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Original Article

Roles of heat shock protein 70 toward hypoxia-inducible factor 1α (HIF-1α) blockade in newborn rats with hypoxia-induced pulmonary hypertension

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Abstract: The aim of the present study was to investigate the roles of adenovirus-mediated heat shock protein 70 (HSP70) in newborn rats with hypoxia-induced pulmonary hypertension (HPH). A total of 128 newborn rats were randomly divided into an HPH group (H) and control group (C), with 8 rats in each group. According to transfection solutions received, group H was subgrouped into three different groups: Group H1, which received saline, group H2, which received transfection of an empty virus, and group H3, which received transfection of HSP70 virus. Subsequently, pulmonary artery pressure (mPAP) was measured in all groups and levels of HSP70, hypoxia-inducible factor 1α (HIF-1α), endothelin-1 (ET-1), and inducible nitric oxide synthase (iNOS) were measured at different time points. mPAP in groups H1 and H2 at 3 days, 7 days, 10 days, and 14 days was significantly higher than in group C ($P<0.05$). Furthermore, immunohistochemical expression intensity of HSP70 in group H3 on days 3, 7, and 10 was significantly enhanced ($P<0.05$), compared to groups H1 and H2. However, expression of HIF-1α, ET-1, and iNOS in group H3 was reduced ($P<0.01$). Intergroup comparisons of HSP70 expression determined that expression of HSP70 in group H3 was enhanced ($P<0.05$), however expression of HIF-1α, ET-1, and iNOS was reduced ($P<0.05$), compared to groups H1 and H2. Results of current study demonstrate that expression of adenovirus-mediated HSP70 in newborn HPH rats may downregulate expression of HIF-1α, ET-1, and iNOS, reducing mPAP. Therefore, adenovirus-mediated HSP70 may be developed as a novel therapeutic method of treating neonatal HPH.

Keywords: Heat shock protein 70, adenovirus, hypoxia-inducible factor 1α, hypoxia induced pulmonary hypertension, newborn rats

Introduction

Hypoxia-induced pulmonary hypertension (HPH) of newborns is a common acute critical illness. Incidence of HPH in newborns is 2-6/1000 live births. Early pulmonary vascular spasms are reversible with timely treatment. In advanced pulmonary vascular remodeling, persistent pulmonary hypertension of the in newborn (PPHN) [1] is often developed. It is difficult to correct the hypoxic respiratory failure and it is difficult to treat the mortality rate of newborns when PPH is high, reaching to 5~30%. It has been demonstrated that hypoxia inducible factor 1α (HIF-1α) is a key transcription factor of hypoxia [2], serving important roles in the pathogenesis of HPH [3-5]. HIF-1α regulates expression of its downstream target genes endothelin 1 (ET-1; a vasoconstrictor) and nitric oxide (NO; a vasodilator) [6, 7]. Current treatments for HPH are symptomatic [8, 9] and exhibit poor efficacy. Treatment of ET-1 antagonists [10] is still in the preclinical phase, restricted in neonates because of only oral dosage forms. Inhalation of NO [11] can be used for treatment of full-term infants with HPH, but the source of gas is difficult to obtain and preserve. Higher levels of medical technology with expensive equipment and some clinical contraindications also limit its application in neonates. Inducing the degradation of HIF-1α, reducing its expression, inhibiting the activities of ET-1 and inducible nitric oxide synthase (iNOS), preventing pulmonary vascular spasms, remodeling and reducing mean pulmonary artery pressure (mPAP), and preventing the development of HPH may be

novel therapeutic strategies for treatment of neonatal HPH. Heat shock protein 70 (HSP70) [12] promotes the degradation of HIF-1α under hypoxic conditions [13]. Therefore, the current study used gene transfection with adenovirus as the vector to increase HSP70 expression in lung tissues. The aim of the current study was to identify whether HSP70 expression down-regulates expression of HIF-1α, ET-1, and iNOS, while reducing mPAP, thus inhibiting the development of HPH.

Materials and methods

Animals

A total of 128 healthy Wistar neonatal rats [7-10 days old; weighing 30-40 g; animal license number, SCXK (Xin2011-0004)] and 16 mother rats [60-70 days old; weighing 230-250 g] were provided by the Animal Experiment Center, the Medical Research Center of the First Affiliated Hospital of Xinjiang Medical University (Xinjiang, China). Rats were randomly divided into two groups (n=8 per subgroup): HPH group (H) and control group (C). According to transfection solutions received, rats in group H were subgrouped into 3 groups: Group H1 received a saline, group H2 received an empty virus, and group H3 received Ad-HSP70 virus. Rats in group H were injected with 5 μl 1010 PFU/ml Ad-HSP70 virus and Ad-GFP (Hanbio Biotechnology, Shanghai, China) viral transfection solution or sterile saline, via the tail veins. Rats were not anesthetized prior to this procedure. The HPH model was established immediately following injections [14]. Transfected neonatal rats were placed, together with their mothers, in a normal-pressure hypoxic cabin infused with 8% nitrogen-oxygen mixture gas. An oxygen concentration monitor (CY-100B, Lihua Science & Technology Co. Ltd., Hangzhou, China) was used for 8 hours of monitoring of oxygen concentrations, maintaining it at 8-10%. All rats were subjected to a 12-hour light-dark cycle and, apart from the absence of hypoxic conditions in group C, rats in all groups were kept under the same conditions (temperature was 21-25°C, relative humidity was 55%-60%, rats were fed with basic feed, sterilized sterile water, and free feeding water). The current study was performed in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health [15]. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Xinjiang Medical University.

Measurement of mPAP

After being kept in hypoxic conditions for 3, 7, 10, and 14 days, neonatal rats in each group (n=8 per subgroup) were conventionally anesthetized (intraperitoneal injection of ketamine (75 mg/kg), atropine (0.375 mg/kg), and diazepam (7.5 mg/kg) mixture by tail veins). They were fixed, sterilized, and underwent endotracheal intubation. One small animal ventilator (HX-200, Taimeng Technology Co. LTd., Chengdu, China) was then connected. Respiratory rate was kept at 120 beats/minute and tidal volume at 4 mL/minute. It was ensured that the thorax remained in symmetric fluctuation and in sync with the ventilator. Subsequently, one U-shaped incision from the right sternal margin of the chest was performed to expose the heart to the pulmonary root. A 4.5 needle was then used to pierce towards the pulmonary arterial root inverse to the direction of blood flow. The pressure sensor was then quickly connected to record mPAP.

Immunohistochemical assay

Newborn rats were killed immediately after the pressure measurement and the right upper lung tissue was quickly cut. Labeling was carried out and by liquid nitrogen was transported to the negative 20-degree refrigerator for preservation. Thickness of resulting sections was 4um and fixed in 4% paraformaldehyde for 24 hours at 4 degrees. This was followed by gradient ethanol dehydration, paraffin embedding, slicing, patching, and hot antigen repair. A total of 8 slices were taken from lung tissue of each newborn rat. Following kit instructions, levels of HSP70, HIF-1α, ET-1, and iNOS in lung tissues were detected using specific antibodies. Phosphate-buffered saline was used instead of the primary antibody as a negative control. For staining, 3,3'-diamino-diphenyl-diamine HCl (0.01 mol/l) was used. Sections were scored in accordance with the degree of the cells positive staining: Colorless, 0 point; Yellow, 1 point; Brown, 2 points; and Tan, 3 points. Sections were also scored according to the proportion of positive cells: 1-25%, 1 point; 26-50%, 2 points; 51-75% 3 points; >75%, 4 points. The product

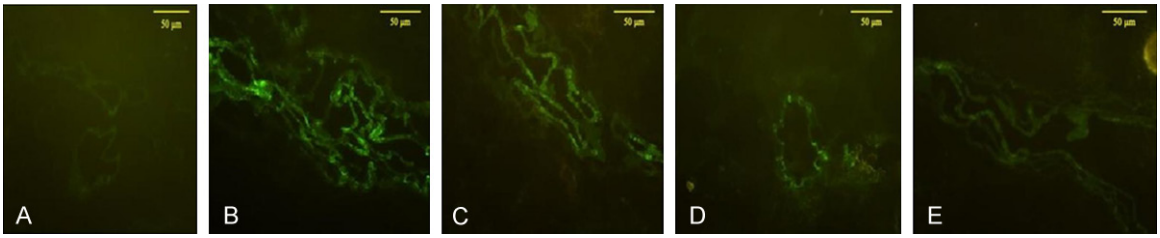

Figure 1. Localization of heat shock protein 70 in lung tissue from rats, as determined using an immunofluorescent microscope (magnification, ×200). A. Control group; B. H3 group, day 3; C. H3 group, day 7; D. H3 group, day 10; E. H3 group, day 14.

Table 1. Changes in mean pulmonary artery pressure in the four groups of newborn rats (n=8 in each group)

Day	Group								*p*-value
	H1		H2		H3		C		
	M	Q	M	Q	M	Q	M	Q	
3	12.00	2.50[a]	13.50	2.00[a]	8.50	4.00[b,c]	9.50	4.75	<0.01
7	15.00	2.00[a]	15.50	1.75[a]	10.50	1.00[b,c]	10.50	1.00	<0.01
10	18.00	1.75[a]	18.00	1.00[a]	13.00	1.00[b,c]	13.00	1.00	<0.01
14	20.00	2.25[a]	22.00	4.25[a]	20.00	5.25[a]	15.50	3.25	<0.05

[a]P<0.05 vs. group C; [b]P<0.05 vs. group H1, [c]P<0.05 vs. group H2. Group H1, HPH group receiving saline group; group H2, an empty virus-transfected HPH group; group H3, a virus heat shock protein 70-transfected group; C, control group. M, median; Q, interquartile range. HPH, hypoxia-induced pulmonary hypertension.

of staining intensity and positive area ratio was used as the final score: 0 point, negative (-); 1-2 points, weak positive (+); 3-4 points, positive (++); >4 points, strongly positive (+++) with 400-fold optical microscope (PIPS-2020 pathological image analysis software: Polaroid, USA).

Western blotting

Following sacrifice, ~100 mg of right lung tissue was rapidly sampled from each rat. This was followed by extraction of total protein using a nucleus and cytoplasm protein extraction kit (78835; Thermo Fisher Scientific, Inc., Waltham, MA, USA), following manufacturer instructions. Lamin A/C was selected as an internal standard of nuclear proteins and β-actin was selected as the internal standard of cytoplasmic proteins. Bicinchoninic acid method (BCA) was used to determine protein concentrations. Proteins were then quantified and degraded and underwent SDS-polyacrylamide gel electrophoresis (30 mA constant current). Proteins were then transferred to PVDF membranes for 1-2 hours at 100 V (wet method, 283BR11735 electrophoresis, electroporation instrument, Bio-Rad Laboratories, Inc., Hercules, CA, USA). They were closed at room temperature for 1 hour and incubated with one antibody: HSP70: 1:1,000, β-actin: 1:400, LaminA/C: 1:1000, HIF-1α: 1:500, iNOS: 1:200, ET-1: 1:500 (Thermo, USA) at 4°C overnight. Subsequently, the secondary antibody (WB7105 Western blot secondary antibody kit, Invitrogen; Thermo Fisher Scientific, Inc.) was added for 1 hour at room temperature and coloration was performed. One Gel imaging analyzer (76S/07865 gel imaging analyzer: BIO-RAD, USA) was used to acquire images and Quantity One software (BIO-RAD, USA) was used to analyze gray values of the target strips.

Statistical analysis

SPSS 20.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Normally distributed measurement data are expressed as mean ± standard deviation. One-way analysis of variance was used for multi-group comparisons and Student-Newman-Keul's test was used to perform paired comparisons between two groups. Non-normally distributed measurement data are expressed using the median and interquartile range. P<0.05 indicates a statistically significant difference.

Results

Transfection of HSP70 genes

Lung tissue slices, obtained from groups H3 and C, were observed using an immunofluorescence microscope (**Figure 1**). Lung tissue from rats in group H3, that underwent hypoxia for 3,

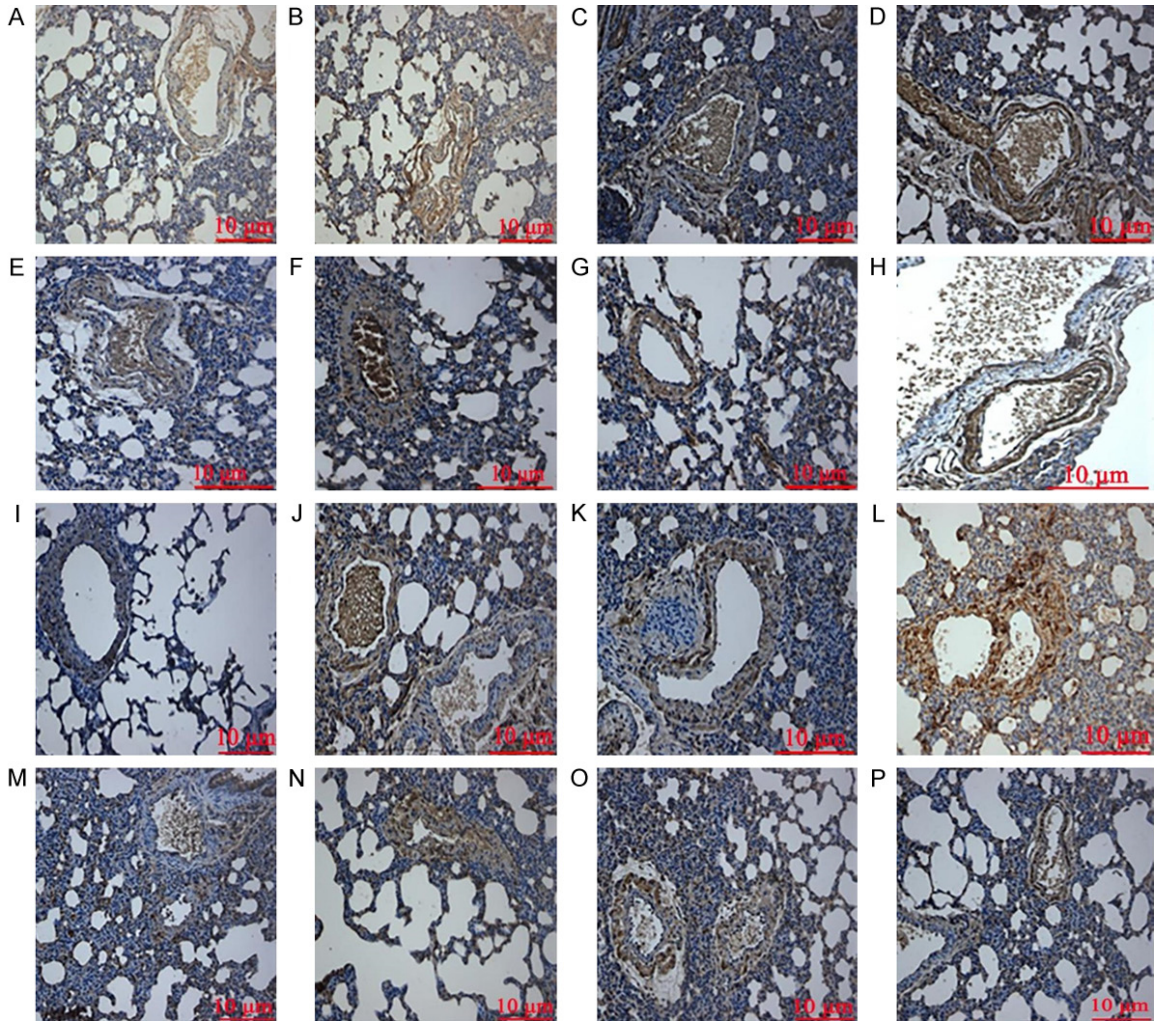

Figure 2. Immunohistochemical staining of HSP70, HIF-1α, ET-1, and iNOS in the lung tissue of newborn rats (magnification, ×400). A. Small pulmonary artery: reaction intensity of HIF-1α was negative; B-D. Small pulmonary artery: reaction intensities of HIF-1α were weakly positive, positive, and strongly positive; E. Small pulmonary artery: reaction intensity of ET-1 was negative. F-H. Small pulmonary artery: reaction intensities of ET-1 were weakly positive, positive, and strongly positive; I. Small pulmonary artery: reaction intensity of iNOS was negative. J-L. Small pulmonary artery: reaction intensities of iNOS were weakly positive, positive, and strongly positive. M. Small pulmonary artery: reaction intensity of HSP70 was negative. N-P. Small pulmonary artery: reaction intensities of HSP70 were weakly positive, positive, and strongly positive. HSP70, heat shock protein 70; HIF-1α, hypoxia-inducible factor 1α; ET-1, endothelin-1; iNOS, inducible nitric oxide synthase.

7, and 10 days (**Figure 1B-D**), exhibited immunofluorescent signal marker protein. However, those in group H3 that experienced hypoxia for 14 days (**Figure 1E**) and lung tissue of rats in group C (**Figure 1A**) exhibited no markers.

mPAP changes

mPAP in groups H1 and H2 on days 3, 7, 10, and 14 were significantly greater than in rats in group C at the same time points ($P<0.05$). However, mPAP in group H3 at days 3, 7, and 10 was significantly lower than in groups H1 and H2 ($P<0.05$; **Table 1**), indicating that adenovirus mediated HSP70 can reduce pulmonary arterial pressure.

Immunohistochemical expression of HSP70, HIF-1α, ET-1, and iNOS

Expression of HSP70, HIF-1α, ET-1, and iNOS in lung tissue taken from rats in Group C, as determined by immunohistochemistry (**Figure 2**), was mostly negative. For groups H1 and H2, the immunoreactive intensities of HSP70 ranged from weakly positive (+) to positive (++), while

Table 2. Immunohistochemical reaction intensities of HSP70, HIF-1α, ET-1, and iNOS in lung tissues of newborn rats

Factor	Day	Intensity	Group				*p*-values
			H1	H2	H3	C	
HSP70	3	-	1	2	0	8	<0.01
		+	2	1	0	0	
		++	3	2	1	0	
		+++	2	3	7	0	
	7	-	0	0	0	7	<0.01
		+	2	1	0	1	
		++	2	3	1	0	
		+++	4	4	7	0	
	10	-	0	0	0	6	<0.05
		+	1	0	0	1	
		++	2	4	2	1	
		+++	5	4	6	0	
	14	-	4	4	4	5	>0.05
		+	1	2	2	1	
		++	2	2	1	1	
		+++	1	0	1	1	
HIF-1α	3	-	7	6	6	8	<0.01
		+	1	1	2	0	
		++	0	1	0	0	
		+++	0	0	0	0	
	7	-	7	2	3	6	<0.01
		+	0	1	1	2	
		++	1	2	1	0	
		+++	0	3	3	0	
	10	-	6	0	0	6	<0.01
		+	1	0	0	1	
		++	1	2	4	0	
		+++	0	5	4	1	
	14	-	5	0	0	4	>0.05
		+	2	0	0	2	
		++	1	1	2	1	
		+++	0	7	6	1	
ET-1	3	-	8	3	2	7	<0.01
		+	0	2	2	0	
		++	0	2	2	1	
		+++	0	1	2	0	
	7	-	7	0	0	6	<0.01
		+	1	1	1	0	
		++	0	2	3	1	
		+++	0	5	4	1	
	10	-	6	0	0	5	<0.01
		+	0	0	0	3	
		++	1	2	1	0	
		+++	1	6	7	0	
	14	-	5	4	4	4	>0.05

those of HIF-1α, ET-1, and iNOS ranged from positive (++) to strongly positive (+++). In group H3, the immunoreactive intensity of HSP70 was strongly positive (+++) and those of HIF-1α, ET-1, and iNOS ranged from weakly positive (+) to positive (++). Their expression was more commonly observed on the endothelial cells of the pulmonary vascular wall (**Figure 2**). There were significant differences in the intensity of HSP70 expression among different HPH subgroups on days 3, 7, and 10. HSP70 expression was significantly upregulated in all HPH subgroups, compared with group C ($P<0.01$), and was significantly enhanced in group H3, compared to groups H1 and H2 ($P<0.01$). Immunohistochemical expression intensities of HIF-1α, ET-1, and iNOS were all significantly upregulated on days 3, 7, and 10 in all HPH subgroups, compared to the control group ($P<0.01$). In contrast, levels of HIF-1α, ET-1, and iNOS in group H3 were all significantly decreased compared with groups H1 and H2 ($P<0.01$; **Table 2**). There were no significant differences in expression intensities of HSP70, HIF-1α, ET-1, and iNOS at 14 days in each group.

Expression of HSP70, HIF-1α, ET-1, and iNOS

There were significant differences in expression of HSP70, HIF-1α, ET-1, and iNOS among the different groups on days 3, 7, and 10 ($P<0.05$). Expression of HSP70 was upregulated in group H3, compared to groups H1 and 2 ($P<0.05$). HIF-1α, ET-1, and iNOS were upregulated in groups H1 and H2, compared to the control group. However, their expression was significantly reduced in group H3, compared to groups H1 and 2 ($P< 0.05$; **Figure 3**).

Discussion

HPH may be reversed by early timely treatment. If not treated in time, 1ate pulmonary vascular remodeling [15,

		+	1	1	2	2	
		++	1	2	2	1	
		+++	1	1	0	1	
iNOS	3	-	8	3	2	7	<0.01
		+	0	1	1	0	
		++	0	2	2	1	
		+++	0	2	3	0	
	7	-	7	0	0	6	<0.01
		+	1	1	1	1	
		++	0	1	2	1	
		+++	0	6	5	0	
	10	-	5	1	0	4	<0.01
		+	1	1	1	2	
		++	1	2	2	1	
		+++	1	4	5	1	
	14	-	5	0	0	6	>0.05
		+	2	0	0	0	
		++	1	3	2	2	
		+++	0	5	6	0	

HSP70, heat shock protein 70; HIF-1α, hypoxia-inducible factor 1α; ET-1, endothelin-1; iNOS, inducible nitric oxide synthase; (-), negative; (+), weakly positive; (++), positive; (+++), strongly positive.

16], even persistent fetal circulation, may appear. It has been demonstrated that HIF-1α promotes expression of pro-vasodilators, causing pulmonary vascular spasms and remodeling, as well as leading to an increase in mPAP [5]. Downregulating expression of HIF-1α in the early stages of hypoxia may, therefore, be a novel effective method of treating HPHN.

HSP70 promote the degradation of HIF-1α [17]. They are a group of highly conserved non-specific intracellular protective proteins [18-20] that can protect cells from damage, promote the degradation and clearance of abnormal proteins, and maintain the normal physiological function of cells. The present study demonstrated that adenovirus-mediated HSP70 can be successfully targeted to pulmonary endothelial cells in neonatal rats, indicating that *in vivo* metabolism was reduced on day 14 of hypoxia. Low expression of HSP70 was detected in the control group, but its expression increased in HPH groups. Hypoxia may activate HSP70, leading to an increase in expression. Expression of HSP70 was highest in Group H3 at all time points, indicating that adenovirus-mediated HSP70 transfection may induce high expression of exogenous HSP70. Positive expression of HSP70 in the HPH groups was detected 3 days following transfection, peaking on day 10 and then declining. On day 14, HSP70 expression in all HPH subgroups decreased. Thus, it may be hypothesized that during prolonged periods of hypoxia, cell structures and functions of lung tissue may be damaged, reducing expression of HSP70. The present study demonstrates that the protective effects of endogenous HSP70 on HPH-induced damage in the lungs are weak and cannot reduce lung injuries. Severe long-term oxidative stress may block the synthesis of proteins required to maintain cell growth. Moreover, the overgeneration of denatured proteins may attenuate the protective abilities of HSP70. The current study demonstrates that high expression of recombinant adenovirus-mediated HSP70 serves a protective role in HPH.

Results of the current study indicate that HPH was successfully formed in groups H1 and H2. HPH was not evident in group H3 on days 3, 7, and 10 of hypoxia, suggesting that high expression of adenovirus-mediated HSP70 may block the development of HPH and induce degradation of HIF-1α. mPAP in group H3 was increased on day 14. HSP70 was downregulated in lung tissue, indicating that when the *in vivo* metabolic expression of adenovirus-mediated HSP70 is weakened, its ability to block the formation of HPH may be reduced. However, persistence of hypoxic conditions may cause an increase in mPAP.

HSP70 may mediate the degradation of HIF-1α proteins via non-VHL-dependent pathways [17]. During hypoxic conditions, HIF-1α may induce expression of ET-1 and iNOS, thus serving a damaging role in HPH [21, 22]. In the current study, immunohistochemical results indicated that HIF-1α was primarily expressed on pulmonary vascular endothelial cells. Immunoreactive intensities of HIF-1α in group H3 ranged from weakly positive (+) to positive (++), while those of ET-1 and iNOS ranged from weakly positive (+) to positive (++). Levels of HIF-1α were upregulated in all HPH subgroups on days 3, 7, and 10. Compared to groups H1 and H2, expression of HIF-1α was downregulated in group H3, indicating that adenovirus-mediated HSP70 in-

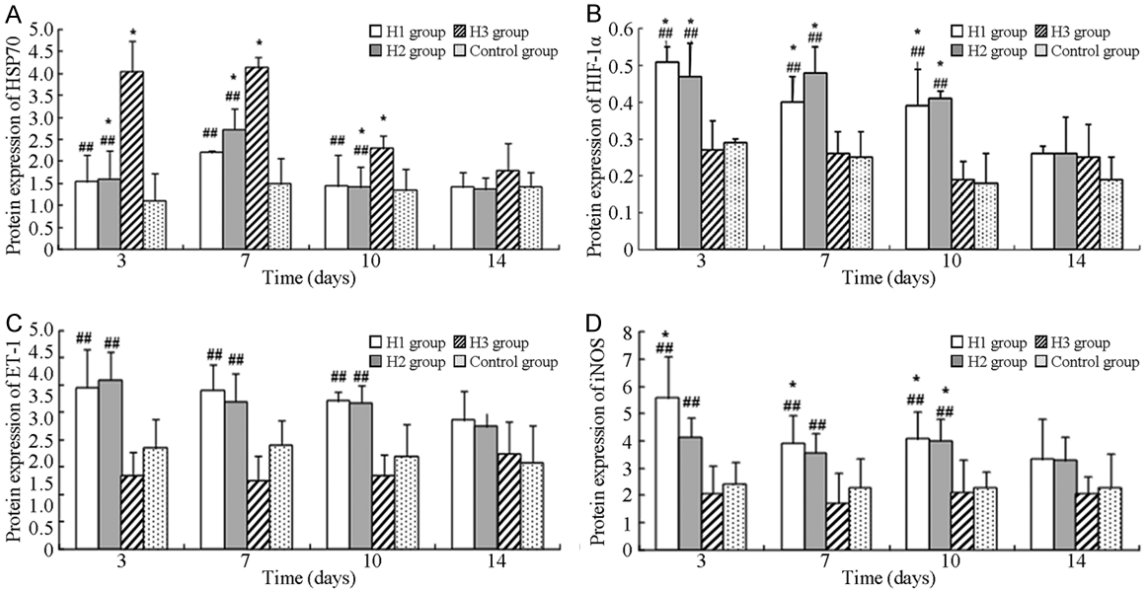

Figure 3. Expression of (A) HSP70, (B) HIF-1α, (C) ET-1, and (D) iNOS in lung tissue of newborn rats. *P<0.05 vs. group C; ##P<0.05 vs. group H3. Group C, control group; group H1, HPH group receiving saline; group H2, HPH group receiving transfection of empty virus; group H3, HPH group transfected with HSP70 virus; HPH, hypoxia induced pulmonary hypertension; HSP70, heat shock protein 70; HIF-1α, hypoxia-inducible factor 1α; ET-1, endothelin-1; iNOS, inducible nitric oxide synthase.

duces high expression of HSP70 and subsequently inhibits expression of HIF-1α. Furthermore, compared to the control group, expression of ET-1 and iNOS in all HPH subgroups on days 3, 7, and 10 was increased, indicating that hypoxia promotes expression of these proteins. Expression of ET-1 and iNOS in group H3 was reduced, compared to groups H1 and H2, indicating that adenoviral-mediated HSP70 induces high expression of HSP70, subsequently inhibiting expression of HIF-1α and downregulating expression of its downstream target genes ET-1 and iNOS.

In conclusion, the present study demonstrates that adenovirus-mediated HSP70 may induce high expression of HSP70 in lung tissues of newborn HPH rats. Furthermore, it was demonstrated that HSP70 exhibits protective effects in pulmonary tissue in HPH by promoting the degradation of HIF-1α and downregulating expression of its downstream target genes ET-1 and iNOS.

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Disclosure of conflict of interest

None.

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