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MAPK and PI3K pathways regulate hypoxia-induced atrial natriuretic peptide secretion by controlling HIF-1 alpha expression in beating rabbit atria

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ABSTRACT

Mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways are pivotal and intensively studied signaling pathways in hypoxic conditions. However, the roles of MAPK and PI3K in the regulation of hypoxia-induced atrial natriuretic peptide (ANP) secretion are not well understood. The purpose of the present study was to investigate the mechanism by which the MAPK/ERK (extracellular signal-regulated kinase) and PI3K signaling pathways regulate the acute hypoxia-induced ANP secretion in isolated beating rabbit atria. An acute hypoxic perfused beating rabbit atrial model was used. The ANP levels in the atrial perfusates were measured by radioimmunoassay, and the hypoxia-inducible factor-1α (HIF-1α) mRNA and protein levels in the atrial tissue were determined by RT-PCR and Western blot. Acute hypoxia significantly increased ANP secretion and HIF-1α mRNA and protein levels. Hypoxia-induced ANP secretion was markedly attenuated by the HIF-1α inhibitors, rotenone (0.5 μmol/L) and CAY10585 (10 μmol/L), concomitantly with downregulation of the hypoxia-induced HIF-1α mRNA and protein levels. PD098059 (30 μmol/L) and LY294002 (30 μmol/L), inhibitors of MAPK and PI3K, markedly abolished the hypoxia-induced ANP secretion and atrial HIF-1α mRNA and protein levels. The hypoxia-suppressed atrial dynamics were significantly attenuated by PD098059 and LY294002. Acute hypoxia in isolated perfused beating rabbit atria, markedly increased ANP secretion through HIF-1α upregulation, which was regulated by the MAPK/ERK and PI3K pathways. ANP appears to be part of the protective program regulated by HIF-1α in the response to acute hypoxic conditions.

1. Introduction

Restricted oxygen availability is a feature of many physiological and pathological conditions. Hypoxia is a phenomenon in most heart diseases [1], such as myocardial hypertrophy [2] and myocardial infarction [3]. To adapt to hypoxia, mammalian cells induce hypoxia-inducible genes, such as vascular endothelial growth factor (VEGF), various glycolytic enzymes and glucose transporter 1 [4]. The expression of these genes is regulated by hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor composed of HIF-1α and HIF-1β [5], which activity is determined by the stability of the α-subunit [6].

Numerous studies have clearly shown that hypoxia potently stimulates atrial natriuretic peptide (ANP) secretion, resulting in cellular adaptation to hypoxia and protection of the ischemic heart [7–9].However, there are conflicting reports regarding the role of HIF-1α in hypoxia-induced ANP secretion [10–12], thus the mechanism by which hypoxia induces ANP secretion via HIF-1α remains to be defined. It has been demonstrated that the expression and activity of HIF-1α is regulated by major signaling pathways including the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway [13,14] and the phosphatidylinositol 3-kinase (PI3K) pathway [15,16]. In previous studies, it has been observed that the MAPK/ERK and PI3K signaling pathways participate in the regulation of the ANP secretion under normoxic conditions [17,18]. However, the roles of the MAPK/ERK and PI3K pathways in hypoxia-induced ANP secretion are not

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clear. Therefore, the purpose of the present study was to investigate the mechanism by which the MAPK/ERK and PI3K pathways regulate the acute hypoxia-induced ANP secretion in isolated beating rabbit atria.

2. Materials and methods

2.1. Preparation of perfused beating rabbit atria

New Zealand white rabbits of either sex were used, and the mean wet weight of the atrium was 182.5 ± 6.8 mg. Isolated perfused beating left atria were prepared using previously described methods [18,19]. Soon after setting up each perfused atrium, transmural electrical field stimulation with a luminal electrode was started at 1.5 Hz (0.3 ms, 30–40 V), and the atrium was perfused with HEPES buffer solution using a peristaltic pump (1 mL/min) that allowed atrial pacing for measurements of the changes in atrial volume (stroke volume), pulse pressure and ANP secretion. The perfused atrium was supplied with sufficient oxygen during the entire processes. The HEPES buffer contained (in mmol/L) 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 10 glucose, 10 HEPES (pH 7.4 with NaOH) and 0.1% bovine serum albumin.

2.2. Acute hypoxic atrial model preparation

The acute hypoxic atrial model was prepared by replacing O_2 with N_2 gas and the normal HEPES buffer was replaced with N_2-saturated HEPES buffer. The inhibitory atrial dynamics and the markedly increased ANP secretion were taken as criterions for successful hypoxic atrial modeling.

2.3. Experimental protocols

Each atrium was perfused for 60 min to stabilize the parameters of ANP secretion and atrial dynamics. The perfusates were collected at 2 min intervals at 4 °C for measurements of ANP levels.

The control cycle (12 min as an experimental cycle) was followed by infusion of hypoxic buffer for four cycles, to determine the changes in atrial dynamics and the ANP levels in the perfusates. For RT-PCR analysis, immediately after perfusion the atrial tissue was frozen and stored at −80 °C until the analysis was performed.

To investigate the mechanism of hypoxia-induced ANP secretion, another series of experiments was performed. After the control period, two treatment cycles were followed by four cycles of infusion of the treatment agent plus hypoxia. The treatment agents used in the present study were as follows: (1) rotenone (0.5 μmol/L; $n = 6$) and CAY10585 (10 μmol/L; $n = 6$), inhibitors of HIF-1α; (2) PD098059 (30 μmol/L; $n = 6$), a MAPK/ERK pathway inhibitor; (3) LY294002 (30 μmol/L; $n = 6$), a PI3K/Akt pathway inhibitor.

2.4. Radioimmunoassay of ANP concentrations

The levels of immunoreactive ANP in the perfusates were measured by specific radioimmunoassays as described previously [19]. The amounts of secreted immunoreactive ANP are expressed as ng/min/g of wet atrial tissue. Most of the secreted ANP was processed ANP.

2.5. Reverse transcription PCR (RT-PCR)

For total cellular RNA extraction, the abovementioned left atrial tissues stored at −80 °C were lyzed with TRIzol reagent (TRIzol® Reagent, Invitrogen, USA) according to the manufacturer's instructions. The concentration of total RNA was measured at 260 nm. Total RNA samples were run on 1% agarose gels to check their integrity. Two micrograms of total RNA per sample were converted to cDNA by reverse transcriptase using a commercially available reverse transcription (RT) kit (PrimeScript® RT-PCR Kit, TaKaRa, China). RT products were amplified by PCR using rabbit HIF-1α and 18S rRNA (housekeeping gene) specific primers. The PCR primers used and the optimal PCR conditions are listed in Table 1. PCR fragments (10 μl) were analyzed on 2% agarose gels stained with ethidium bromide. Band density was measured by the Image J software. The relative level of the HIF-1α mRNAs was calculated relative to the density of the 18S rRNA level for each sample.

2.6. Western blot analysis

Semiquantitative analysis of proteins of the abovementioned left atrial tissues was per-formed by western blotting. The supernatant was mixed with RIPA Lysis buffer (P0013B, Beyotime institute of Biotechnology, China) and heated for 5 min at 95 °C. The protein concentrations were determined by the BCA method. Extracts were then separated on a SDS–PAGE (8% gel). After electrophoresis, proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) filter membrane (0.45 mm, Beyotime institute of Biotechnology, China) which were blocked with 5% non-fat dry milk in TBST [(mmol/l): Tris–HCl 20 (pH 7.6), NaCl 137, and 0.1% Tween 20]. Primary antibodies to HIF-1α and GAPDH (ab65979 and ab9485,Abcom,-Hong Kong) were diluted in 5% milk/TBST and incubated overnight at 4 °C (1:2000). IgG horseradish peroxidase-linked secondary antibodies was diluted 1:2000. After extensive washing with TBST, the complexes were visualized using enhanced chemiluminescence plus reagent (ECM kit,Boster,China) according to the manufacturer's instructions. The specific bands were quantified using Image J software.

2.7. Statistical analysis

The significance of differences between values was determined by one-way ANOVA followed by Dunnett's multiple comparison test. An unpaired *t*-test was also applied. Statistical significance was defined as $P < 0.05$. All data are presented as mean ± SEM.

Table 1
PCR conditions and primer sequences for HIF-1α and 18S rRNA.

Gene	Sequence	Product (bp)	PCR program (35 cycle)
HIF-1α	Fw: 5′-CCACAGGACAGTACAGGATG-3′ Rev: 5′-TCAAGTCGTGCTGAATAATACC-3′	150 bp	94 °C (3′)/94 °C (30″), 57 °C (30″), 72 °C (1′)/72 °C (5′)
18S rRNA	Fw: 5′-CGGCGACGACCCATTCGAAC-3′ Rev: 5′-GAATCGAACCCTGATTCCCCGTC-3′	99 bp	94 °C (3′)/94 °C (30″), 64 °C (30″), 72 °C (1′)/72C (5′)

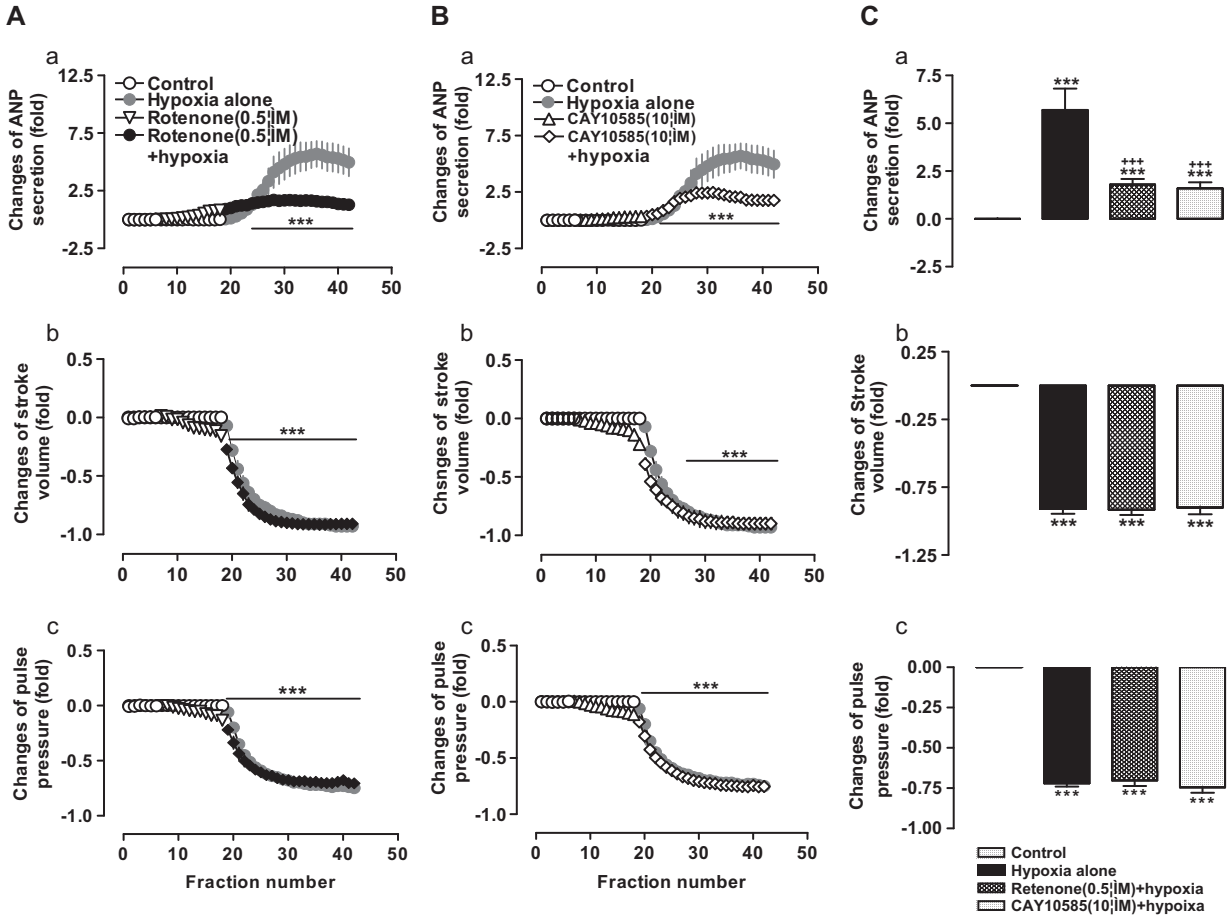

Fig. 1. Effects of HIF-1α inhibitors, rotenone (0.5 μmol/L) and CAY10585 (10 μmol/L), on hypoxia-induced ANP secretion (Aa,Ba, and Ca), stroke volume (Ab, Bb, and Cb) and pulse pressure (Ac, Bc, and Cc). Values of Ca–c are expressed as changes in the mean of the samples exposed to hypoxia compared with the mean of control values. Data are mean ± SEM ($n = 6$). $^{***}P < 0.001$ vs. control period; $^{+++}P < 0.001$ vs. hypoxia alone.

3. Results

3.1. Effect of acute hypoxia on atrial ANP secretion and on atrial dynamics

In the present study, acute hypoxia significantly increased ANP secretion by about 5.7-fold ($n = 6$, $P < 0.001$ vs. control; in Fig. 1Aa) in isolated perfused beating rabbit atria. ANP secretion peaked in the fourth experimental cycle (i.e., the third cycle of hypoxia, about 36 min after hypoxia), and then slightly recovered, though it retained high levels. The atrial dynamics were clearly decreased by acute hypoxia (n = 6, $P < 0.001$ vs. control; Fig. 1Ab–c). The data suggested that the acute hypoxic model of isolated perfused beating rabbit atria was successful.

3.2. Effects of HIF-1α inhibitors on the hypoxia-induced atrial ANP secretion and HIF-1α activation

To investigate the effect of HIF-1α on the hypoxia-induced ANP secretion, another series of experiments were performed in beating rabbit atria. As shown in Fig. 1, in the presence of the HIF-1α inhibitors, rotenone (0.5 μmol/L) or CAY10585 (10 μmol/L), although hypoxia still increased ANP secretion ($n = 6$, $P < 0.001$ vs. control; Fig. 1Aa and Ba), these inhibitors significantly attenuated the effect of hypoxia-induced ANP secretion (from 5.7-fold by hypoxia alone to 1.7 and 2.5-fold with inhibitors plus hypoxia, respectively; $n = 6$, $P < 0.001$ inhibitors plus hypoxia vs. hypoxia alone; Fig. 1Ca) without changing the hypoxia-suppressed atrial dynamics ($n = 6$, $P < 0.001$ vs. control; Fig. 1Ab–c, Bb–c and Cb–c).

Moreover, to determine the effects of rotenone and CAY10585 on the hypoxia-induced HIF-1α mRNA and protein levels in atrial tissues, RT-PCR and Western blot were performed with the corresponding atrial samples. The data showed that HIF-1α mRNA and protein levels were significantly increased by hypoxia ($n = 6$, $P < 0.001$, $P < 0.01$ vs. control, respectively; Fig. 2A and B), but this increase was reversed by rotenone ($n = 6$, $P < 0.01$ rotenone plus hypoxia vs. hypoxia alone, respectively; $P > 0.05$ vs. control; Fig. 2A and B) and CAY10585 ($n = 6$, $P < 0.001$, $P < 0.01$ CAY10585 plus hypoxia vs. hypoxia alone, respectively; $P > 0.05$ vs. control; Fig. 2A and B). The data indicate that HIF-1α is directly involved in the regulation of hypoxia-induced ANP secretion in isolated acute hypoxic beating atria.

3.3. Effects of the MAPK/ERK and PI3K signaling pathways on the hypoxia-induced ANP secretion and HIF-1α activation

To investigate the effects of the MAPK/ERK and PI3K signaling pathways on the hypoxia-induced ANP secretion, a series of experiments were performed on hypoxic atria with PD098059 (30 μmol/L) or LY294002 (30 μmol/L). These inhibitors alone did not modulate the ANP secretion or the atrial dynamics ($n = 6$, $P > 0.05$ vs. control; Fig. 3). In the presence of PD098059 markedly attenuated the hypoxia-induced ANP secretion ($n = 6$, $P < 0.01$ vs. control period, $P < 0.001$ vs. hypoxia alone; Fig. 3Aa and Ca) and the

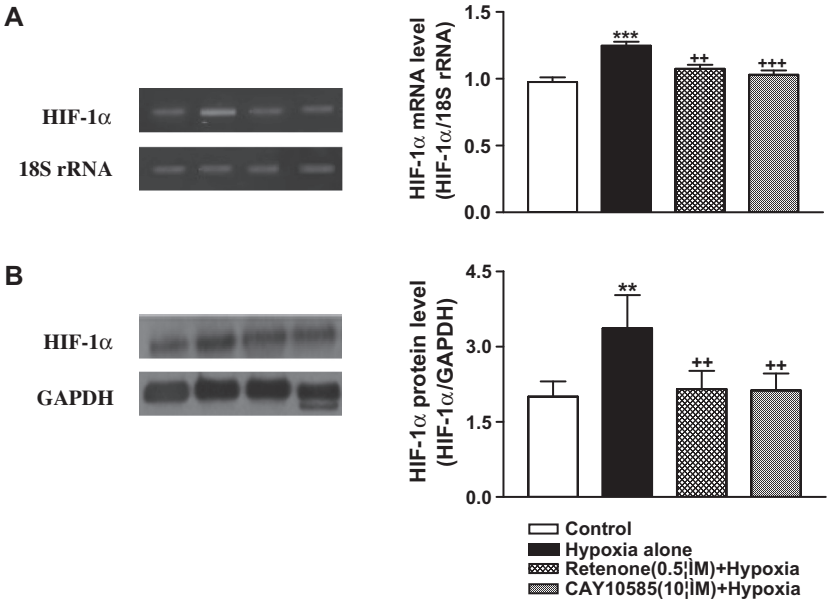

Fig. 2. Effects of HIF-1α inhibitors, rotenone (0.5 μmol/L) and CAY10585 (10 μmol/L), on the hypoxia-induced atrial HIF-1α mRNA and protein levels. Data are mean ± SEM ($n = 6$). $^{***}P < 0.001$ vs. control group; $^{++}P < 0.01$, $^{+++}P < 0.001$ vs. hypoxia alone.

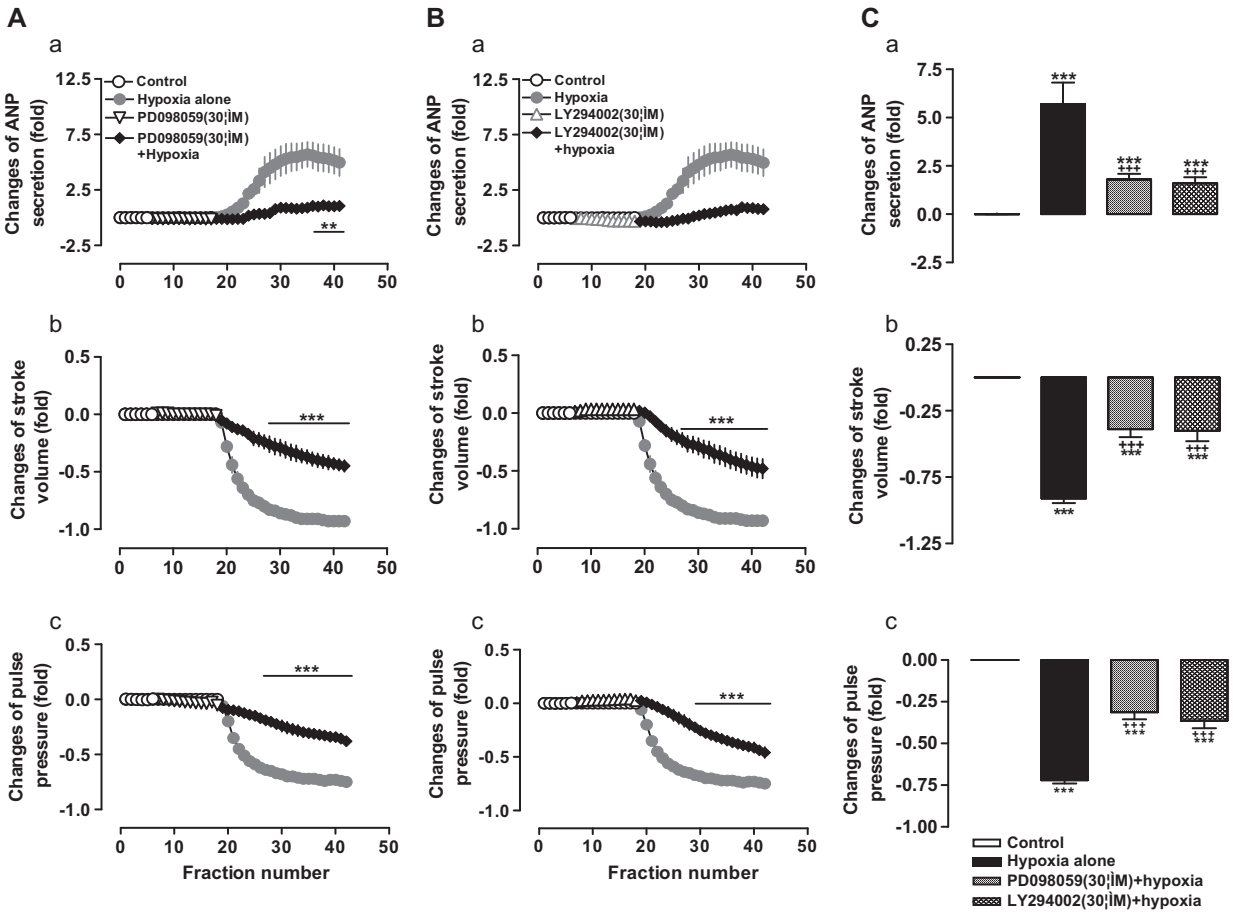

Fig. 3. Effects of the MAPK/ERK inhibitor, PD098059 (30 μmol/L), and the PI3K inhibitor, LY294002 (30 μmol/L), on the hypoxia-induced ANP secretion (Aa, Ba and Ca), stroke volume (Ab, Bb and Cb) and pulse pressure (Ac, Bc and Cc). Values in Ca–c are expressed as changes in the mean of samples exposed to hypoxia compared with the mean of control values. Data are mean ± SEM ($n = 6$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control period; $^{+++}P < 0.001$ vs. hypoxia alone.

hypoxia-suppressed atrial dynamics ($n = 6$, $P < 0.001$ vs. control period, $P < 0.001$ vs. hypoxia alone; Fig. 3Ab–C and Cbc). Furthermore, LY294002 was almost blocked the hypoxia-induced ANP secretion ($n = 6$, $P > 0.05$ vs. control period, $P < 0.001$ vs. hypoxia

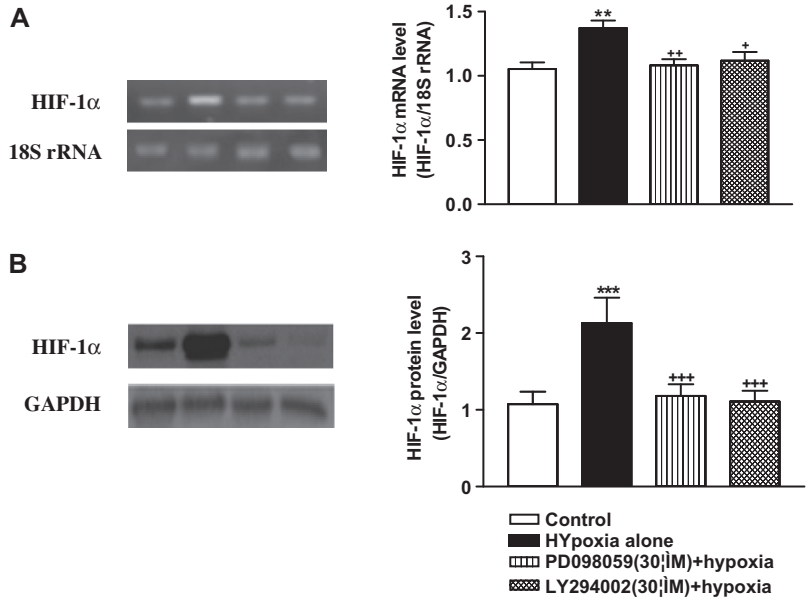

Fig. 4. Effects of the MAPK/ERK inhibitor, PD098059 (30 μmol/L), and the PI3K/Akt inhibitor, LY294002 (30 μmol/L), on the hypoxia-induced atrial HIF-1α mRNA and protein levels. Data are mean ± SEM ($n = 6$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ vs. hypoxia alone.

alone; Fig. 3Ba and Ca), and the hypoxia-suppressed atrial dynamics were significantly attenuated ($n = 6$, $P < 0.001$ vs. control period, $P < 0.001$ vs. hypoxia alone; Fig. 3Bb–c and Cb–c).

To investigate the mechanism by which the MAPK/ERK and PI3K signaling pathways regulate hypoxia-induced ANP secretion, the HIF-1α mRNA and protein levels were performed with the corresponding atrial tissues by RT-PCR and Western blot. As shown in Fig. 4, acute hypoxia significantly increased atrial HIF-1α mRNA and protein levels ($n = 6$, $P < 0.01$, $P < 0.001$ vs. control, respectively; Fig. 4A and B). This increase was completely abolished by PD098059 ($n = 6$, $P < 0.01$, $P < 0.001$ vs. hypoxia alone, respectively) and LY294002 ($n = 6$, $P < 0.05$, $P < 0.001$ vs. hypoxia alone, respectively) on HIF-1α mRNA and protein levels. These findings suggest that the MAPK/ERK and PI3K signaling pathways regulate hypoxia-induced ANP secretion by controlling the expression of HIF-1α in isolated beating acute hypoxic atria.

4. Discussion

The present study demonstrated that acute hypoxia markedly increased ANP secretion in isolated perfused beating rabbit atria. Hypoxia-induced ANP secretion was regulated by the MAPK/ERK and PI3K signaling pathways by controlling HIF-1α expression.

HIF-1 is a transcription factor that regulates oxygen homeostasis and plays key roles in development, physiology and diseases. HIF-1 activity is induced in response to hypoxia and mediates the adaptive response to hypoxia by regulating the expression of multiple genes that encode key components of the response pathway [20]. It has been demonstrated that hypoxia induces HIF-1α activity, which is involved in the regulation of cardiac natriuretic peptide secretion [10–12]. The present study showed that acute hypoxia markedly increased ANP secretion and HIF-1α mRNA and protein levels concomitantly with significantly decreasing atrial dynamics. The hypoxia-induced ANP secretion was markedly attenuated by the HIF-1α inhibitors, rotenone and CAY10585, along with downregulation of the hypoxia-induced HIF-1α mRNA and protein levels. Consequently, these findings indicate that HIF-1α participates in the regulation of hypoxia-induced ANP secretion in isolated beating rabbit atria. The results of the present study are consistent with a previous study that showed that HIF-1α is a direct modulator of hypoxia-induced ANP secretion [12], but are inconsistent with previous reports, which demonstrated that hypoxia-induced ANP secretion is indirectly [21] or not modulated by HIF-1α [10]. The reason for these conflicting observations is not fully understood, but may be related to different experimental models and/or methods.

It has been demonstrated that the intensively studied MAPK and PI3K signaling pathways are pivotal pathways [22,23]. These signaling pathways are at the heart of a molecular signaling network that governs growth, proliferation, differentiation and survival in many, if not all, cell types. They are deregulated in various diseases, ranging from cancer to immunological, inflammatory and degenerative syndromes, and thus represent an important drug target. Furthermore, previous studies have shown that hypoxia activates the MAPK and PI3K signaling pathways to modulate HIF-1α activity in the heart [24,25]. Consistent with previous studies [24,25], the present study also observed that acute hypoxia significantly increased atrial HIF-1α mRNA and protein levels in isolated beating rabbit atria, which was completely abolished by MAPK/ERK inhibitor PD098059 and PI3K inhibitor LY294002. Moreover, in the present study, the hypoxia-induced ANP secretion was almost blocked by LY294002 and markedly attenuated by PD098059 via modulation of the HIF-1α mRNA and protein levels. These findings indicate that the MAPK/ERK and PI3K signaling pathways are vital in the regulation of hypoxia-induced ANP secretion in isolated perfused beating rabbit atria, which is consistent with previous studies showing that the MAPK/ERK and PI3K signaling pathways participate in the regulation of G protein-coupled receptors and receptor tyrosine kinases that mediate ANP secretion under normoxic conditions [17,18].

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