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Nur77 induced by HIF-1α mediates vascular remodeling in hypoxic pulmonary hypertension

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Abstract

Nur77 is a member of the NR4A subfamily of orphan nuclear receptors that is expressed and has a function within the immune system. This study aimed to investigate the role of Nur77 in hypoxic pulmonary hypertension. SPF male SD rats were exposed in hypobaric chamber simulating 5000 m high altitude for 0, 3, 7, 14, 21 or 28 days. Rat pulmonary artery smooth muscle cells (RPASMCs) were cultured under normoxic conditions (5% CO_2-95% ambient air) or hypoxic conditions (5% O_2 for 6 h, 12 h, 24 h, 48 h). Hypoxic rats developed pulmonary arterial remodeling and right ventricular hypertrophy with significantly increased pulmonary arterial pressure. The levels of Nur77, HIF-1α and PNCA were upregulated in pulmonary arterial smooth muscle from hypoxic rats. Silencing of either Nur77 or HIF-1α attenuated hypoxia-induced proliferation. Silencing of HIF-1α down-regulated Nur77 protein level, but Nur77 silence did not reduce HIF-1α. Nur77 was not co-immunoprecipitated with HIF-1α. This study demonstrated that Nur77 acted as a downstream regulator of HIF-1α under hypoxia, and plays a critical role in the hypoxia-induced pulmonary vascular remodeling, which is regulated by HIF-1α. Nur77 maybe a novel target of HPH therapy.

Keywords: Orphan nuclear receptor Nur77; Hypoxia-induced factor-1α; Hypoxia; Pulmonary hypertension

1. Introduction

Hypoxic pulmonary hypertension (HPH) is a complex, multidisciplinary disorder, characterized by pulmonary artery constriction at early phase and profound pulmonary vascular remodeling. The restricted flow through the pulmonary arterial circulation results in increased pulmonary vascular resistance, ultimately in right heart failure, and premature death [1]. The histopathological hallmark of pulmonary hypertension (PH) is pulmonary vascular remodeling as well as the complex vascular plexiform lesion formation [2]. Hypoxia-induced pulmonary vascular remodeling is marked by structural alterations in small arteries due to excessive proliferation, migration and apoptotic resistance of pulmonary artery smooth muscle cells (PASMCs) [3, 4]. Despite many years of researches about the effect of hypoxia on the development of PH, the underlying molecular mechanisms of HPH are not completely understood.

Nur77, one of the members of the nuclear receptor 4A (NR4A) family, is an immediately-early gene that could be activated by many physiological stimuli [5, 6]. Accumulating evidences have shown that Nur77 plays critical roles in regulating cell proliferation, differentiation, migration, and apoptosis [7-10]. It has been verified that Nur77 is expressed aberrantly in lung cancer cell lines, and ARDS [11-13]. Moreover, in vascular smooth muscle cells, the expression of Nur77 was significantly induced by mitogenic stimuli [8, 14]. From two monocrotaline-induced pulmonary arterial hypertension (PAH) experimental models, the expression of Nur77 was inconformity [15, 16]. Therefore, the functional role of Nur77 in the development of HPH needs further exploration.

Hypoxia-induced factor-1α (HIF-1α), a master regulator of transcriptional responses to hypoxia, plays a smooth-specific and cell-autonomous role in the pathogenesis of HPH [17]. The direct downstream regulation of Nur77 by HIF-1α has been confirmed in renal carcinoma cell lines [18], and in turn, Nur77 has been reported to stabilize HIF-1α [19, 20]. Hence, we hypothesized that Nur77 mediates vascular remodeling in hypoxic pulmonary hypertension,

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which is induced by HIF-1α, and expect to explore a novel potential therapeutic target in the treatment of HPH.

2. Materials and Methods

2.1. Animals

This study was approved by the Institutional Review Board of the Third Military Medical University. Seventy-eight SPF male Sprague-Dawley (SD) rats (250-300g) were purchased from the Laboratory Animal Center, Third Military Medical University. Care and use of the animals complied with the Chinese Association for Laboratory Animal Science Policy. All rats were evenly randomized into six groups: normoxia group and hypoxia groups maintained at an altitude of 5000 m for 3 days, 7 days, 14 days, 21 days and 28 days.

2.2. Hemodynamic Measurement

SD rats were anesthetized with an intraperitoneal injection of chloral hydrate (300 mg/kg). A PE-50 pressure catheter (OD 0.9 mm, ID 0.5 mm, length 15 cm) was inserted via the right jugular vein, adjusted through the right atrium and the right ventricle and ultimately positioned at the pulmonary artery. The pulmonary artery pressure and right ventricular pressure were measured with a pressotransducer connected to the catheter by a NO.7 syringe needle.

2.3. Histology Analysis and Immunostaining

After hemodynamic assessment, the lungs of all rats were harvested for histology analysis and immunostaining. The paraffin-embedded lung section was stained with anti-Nur77 antibody (1:100; #sc-365113; Santa Cruz, Santa Cruz, CA, USA), anti-HIF-1α antibody (1:200; #ab2185; Abcam, Cambridge, MA, USA), anti-proliferating cell nuclear antigen (PCNA) (1:200; #2586; CST, Danvers, MA, USA), and anti-smooth muscle actin (SMA). The stain sections were visualized with light microscopy (Nikon, Tokyo, Japan).

2.4. Right Ventricular Hypertrophy Index

To assess the degree of right ventricular hypertrophy, the right ventricle (RV) was dissected from the left ventricle (LV) and the septum (S), and weighed separately. The right ventricular hypertrophy index (RVHI) was calculated by the ratio of RV/(LV+S).

2.5. Cell Culture and Experiments

The primary rat pulmonary artery smooth muscle cells (RPASMCs) were purchased from the Beijing Bena Culture Collection Biotechnology Research Institute. All cells were cultured in smooth muscle cell medium containing smooth muscle cell growth supplement. Cells were maintained under normoxic conditions (5% CO_2-95% ambient air) or hypoxic conditions (5% O_2 for 24 h). The small interfering RNAs (siRNAs) against Nur77 (human 5'-GGGCATGGTGAAGGAAGTT-3') or HIF-1α (human 5'-GCUGAUUUGUGAACCCAUUTT-3') and the non-target control siRNA were designed and synthesized by GenePharma (China).

2.6. Cell Proliferation Experiment

Cell proliferation was assessed by a Counting Kit (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan), and the RPASMCs were exposed to normoxia or hypoxia for 6 h, 12 h, 24 h and 48 h according to the manufacturer's instruction.

2.7. RNA Isolation and RT-qPCR Analysis

For RT-qPCR analysis, total RNA was extracted from the separated pulmonary artery tissues and cultured RPASMCs using TRIzol reagent (TaKaRa, Dalian, China) following the manufacturer's instruction. mRNA levels were measured using an SYBR Green real-time quantitative PCR kit (TaKaRa, Dalian, China) and analyzed by an ABI Prism Fast 7500 system (Applied Biosystems, Foster City, CA, USA). 18s rRNA mRNA served as an internal control. For pulmonary arteries tissues, the sequences of the primers are as follows: Rat Nur77 forward 5'-GCTCATCTTCTGCTCAGGCCT-3' and reverse 5'-CAGACGTGACAGGCAGCTGGC-3'; HIF-1α forward 5'-GGACAAGTCACCACAGGACA-3' and reverse 5'-GGGAGAAAATCAAGTCGTGC-3'; PCNA forward 5'-ATCCTGAAGAAGGTGCTGGA-3' and reverse 5'-GCTGCACTAAGGAGACGTGA-3'; 18s rRNA forward 5'-TGAGAAACGGCTACCACATCC-3' and reverse 5'-GCACCAGACTTGCCCTCCA-3'. And for RPASMCs, the sequences of the primers are as follows: human Nur77 forward 5'-AACCCAAGCAGCCTCCAGAT-3' and reverse 5'-CTGCCCACTTTCGGATAACG-3'; HIF-1α forward 5'-GGACAAGTCACCACAGGACA-3' and reverse 5'-GGGAGAAAATCAAGTCGTGC-3'; PCNA forward 5'-ACACTAAGGGCCGAAGATAACG-3' and reverse 5'-ACAGCATCTCCAATATGGCTGA-3'; 18s rRNA forward 5'-CGGCTACCACATCCAAGGAA-3' and reverse 5'-CTGGAATTACCGCGGCT-3'.

2.8. Western Blot Assay and Coimmunoprecipitate

For protein expression analysis, the frozen separated pulmonary arteries tissues [21] and cultured RPASMCs were lysed in ice-cold RIPA lysis buffer (1% Triton X-100, 1% deoxycholate, 0.1%SDS). For coimmunoprecipitate analysis, the RPASMCs were isolated by 1% NP-40 lysis buffer. The primary antibodies were used as follows: anti-Nur77 antibody (1:500; #sc-365113; Santa Cruz, Santa Cruz, CA, USA), anti-HIF-1α antibody (1:1000; #04-1006; Millipore, Billerica, MA, USA), anti-PCNA antibody (1:1500; #2586; CST, Danvers, MA, USA) and anti-β-actin antibody (1:500; Santa Cruz, Santa Cruz, CA, USA).

2.9. Statistical Analysis

Quantitative data are presented as mean ± SD (standard deviation). Statistical analysis was carried out using Statistic Package for Social Science (SPSS) 22.0 (IBM, Armonk, NY, USA) and GraphPad Prism 5.0 (La Jolla, CA, USA). Comparisons between 2 groups were analyzed with Student's t-test, and one-way analysis of variance with Dunnett's was used to analyse multiple groups. Differences were considered to be significant at $P<0.05$.

3. Results

3.1. Hypobaric hypoxia increases the pulmonary artery pressure and induces pulmonary vascular remodeling

To verify that the hypoxic pulmonary hypertension rat model was established and get the degree of pulmonary hypertension, we used a catheter to measure the pulmonary artery pressure. Mean pulmonary artery pressure (mPAP) and pulmonary artery systolic pressure (PASP) were elevated significantly after 7 days of exposure to hypoxia

(Figure 1A). Pathological staining showed that pulmonary artery walls were thickened after 7 days of exposure to hypoxia (Figure 1B).

Hypoxia-induced pulmonary artery hypertension was indirectly verified by right ventricular remodeling. The mean right ventricular pressure (mRVP) also was elevated significantly after 7 days (Figure 1C), and right ventricular systolic pressure was elevated after 14 days exposed to hypoxia. Right ventricle weight was increased remarkably after 14 days of exposure to hypoxia. Right ventricular hypertrophy index (RVHI) was increased significantly after 14 days of exposure to hypoxia (Figure 1C).

3.2. Hypobaric hypoxia upregulated Nur77 expression in pulmonary

The protein and mRNA levels of Nur77 were increased in pulmonary after 3 days of exposure to hypoxia (Figure 2A, B). However, the mRNA levels of Nur77 were decreased after 28 days in hypoxic pulmonary hypertension rats (Figure 2C). The protein and mRNA levels of PCNA were increased after 3 days and 7 days exposed to hypoxia. The same with Nur77, the protein and mRNA expressions of HIF-1α increased after 3 days of exposure to hypoxia. However, the mRNA expression decreased after 28 days in hypoxic pulmonary hypertension rats (Figure 2A, B, C).

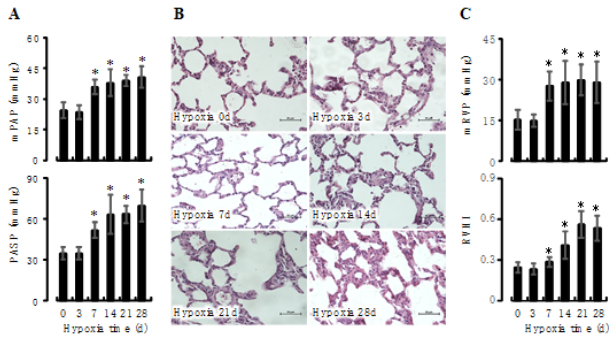

Fig. 1. Effect of hypoxia on pulmonary artery pressure and pulmonary vascular wall. (A) Mean pulmonary artery pressure (mPAP) and pulmonary artery systolic pressure (PASP) elevate as hypoxia time prolongs. (B) Pathological change of pulmonary artery walls thickens as hypoxia time prolongs (stained for hematoxylin-eosin, magnification, ×40). (C) Mean right ventricular pressure (mRVP) and right ventricular hypertrophy index (RVHI) elevate as hypoxia time prolongs. Data are expressed as mean ± SD; $n = 13$. *$P < 0.05$. mPAP, mean pulmonary artery pressure; PASP, pulmonary artery systolic pressure; mRVP, mean right ventricular pressure; RVHI, right ventricular hypertrophy index.

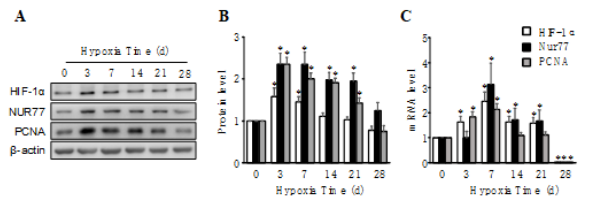

Fig. 2. Effect of hypoxia on HIF-1α, Nur77 and PCNA expression. (A) and (B) The protein expressions of HIF-1α, Nur77 and PCNA as hypoxia time prolongs. (C) The mRNA expressions of HIF-1α, Nur77 and PCNA as hypoxia time prolongs. Data are expressed as mean ± SD; $n = 13$. *$P < 0.05$. HIF-1α, hypoxia-induced factor-1α; PCNA, proliferating cell nuclear antigen.

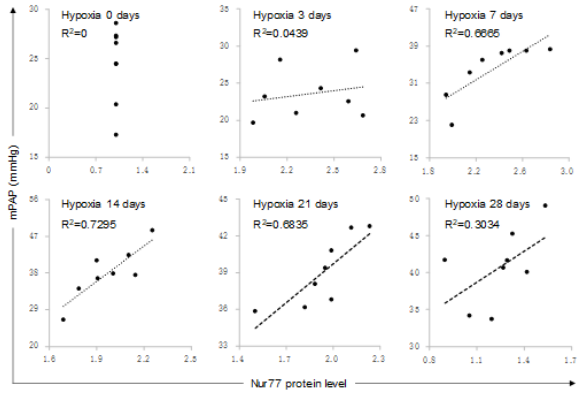

Fig. 3. The correlation between Nur77 protein level and mPAP in 0, 3, 7, 14, 21, 28 days after exposure in hypobaric chamber. mPAP, mean pulmonary artery pressure. R^2, coefficient of determination.

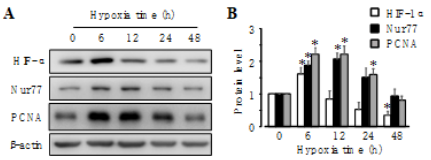

Fig. 4. HIF-1α, Nur77 and PCNA expression in RPASMCs as hypoxia time prolongs. Data are expressed as mean ± SD; $n = 13$. *$P < 0.05$.

3.3. Hypoxia-induced Nur77 expression was relevant to pulmonary vascular remodeling and hypoxia pulmonary hypertension

The protein levels of Nur77 were related to mPAP in 7, 14, and 21 days after exposed in hypobaric chamber, but not in 0 and 28 days (Figure 3).

3.4. Hypoxia-induced Nur77 expression was synchronized with PCNA in RPASMCs

Cultured RPASMCs expressed Nur77, and which was increased after culturing in 5%O_2 chamber for 6 hours (Figure 4A, B), so were HIF-1α and PCNA (Figure 4A, B). The change of Nur77 was synchronized with PCNA and HIF-1α (Figure 4B).

3.5. Nur77 played a downstream regulator of HIF-1α in hypoxic pulmonary hypertension

Coimmunoprecipitation analysis showed that Nur77 and HIF-1α did not directly combine in vivo (Figure 5A). Further, under hypoxic conditions, the expression of Nur77 was decreased when silencing HIF-1α by siRNA, and the expression of PCNA was decreased as well in RPASMCs (Figure 5B). On the contrary, the expression of HIF-1α was changed little when silencing Nur77 by siRNA, while the expression of PCNA was decreased going with the deregulation of Nur77 (Figure 5C).

4. Discussion

This study determined that Nur77 mediates vascular remodeling in hypoxic pulmonary hypertension, which is induced by HIF-1α. In our experiment, we demonstrated that hypoxia could induce pulmonary artery hypertension. We successfully established the pulmonary hypertension

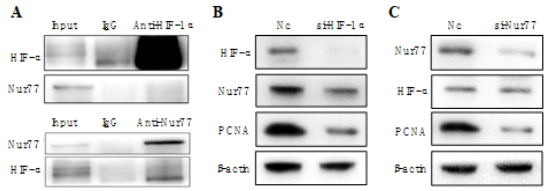

Fig. 5. The relationship of HIF-1α and Nur77 in hypoxic pulmonary hypertension. (A) Coimmunoprecipitation analysis shows that Nur77 and HIF-1α did not directly combinate. (B) and (C) The expression of HIF-1α, Nur77 and PCNA when silencing HIF-1α or Nur77. Anti-HIF-1α, HIF-1α antibody; Anti-Nur77, Nur77 antibody; Nc, normal control; si-HIF-1α, siRNA against HIF-1α; si-Nur77, siRNA against Nur77.

rats model by hypoxic conditions, since mean pulmonary artery pressure elevated and right ventricular thickened as hypoxia time prolonged.

4.1. Nur77 and proliferation

Then, we found that Nur77 mediated vascular remodeling in hypoxic pulmonary hypertension, which was characterized by excessive proliferation of RPASMCs. Hypoxia-induced the high expression of Nur77 in the hypoxic pulmonary hypertension rats and RPASMCs. The silence of Nur77 decreased the proliferation of RPASMCs. At the same time, pathological changes showed that pulmonary artery wall thickened and remodeled, along with increased PCNA expression as hypoxia time prolongs.

4.2. Hypoxia and Nur77

Nur77 is highly expressed in multiple cancer cell lines and tumors, including lung, colon, bladder, and pancreatic tumors [11, 22]. Conversely, investigations of hematologic neoplasms have indicated that Nur77 promotes cell apoptosis [23], implying that Nur77 has cell-specific bidirectional functions. Although the expression of Nur77 was inconformity as reported above [15, 16], in our study, hypoxia increased Nur77 protein and mRNA levels in both isolated pulmonary arteries and cultured RPASMCs. As was shown in the present study, hypoxia-induced excessive proliferation of RPASMCs, which was consistent with the significantly increased expressions of PCNA in the pulmonary arteries and cultured RPASMCs. In turn, siRNAs against Nur77 attenuated the increased protein and mRNA levels and decreased the proliferation of RPASMCs. These findings suggest that Nur77 is involved in the development of HPH by promoting pulmonary vascular proliferation.

4.3. HIF-1 and proliferation

In consistency with Nur77 expression, we demonstrated that HIF-1α was upregulated in the hypoxic pulmonary hypertension rats and RPASMCs. And the silence of HIF-1α decreased the proliferation of RPASMCs. HIF-1α, a master transcription factor in hypoxia, regulates multiple genes responsible for energy metabolism, proliferation, migration and apoptosis [24, 25]. In HPH, HIF-1α acted as a regulator of vascular remodeling [18].

4.4. Nur77 and HIF-1

Interestingly, protein and mRNA levels of Nur77, HIF-1α and PCNA decreased in the 28th day rather than increased. High-altitude adaptation to hypoxia might be responsible for the phenomenon. A high-frequency missense mutation in the EGLN1 gene, which encodes prolyl hydroxylase 2 (PHD2) contributes to high-altitude adaptation. PHD2 triggers the degradation of HIFs, which mediate many physiological responses to hypoxia [26-28]. Certain mechanism still needs further investigation.

At the end of the experiment, we verified that Nur77 played a downstream regulator of HIF-1α in hypoxia-induced RPASMCs by gene silencing. HIF-1α did not directly interact with Nur77 as was shown in coimmunoprecipitate, demonstrating a possibly third substance between the HIF-1α and Nur77. And HIF-1α upregulated the expression of Nur77 in RPASMCs. The knockdown of Nur77 with siRNA did not affect the expression of HIF-1α, while siRNA against HIF-1α decreased the expression of Nur77 in the RPASMCs. Deregulation of Nur77 or HIF-1α decreased the expression of PNCA. Therefore, Nur77 plays a critical role in hypoxia-induced vascular remodeling, which was regulated by HIF-1α. The interaction between Nur77 and HIF-1α has been investigated firstly in renal carcinoma cell lines [18], indicating that Nur77 acted as a transcription target of HIF-1α. Recently, a study involving non-small cell lung cell lines revealed that hypoxia-induced down-regulation of Nur77 was mediated by HIF-1α [11].

5. Conclusion

Our findings provide experimental evidence for the role of Nur77 in hypoxia-induced vascular remodeling. In the future, Nur77 may become a novel target of HPH therapy.

Ethical approval

All animal housing and experiments were conducted in strict accordance with the institutional guidelines for care and use of laboratory animals. and certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki.

Data availability

The datasets used and analyzed during the current study were available from the corresponding author on reasonable request.

Author Contributions

Congzheng Mao: design of the work; project administration; data acquisition; writing original draft. Jian Huang: data acquisition; writing original draft; methodology; writing review and editing; Haichao Liu: methodology and statistical analysis, writing and editing. Yi Liu: methodology and data curation; validation. Zhenhong Hu: statistical analysis, writing and editing; Ruijuan Xu: methodology and data curation; validation. All authors approved the submitted version.

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Conflict of interest

On behalf of all co-authors, we declare no competing fi-

nancial interests in the submission of this manuscript, and manuscript is approved by all authors for publication.

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