PDE4D or anti-PHD2 (upper panel). The experiments were also performed in the reverse direction and similar results were obtained (lower panel). (E) The effect of PHD2 overexpression or knockdown on the PDE4D activity. IBMX (100 μM) was used as a positive control for the inhibition of PDE4D activity. $^{*}p < 0.05$ vs. control. OE: overexpression. KD: knockdown.

3.4. Proteasome inhibition blocked the downregulation of PDE4 that was induced by PHD2 overexpression

Hydroxylated PHD targets can be recognized by von Hippel–Lindau (VHL) tumor suppressor protein, combined to ubiquitin ligase E3 and subject to degradation by proteasome-mediated proteolysis [16]. We hypothesized that after its hydroxylation by PHD2, PDE4D was degradated through the ubiquitin–proteasome pathway. In PHD2-overexpressing NRCM, the proteasome inhibitor MG132 blocked the downregulation of the PDE4D expression

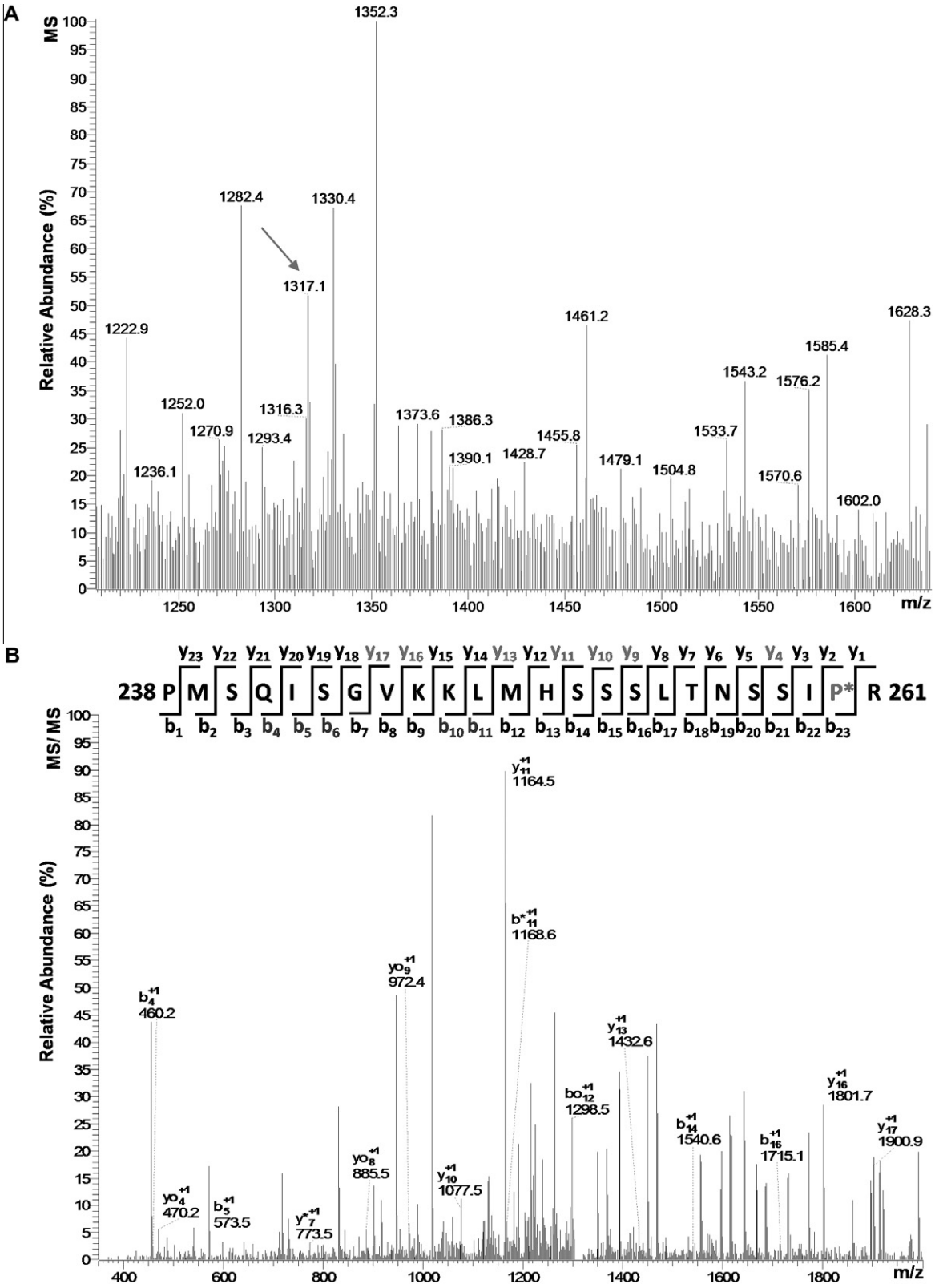

Fig. 3. The hydroxylated proline sites (P^{260}) of rat PDE4D. H9c2 cells were transfected with mock vector or PHD2. PDE4D was purified from the transfected cells, digested with trypsin and analyzed by LC–MS. (A) The monoisotopic precursor ion at $m/z = 1317.1$ (indicated with a red arrow) corresponds to the $[M+2H]^{2+}$ of the hydroxylated PMSQISGVKKLMHSSSLTNSSIPR peptide from the H9c2 cells. (B) Tandem mass spectrum of the precursor ion at $m/z = 1317.1$. The red "P" with an asterisk indicates a hydroxylated proline residue. The peak heights show the relative abundances of the corresponding fragment ions, with the annotations identifying the matched N terminus-containing ions (b ions) in blue and the C terminus-containing ions (y ions) in red. For clarity, only the major peaks are labeled.

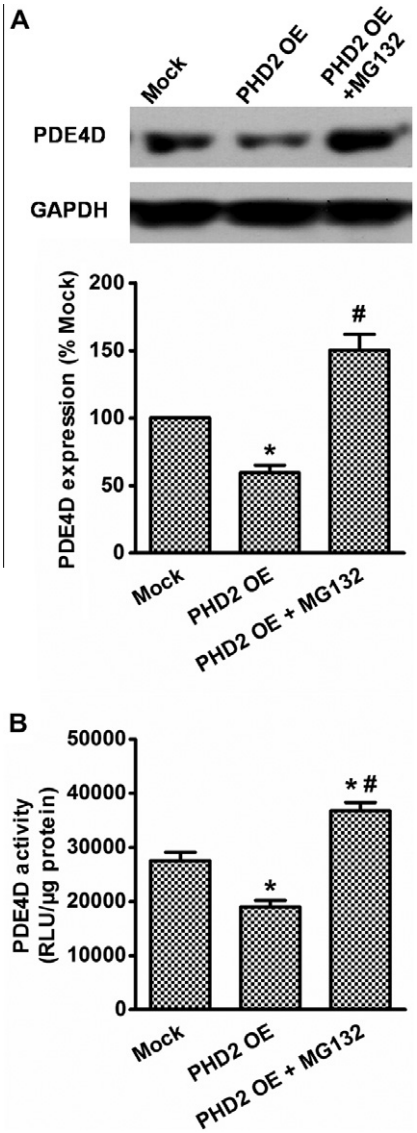

Fig. 4. The effect of proteasome inhibition on the PDE4D expression and activity. (A) MG132 increased the PDE4D expression in PHD2-overexpressing NRCM. NRCM were transfected with mock vector or PHD2 for 48 h and then incubated for 18 h in the presence or absence of MG132 (10 μM). A typical example of the Western blot analysis (upper panel) and the summarized data (lower panel) are given. GAPDH was used as a loading control. (B) The effect of MG132 treatment on the PDE4D activity in PHD2-overexpressing NRCM. $^{*}p < 0.05$ vs. mock. $^{\#}p < 0.05$ vs. PHD2-overexpressing group. OE: overexpression.

(Fig. 4A). Moreover, the PDE assay indicated that the decrease in PDE4D activity induced by PHD2 overexpression could be restored through the addition of MG132 (Fig. 4B). These findings suggest that the ubiquitin–proteasome pathway may be involved in the PHD2-mediated degradation of PDE4D.

4. Discussion

The observations herein demonstrate that PHD2 can regulate the intracellular cAMP level in cardiomyocytes through its interaction with PDE4D. This interaction leads to the hydroxylation of the specific proline sites on PDE4D, which was then targeted for degradation by the ubiquitin–proteasome pathway, resulting in the decrease of PDE4D protein expression and activity.

The PHD proteins, which are a subfamily of iron-dependent dioxygenases, play a key role in the regulation of the HIF abundance and oxygen homeostasis *in vivo*. Several differences have been found in the molecular structure and tissue distribution of the three PHD isoforms, each with relatively specific functions [17]. PHD2 is the primary HIF prolyl hydroxylase under normal conditions [18], whereas PHD1 and PHD3 may regulate the stability of HIF under specific conditions, such as prolonged hypoxia, or in certain cells/tissues [19]. Functionally, different PHD isoforms may differentially participate in specific pathophysiological processes. PHD2 is involved in the regulation of angiogenesis, tumor invasion, and hematopoiesis [9]. PHD3 is able to regulate the apoptosis of sympathetic neurons [20] and is also related to doxorubicin-induced cardiomyocyte death [21]. The current study demonstrates that only PHD2 significantly regulates the intracellular cAMP level under normal conditions (Fig. 1). Although PHD3 is highly expressed in cardiomyocytes, our study showed that this protein does not seem to be related to the cAMP content. However, whether the intracellular cAMP can be regulated by PHD3 or PHD1 under other conditions (such as hypoxia) remains to be addressed in future studies.

The cAMP molecule is generated mainly through the triggering of the agonist-induced activation of GPCRs and the subsequent activation of AC at the inner face of the plasma membrane [22]. PDE mediates the degradation of cAMP and is responsible for the regulation of the intracellular cAMP gradients [23]. Thus, the intracellular cAMP level depends on the balance between synthesis by AC and degradation by PDE. We found that the overexpression of PHD2 increased the intracellular cAMP level (Fig. 1A), which suggests that PHD2 positively regulates the cAMP content. Because no change was detected in the AC expression or activity (Fig. 2A and B), the PDE-mediated degradation may be involved in the increase of the cAMP level in cells that overexpress PHD2.

The activity of PDEs can be regulated via protein–protein interactions or through the binding of small ligands [23,24]. Ca^{2+}/calmodulin has been found to associate with PDE1 to modulate its activity [25]. Another well-studied PDE-associated protein, β-arrestin, recruits PDE4D to β2-AR in the plasma membrane and regulates cAMP level [26]. Moreover, the posttranslational modification of PDE also plays a role in the regulation of its activity. To date, the only known posttranslational modification of PDEs is its phosphorylation by kinases, such as protein kinase A [27,28] and the extracellular signal-regulated protein kinase [29,30]. In this study, we first demonstrated that PHD2 could regulate PDE4D protein expression and activity through hydroxylation modification (Fig. 2C and E and Fig. S2). This finding was further corroborated by the formation of a complex between PHD2 and PDE4D (Fig. 2D) and the identification of specific hydroxylation sites on the PDE4D protein (Fig. 3).

In summary, we found that PHD2 modulates the intracellular cAMP level by interacting with PDE4D. The identification of PHD2 as a novel regulator of PDE in this study offers new insights into the cAMP signaling system and may provide potential alternatives for the development of novel therapeutic agents, particularly for the treatment of cardiovascular diseases.

Conflicts of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.005.

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