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Arginine modulates endothelial phenotypes in experimental bronchopulmonary dysplasia-associated pulmonary hypertension: potential involvement of RARS1–HES1 signaling

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Abstract

Background: Bronchopulmonary dysplasia (BPD) is a complex chronic lung disease caused by the interaction between lung development and prenatal and postnatal injury events. Up to one-third of infants with BPD further develop pulmonary vascular dysfunction, leading to chronic pulmonary hypertension (PH). Infants with BPD-PH face increased mortality, a higher risk of adverse neurodevelopmental outcomes, and a lack of effective treatments.

Methods: A total of 30 study participants were recruited from the Children's Hospital Affiliated to Shandong University, with the data collection period spanning from January 2023 to May 2025. Through metabolite analysis, we observed serum arginine concentrations were lower in the BPD-PH group than in the comparison group, but the between-group difference did not reach statistical significance (P = 0.0581). Subsequently, in vitro experiments were conducted to investigate the effects of arginine on the proliferation and migration abilities of vascular endothelial cells and to elucidate the underlying molecular mechanisms. Finally, in vivo experiments were performed to validate the protective role of arginine in BPD-PH.

Results: The results of the in vitro experiments indicate that arginine inhibits the proliferation and migration of vascular endothelial cells. Molecular mechanism studies revealed that arginine reduces the expression of α-smooth muscle actin, hypoxia-inducible factor-1α, and related proteins through the arginyl-tRNA synthetase-1 (RARS1)-hairy and enhancer of split homologue-1 (HES1) pathway, RARS1 and HES1 were detected in the same immunoprecipitated protein complex, supporting a possible involvement of RARS1–HES1 signaling. In neonatal rats exposed to hyperoxia, arginine supplementation was associated with changes in selected lung-tissue mRNA markers. Pulmonary hemodynamics, right-ventricular remodeling, vascular morphometry, and alveolar structure were not assessed.

Conclusion: Our results indicate that arginine alleviates BPD-PH by promoting vascular endothelial cell remodeling through the RARS1-HES1 pathway. Arginine supplementation may represent a novel therapeutic alternative for preventing BPD.

Keywords: Arginine, Arginyl-tRNA synthetase-1, Bronchopulmonary dysplasia, Hairy and enhancer of split homologue-1, Pulmonary hypertension

Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung disease caused by restricted and impaired lung development in preterm infants, characterized by simplified development of the distal pulmonary acini and reduced ventilation and gas exchange functions. The development of the alveolar and perivascular microenvironment is halted, ultimately leading to the formation of BPD. The direct consequence of this pathophysiological process is that respiratory function cannot meet the body's growth and developmental needs, clinically manifested as oxygen dependence and multisystem complications following hypoxia. The remodeling of lung structure also becomes an important structural basis for the development of chronic lung diseases in adulthood. With the survival of preterm infants through advanced neonatal care techniques and effective respiratory support devices, the incidence of BPD has been increasingly rising.[1–3] Notably, a significant proportion of these patients develop concurrent pulmonary hypertension (PH), and there is a significant negative correlation between gestational age and the incidence of PH, which has become a major clinical challenge in the field of neonatal intensive care.[4] The prevalence of BPD-PH in the infant population can reach 19.4%–40.0%, accompanied by a mortality risk of up to 38%, making it one of the leading causes of neonatal morbidity and mortality.[5] The etiology of BPD-PH is complex, arising from the interaction between lung development and prenatal as well as postnatal injury events. This ultimately leads to impaired gas exchange, enhanced pulmonary vasoconstriction, pulmonary vascular remodeling, and consequently elevated pulmonary arterial pressure.[6] BPD leads to significantly impaired pulmonary vascular development, resulting

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The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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in persistent airway and pulmonary vascular disease. Vascular endothelial cell dysfunction plays a key role in this process, and the proliferation of vascular endothelial cells is a major characteristic leading to arterial remodeling. Dysfunctional vascular endothelial cells regulate multiple aspects of PH, including pulmonary vascular tone, inflammation, coagulation, metabolism, and remodeling. Given the growing body of evidence suggesting that vascular endothelial cells are critical mediators in the onset and progression of PH, new therapies for PH may aim to target multiple aspects of vascular endothelial cell dysfunction and signaling.[7]

Amino acids are among the most important metabolites within cells. Beyond their role in protein synthesis, numerous amino acids are also involved in regulating various critical signaling pathways. L-arginine is a semiessential amino acid, provided through diet and synthesized from L-citrulline. Current research is exploring therapeutic strategies involving supplementation with L-arginine or improving its bioavailability. The manner is based on alterations in L-arginine/NO homeostasis. As an important signaling molecule, NO mediates vasodilation and inhibits platelet aggregation and inflammatory responses among other functions and is crucial for maintaining vascular health in the body.[8] In studies about atherosclerosis models, L-arginine supplementation significantly increased vascular NO levels and reduced arterial plaque area.[9] Administration of L-arginine has been shown to lower blood pressure and reverse vascular remodeling.[10] Additionally, L-arginine supplementation reduced infarct size in rats following myocardial ischemia-reperfusion injury.[11] Studies in aged animal models have found that aging leads to increased arginase activity, and exogenous L-arginine supplementation can partially reverse vascular aging.[12] However, the role of arginine in BPD-PH has not been studied, and its effects warrant further investigation.

It has been found to play a role in various lung-related diseases and conditions, such as asthma, chronic obstructive pulmonary disease, cystic fibrosis, PH, and BPD.[13] However, recent studies have revealed that aminoacyl-tRNA synthetase (ARS) is a highly conserved enzyme family with approximately 30 subtypes in living organisms. Amino acids can promote the binding of ARS to its substrates. The classical function of ARS is to catalyze the formation of aminoacyl-tRNA substrates for protein synthesis.[14] Noncanonical functions include transcriptional regulation, RNA splicing, immune modulation, and cellular stress response.[15]

Enhancer of split homologue-1 (HES1) is a transcriptional repressor belonging to the basic helix-loop-helix family of transcription factors.[16] HES1 plays a key role by regulating processes such as smooth muscle differentiation, angiogenesis, arteriovenous fate determination, and vascular morphogenesis. It is an important transcription factor influencing vascular endothelial cell remodeling in PH.[17, 18] Studies have shown that HES1 can participate in regulating the transcriptional expression of various downstream molecules: α-smooth muscle actin (α-SMA) promotes the proliferation and migration of vascular smooth muscle cells.[19] Vascular endothelial growth factor (VEGF) promotes angiogenesis by enhancing vascular permeability, endothelial cell migration, proliferation, and blood vessel formation.[20] Transient receptor potential channel 1 (TRPC1) significantly affects the migration and proliferation of apical vascular endothelial cells, leading to defects in angiogenesis.[21, 22] Hypoxia-inducible factor-1α (HIF-1α) can activate VEGF, thereby promoting angiogenesis and vasodilation.[23] The cyclin-dependent kinase regulator P27Kip1 is a growth inhibitor capable of inducing cell cycle arrest.[24] A deeper understanding of these mechanisms will provide better insights into disease pathogenesis and lung repair and regeneration, which will aid in the discovery of new therapeutic targets. Currently, the molecular mechanisms underlying the regulatory network between arginine and HES1 have not been documented in the literature. Whether arginine can alleviate BPD-PH through noncanonical functions of ARS remains unknown.

Our study has reported that arginine improves vascular endothelial cell function and effectively alleviates BPD-PH by promoting the binding of arginyl-tRNA synthetase-1 (RARS1) to HES1 and regulating the arginylation modification of HES1. This finding provides a novel therapeutic strategy for mitigating BPD-PH.

Materials and methods

Human serum samples

Peripheral blood samples were collected from 2 groups: newborns (n = 10) and newborns with BPD-PH (n=20). On the basis of a confirmed diagnosis of bronchopulmonary dysplasia (BPD), i.e., the presence of oxygen dependence, pulmonary hypertension (PH) is confirmed by hemodynamic or echocardiographic evidence. The samples were obtained from the Children's Hospital Affiliated to Shandong University. A total of 30 study participants were recruited, with the data collection period spanning from January 2023 to May 2025. To ensure homogeneity of the study population, newborns with congenital malformations, genetic metabolic diseases, malignant tumors, or serious primary diseases were excluded. All newborns diagnosed with BPD-PH met the clinical diagnostic criteria. This study was approved by the Ethics Committee of Children's Hospital Affiliated to Shandong University (QLET-IRB/P-2021055) and confirmed that all methods were carried out in accordance with relevant guidelines and regulations. Written informed consent was obtained from the parents or legal guardians. Since this is the exploration of this mechanism, the sample size was determined based on feasibility and the actual situation of clinical recruitment.

Animals

Animal pups and their mothers were bred in Shandong First Medical University Animal Center, under specific pathogen-free conditions with a 12-hour/12-hour light/dark cycle, ambient temperature of 22 ± 1 °C, humidity of 30%–50%, and ad libitum access to food and water. All procedures and protocols in this study were approved by the Ethics Committee of the Jinan Children's Hospital, Qilu Children's Hospital of Shandong University. All experiments were carried out in accordance with the principles outlined in the Children's Hospital Affiliated to Shandong University, QLET-IRB/P-2021055 Guide for the Care and Use of Laboratory Animals.

Pulmonary hypertension model and drug administration

The experiment used a neonatal rat model of BPD-PH induced by hyperoxia exposure. The PH group was continuously exposed to a 70% hyperoxic environment from Day 1 to Day 5 after birth, then transferred to a 21% normoxic environment from Day 6 to Day 14, and injected with an equivalent amount of phosphate-buffered saline (PBS) as the PH-Arg group received arginine.[25, 26] The PH-Arg group rat were established as a BPD-PH neonatal rat model in the same manner as the PH group, and were intraperitoneally injected with an arginine solution (50 mg/kg) 3 times on Days 6, 8, and 9, respectively. To minimize experimental bias and ensure impartiality, animals were randomly allocated to groups, with a male-to-female ratio of 1:1, and all animals were subjected to a randomized grouping method. Each group consisted of 4 rat for subsequent indicator testing. They were from the same litter and were subjected to hyperoxic exposure in the same batch of oxygen chambers. Animal

experiments were divided into 3 groups: control, PH, and PH+Arg. Researchers involved in testing and molecular experiments were blinded. Each intervention group included an appropriate control group. This study was confirmed in accordance with Animal Research: Reporting of In Vivo Experiments guidelines.

Cell culture

hCMEC/D3 and 293T cells were purchased from Procell (Wuhan, China). hCMEC/D3 is a human brain microvascular endothelial cell line. This cell line originates from brain endothelial cell-rich tissue resected during epilepsy surgery. The cells exhibit an epithelial-like morphology, adhere to surfaces for growth, and possess a wide range of endothelial phenotypic characteristics. hCMEC/D3 has a subcultivation ratio of 1:2 and a subcultivation cycle of 2–3 days. The concentration of L-NAME used for the cell experiments in this study was 100 μM. The 293T cell line is a commonly used human embryonic kidney cell line. Both cell types were cultured in Dulbecco's Modified Eagle Medium complete medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2 and ambient oxygen. Humidity was maintained above 90% to prevent medium evaporation.

Cell proliferation assay

Cells were seeded in 96-well plates at an optimal density 24 hours prior. Cell concentration was adjusted to achieve 70% confluence at the time of experimentation, ensuring active growth. The original medium was replaced with drug-containing medium at varying concentration gradients (with controls). After adding 10 μL CCK-8 solution per well and incubating for 2 hours, absorbance was measured at 450 nm. Each concentration included triplicate wells for reliability.

Scratch assay

Log-phase cells were seeded in 6-well plates to reach 100% confluence overnight. A sterile 200-μL pipette tip was used to create perpendicular scratches against the plate lid. Cells were gently washed 3× with PBS, followed by the addition of 2 mL serum-free medium with drug gradients/controls. Scratch closure was imaged under an inverted microscope at multiple time points. Four fields per well were captured and averaged as a single value, and each experiment was performed with four independent cell culture replicates. The scratch area was quantified using ImageJ (National Institutes of Health, https://imagej.net/ij/). Data statistical generation method: (initial scratch area – scratch area after drug treatment)/initial scratch area×100%.

Western blot

Cells were lysed in Radio Immunoprecipitation Assay buffer containing protease inhibitors. After centrifugation (12,000 rpm, 20 minutes, 4 °C), supernatants were collected. Protein concentration was quantified using a bicinchoninic acid kit (Beyotime, Shanghai, China). Proteins (20 μg/lane) were denatured in loading buffer (10 minutes, 100 °C), separated by SDS-PAGE (80 V stacking gel, 120 V separating gel), and transferred to methanol-activated polyvinylidene fluoride membranes. Membranes were blocked with 5% skim milk (2 hours), incubated with primary antibodies (4 °C overnight), washed with TBST (0.2% Tween-20), and incubated with secondary antibodies (1 hour, room temperature). Protein bands were detected using enhanced chemiluminescence and analyzed with ImageJ. Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA).

Co-immunoprecipitation (Co-IP)

Posttransfection cells were washed 3× with PBS and lysed in ice-cold buffer containing protease/phosphatase inhibitors. Lysates were centrifuged (12,000 rpm, 20 minutes, 4 °C), and supernatants were incubated with 10 μL protein A/G agarose beads and 2 μL antibody (4 °C overnight). IgG antibody served as the negative control. Beads were washed 5× with buffer, and bound proteins were eluted with loading buffer for subsequent Western blot analysis.

Real-time polymerase chain reaction

RNA was extracted from both rat lung tissues and cells according to the instructions provided with the following kit. Total RNA was extracted using the SPARKeasy Kit (AC0202-B, Spark, Jinan, China) and quantified fluorometrically. cDNA was synthesized using a Takara reverse transcription kit. Quantitative polymerase chain reaction was performed with SYBR Green premix on a CFX96 system under cycling conditions: 95 °C for 5 minutes; 40 cycles of 95 °C for 15 seconds, 58 °C for 30 seconds, and 72 °C for 20 seconds. β-actin mRNA served as the internal control. The ΔCt values of each group were analyzed, and the mRNA expression of different groups was normalized to $2^{-\Delta Ct}$ (Table 1). Statistical tests were performed on ΔCt values; the graphs show the transformed relative expression levels.

SiRNA knockdown

To simultaneously knock down RARS1 and HES1, si-RARS1, si-HES1 (final concentration, 50 nM), and Lipofectamine 2000 were separately diluted in Opti-MEM and incubated at room temperature for 5 min. The siRNA dilutions and the Lipofectamine 2000 dilution were then mixed at a 1:1 ratio, incubated at room temperature for 20 min, and added dropwise to the cells. After 6 h, the medium was replaced with fresh complete medium. Subsequent experiments were performed 24 h after transfection. The control group simultaneously received siNC transfection. The sense sequences of the siRNAs were as follows: si-NC, 5'-UUCUUCGAACGUGUCACGUTT-3'; si-RARS1, 5'-CCCGCCAAGAGUUAAAUAATT-3'; HES1, 5'-ACGAAGAGCAAGAAUAAUTT-3'. The knockdown validation results for RARS1 and HES1 are shown in Supplementary Figure 1, https://links.lww.com/ECCM/A146.

Table 1

Primers Used in This Study

Primer	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
p27kip1(rat)	TAATTGGGGCTCCGGCTAACT	TGCAGGTCGCTTCCTTATTCC
HIF1-α(rat)	CACCACAGGACAGTACAGGAT	CGTGCTGAATAATACCACTCACA
TRPC1(rat)	AGGAACTAGCCCGGCAATGTA	GCTCGTCACTAGACGTATGGTTT
β-actin(human)	CACTCTTCCAGCCTTCCTTC	GTACAGGTCTTTGCGGATGT
β-actin(rat)	ATATCGCTGCGCTGGTCGTC	AGGATGGCGTGAGGGAGAGC

Statistical analysis

All data were derived from at least 2 independent experiments. All obtained data were included in the statistical analysis, with no missing or excluded data. Statistical analysis in this study was performed using GraphPad Prism 8 software (GraphPad Software). Continuous data were presented as mean ± standard deviation (SD). When the data pass the normality test, we use the Student *t* test to analyze the differences in the data. Categorical data are presented as percentages and analyzed using the chi-square test or Fisher exact test as appropriate. For comparisons involving more than two groups, one-way or two-way analysis of variance was used as appropriate, followed by multiplicity-adjusted post hoc comparisons. Biological replicates, technical replicates, and the unit of analysis are reported separately for each experiment. A significance level of $P < 0.05$ was considered statistically significant.

Results

Serum arginine levels are reduced in infants with bronchopulmonary dysplasia-associated pulmonary hypertension

Serum arginine levels were measured in infants with BPD-PH and non-BPD-PH controls. As shown in Figure 1A and B, BPD-PH had a shorter gestational age, falling into the category of very preterm birth, and a lower birth weight compared with non-BPD-PH infants. Compared with the non-BPD-PH control group, the serum arginine concentration of BPD-PH infants was lower, but there was no statistical significance.

Arginine promotes phenotypic transformation of endothelial cells

Following the observation of differences in arginine levels, we supplemented cell cultures with arginine to validate its effect on BPD-PH and assessed changes in cell migration and proliferation capabilities. As shown in Figure 2A and B, the addition of arginine significantly reduced both cell proliferation and migration abilities. Given that phenotypic remodeling of blood vessels is also a major factor in BPD-PH, we further examined the mRNA levels of TRPC1, HIF-1α, and P27Kip1, as well as changes in the protein expression of α-SMA and VEGF. The results, presented in Figure 2C and D, demonstrated that arginine increased the expression of VEGF and P27Kip1, while decreasing the levels of α-SMA, TRPC1, and HIF-1α. These findings suggest that in the hCMEC/D3 cell model arginine supplementation can alleviate vascular endothelial cell dysfunction, characterized by enhanced contractility and reduced proliferation, migration, and secretory capabilities. This suggests that arginine alleviates the phenotypic features of PH at the cellular level.

L-NAME antagonize arginine-mediated endothelial transformation

L-NAME is an inhibitor of nitric oxide synthase. Its attenuation of arginine-associated effects suggests that nitric oxide signaling may contribute to the observed cellular changes; it does not specifically establish involvement of the RARS1–HES1 pathway.[27] Since arginine promotes phenotypic transformation of endothelial cells, we added its NOS inhibitor to further validate the role of arginine. As shown in Figure 3A and B, treatment with L-NAME alone had no significant effect, whereas arginine inhibited cell migration and proliferation; the addition of arginine and L-NAME enhanced cell migration and increased cell proliferative capacity. As shown in Figure 3C and D, treatment with L-NAME alone had no significant effect; compared with the arginine group, the addition of arginine and L-NAME led to decreased levels of VEGF and P27Kip1, while the expression of α-SMA, TRPC1, and HIF-1α was increased. These findings indicate that the addition of L-NAME abolishes the effects of arginine, further supporting the conclusion that the promotion of endothelial cell phenotypic transformation is specifically mediated by arginine.

Arginine may promote the binding of arginyl-tRNA synthetase-1 to hairy and enhancer of split homologue-1

To investigate the molecular mechanism by which arginine functions, we explored whether arginine regulates transcription by mediating the arginylation of HES1 via RARS1. We performed molecular docking predictions for the RARS1-HES1 interaction. As shown in Figure 4A, RARS1 was predicted to interact with HES1. We overexpressed both RARS1 and HES1 proteins in 293T cells. The results shown in Figure 4B and C suggest that RARS1 appears to bind directly

A

Sample information	Non-BPD-PH (n=10)	BPD-PH (n=20)	P value
Sex (M: F)	3: 7	11: 9	/
Weight (kg)	1.25±0.31	0.90±0.14	0.0002
Gestational weeks	29.86±2.90	27.29±1.38	0.0025
Mode of birth (natural labour: abdominal delivery)	6: 4	7: 13	/
Feeding intolerance	70%	80%	0.6573
NRDS	100%	100%	> 0.9999
Infect	90%	95%	> 0.9999
Anemia	30%	35%	> 0.9999

B

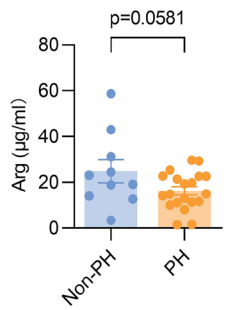


Figure 1. Clinical characteristics and serum arginine concentrations in the study groups. (A) Sample information. Table displays mean ± SD. Statistical significance was determined using a 2-tailed unpaired Student *t* test. Sex and mode of delivery were reported descriptively only. (B) The level of arginine in serum was quantified by LC-MS/MS. Graph displays mean ± SD. The *P* values for body weight and gestational age data were determined using a 2-tailed unpaired Student *t* test, while the *P* values for the remaining data were obtained using the Fisher's exact test. LC-MS/MS, liquid chromatography-tandem mass spectrometry, PH, pulmonary hypertension; SD, standard deviation.

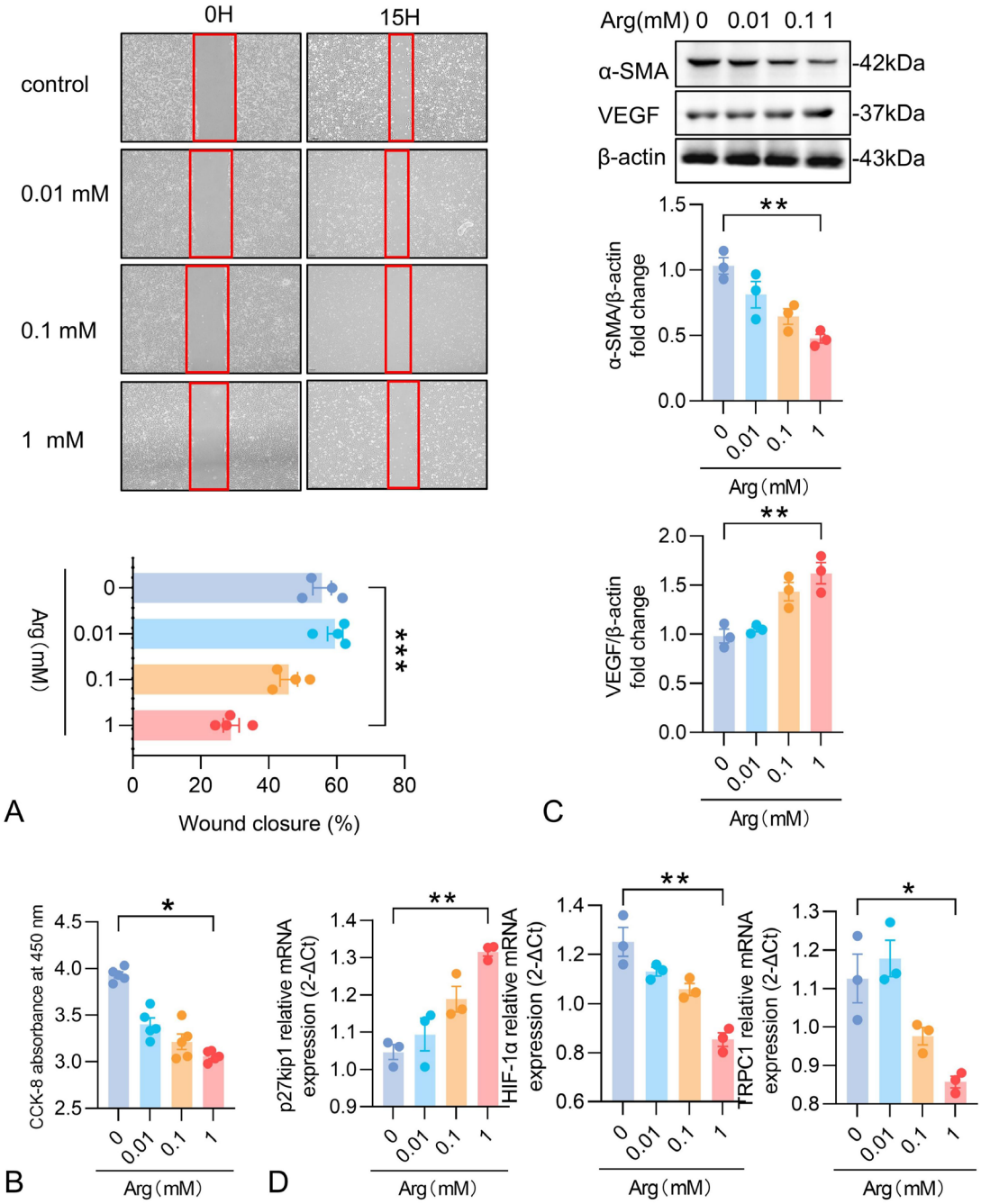


Figure 2. Arginine (Arg) promotes endothelial cell stabilization. (A) hCMEC/D3 cells were treated with or without Arg (0.01, 0.1, or 1 mM) for 15 hours. Scratch wounds were created at 0 hours and imaged under ×10 magnification, with the same fields reimaged after 15 hours, n = 4 in each group. n represents the number of independent biological experiments. (B) Cells treated as in (A) were incubated with CCK-8 solution, and absorbance was measured at 450 nm. n = 5 in each group. n represents the number of independent biological experiments. (C) Protein expression of smooth muscle actin α-SMA and vascular endothelial growth factor (VEGF) in cells treated as in (A), n = 3 in each group. n represents the number of independent biological experiments. (D) mRNA levels of calcium channel protein TRPC1, hypoxia-inducible factor HIF-1α, and cyclin-dependent kinase inhibitor P27Kip in cells treated as in (A), n = 3 in each group. n represents the number of independent biological experiments. *P < 0.05, **P < 0.01, and ***P < 0.001, one-way ANOVA was used to analyze significant differences, Dunnett's post hoc test was used to compare the 0 mmol/L control group with the 1 mmol/L arginine group. Graph displays mean ± standard deviation. HIF-1α, hypoxia-inducible factor-1α; α-SMA, α-smooth muscle actin; TRPC1, transient receptor potential channel 1; VEGF, vascular endothelial growth factor.

to HES1, and this binding tends to be enhanced upon the addition of arginine. These findings imply that arginine may serve as a substrate for RARS1, potentially facilitating the arginylation of HES1 by RARS1, and may participate in the phenotypic transformation of endothelial cells through transcriptional regulation.

Knockdown of arginyl-tRNA synthetase-1 and hairy and enhancer of split homologue-1 expression reverses the effects of arginine, promoting their binding

To further validate that arginine exerts its biological functions through RARS1 and HES1, we treated cells with arginine while simultaneously knocking down the expression of HES1 and

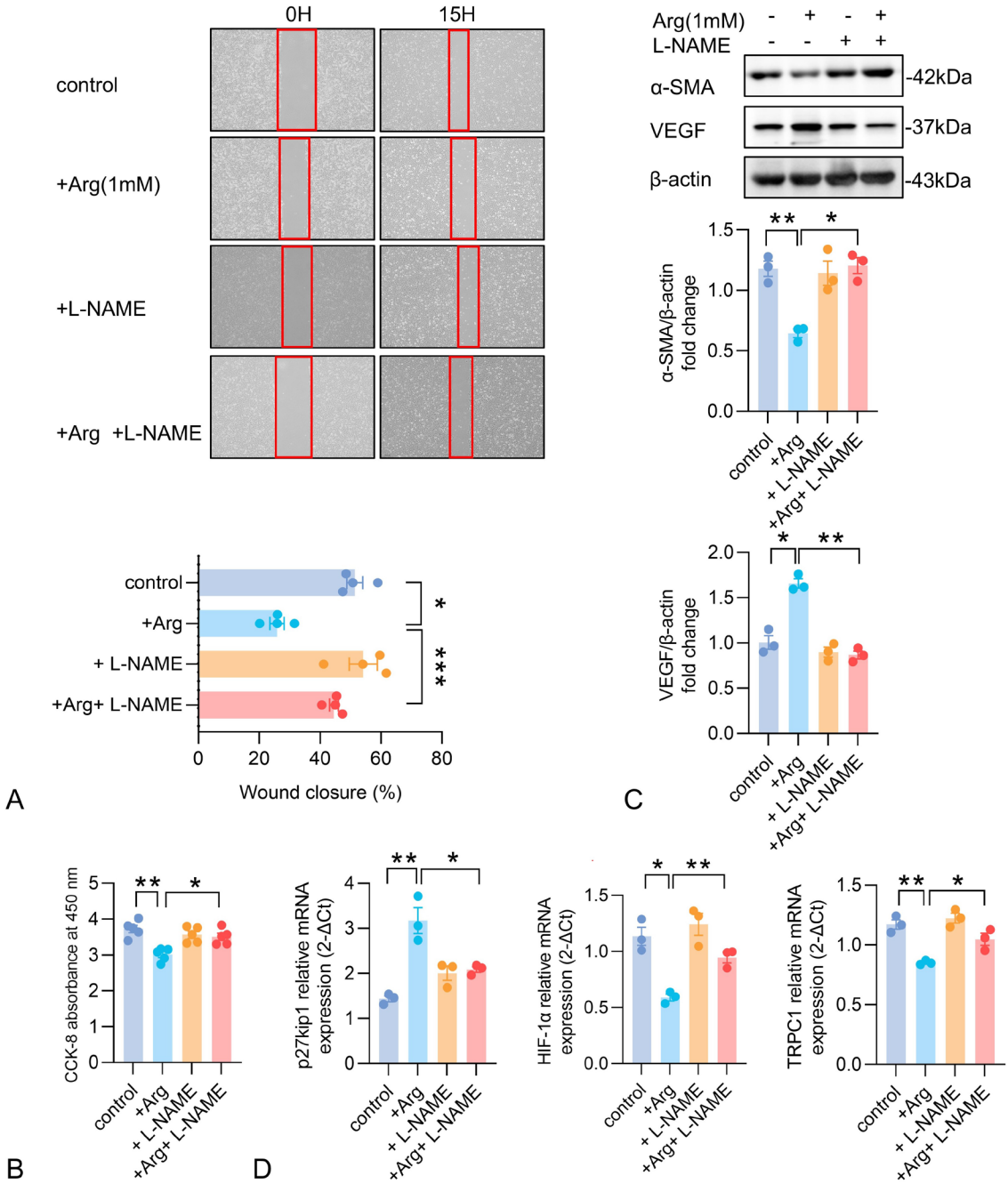


Figure 3. L-NAME abrogates arginine's function. (A) hCMEC/D3 cells were treated with Arg, L-NAME (NO synthase inhibitor), or Arg+L-NAME for 15 hours. Wound healing assays were performed at 0 and 15 hours (×10 magnification), n = 4 in each group. n represents the number of independent biological experiments. (B) CCK-8 assay for cells treated as in (A), n = 5 in each group. n represents the number of independent biological experiments. (C) Western blot of α-SMA and VEGF in cells treated as in (A), n = 3 in each group. n represents the number of independent biological experiments. (D) qPCR analysis of TRPC1, HIF-1α, and P27Kip mRNA levels in cells treated as in (A), n = 3 in each group. n represents the number of independent biological experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Data in Figure 3 were analyzed using two-way ANOVA, with arginine treatment and L-NAME treatment as the two factors. The arginine × L-NAME interaction was tested, followed by multiplicity-adjusted simple-effects comparisons using the Šidák method. Graph displays mean ± standard deviation. Arg, arginine; HIF-1 α, hypoxia-inducible factor-1α; L-NAME, nitro-L-arginine methyl ester; qPCR, quantitative polymerase chain reaction; α-SMA, α-smooth muscle actin; TRPC1, transient receptor potential channel 1; VEGF, vascular endothelial growth factor.

RARS1. As shown in Figure 5A and B, compared with the control group treated with arginine alone, the knockdown of both genes enhanced cell migration and proliferation capabilities. As demonstrated in Figure 5C and D, the levels of VEGF and P27Kip1 decreased, while the expression of α-SMA, TRPC1, and HIF-1α increased compared with the versus the hyperoxia model group. Knockdown of RARS1 and HES1 appeared to attenuate or counteract the promoting effect of arginine on endothelial cell phenotypic transformation, and these results suggest that arginine might be involved in this process through RARS1 and HES1.

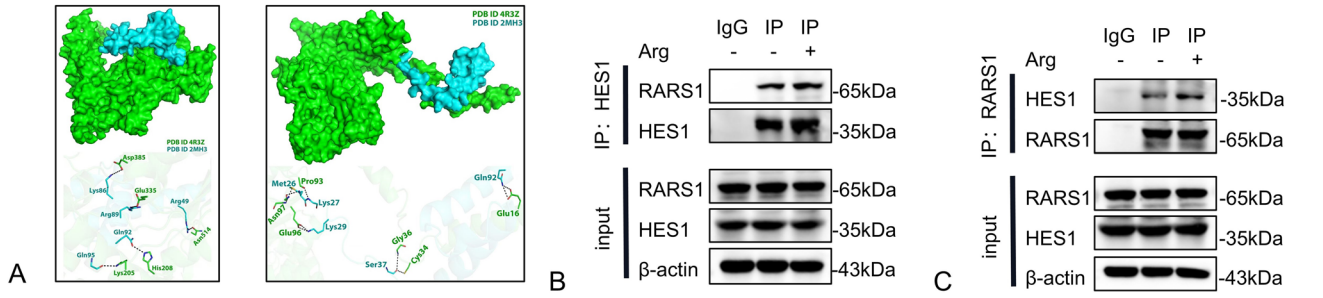


Figure 4. RARS1 and HES1 were detected in the same immunoprecipitated protein complex. (A) Molecular docking prediction of HES1 and RARS1 interaction. Molecular docking prediction done by the HADDOCK website (https://rascar.science.uu.nl/haddock2.4/). (B) Co-immunoprecipitation (Co-IP): 293T cells overexpressing HES1 and RARS1 were treated with or without 1 mM Arg for 15 hours, followed by HES1 IP to detect RARS1. (C) Reverse Co-IP: 293T cells treated as in (B) were subjected to RARS1 IP to detect HES1. n = 1× independent experiments. HES1, hairy and enhancer of split homologue-1; RARS1, arginyl-tRNA synthetase-1.

Arginine alleviates symptoms of pulmonary hypertension in vivo

Given that the addition of arginine promotes phenotypic transformation of endothelial cells in vitro, we established an in vivo PH model in neonatal rats and subsequently collected lung tissues. As shown in Figure 6A–C, compared with the model group, the mRNA levels of TRPC1 and HIF-1α decreased, while the mRNA expression of P27Kip1 increased in the arginine-treated group. These results demonstrate that exogenous arginine supplementation promotes the phenotypic transformation of endothelial cells toward a contractile type in the PH model, consistent with the in vitro experimental findings.

Discussion

In this study, we first collected serum samples from infants with BPD-PH and non-BPD-PH to measure arginine metabolite levels. The results showed that serum arginine levels were lower in the affected infants than in the nonaffected group, suggesting that arginine may play a certain role in BPD-PH. Consequently, we added exogenous arginine, which inhibited cell proliferation and migration and alleviated vascular endothelial cell dysfunction, thereby validating the functional role of arginine. Furthermore, the addition of a NOS inhibitor of arginine was shown to inhibit its effects. In mechanistic investigations, RARS1 and HES1 were detected in the same immunoprecipitated protein complex, supporting a possible involvement of RARS1–HES1 signaling. Additionally, knocking down the expression of HES1 and RARS1 reversed the effects of arginine. Finally, in an in vivo model of BPD-PH in rats, supplementation with arginine alleviated the disease's impact on vascular endothelial cell function. Our findings contribute to a better understanding of the complex pathophysiological mechanisms underlying BPD-PH and provide insights for targeted therapeutic interventions.

Previous studies have indicated that citrulline can attenuate PH in lung injury in neonatal rats, while arginine can be converted back to citrulline via nitric oxide (NO) synthase, simultaneously releasing NO.[28] Therefore, we propose that arginine also plays a critical role in the development and progression of BPD-PH.[29] Clinical researchers have found that NO is produced by the NO synthase family, all of which utilize L-arginine as a substrate. NO production in the lungs and airways plays a regulatory role in airway and vascular smooth muscle tone during lung development.[30] We suggest that arginine not only serves as a substrate for NO production but may also function through arginylation modification, regulating HES1 via RARS1 to exert diverse effects. On the other hand, posttranslational protein arginylation is an important regulator of numerous physiological pathways in cells. N-terminal arginylation plays a crucial role in early angiogenesis, a process mediated by arginine tRNA transferases.[31, 32] Consistent with this, we found a possible protective role of RARS1 in arginylation modification in pulmonary vascular regulation.

Limitations

This study has several limitations. First, the sample size investigated was small, which does not fully validate the safety and efficacy of arginine as a therapeutic agent. Second, the therapeutic effects of arginine were only verified in animal models, and its actual therapeutic impact on patients remains uncertain. Finally, our in vitro experiments used only one cell type, and using brain microvascular endothelial cells to model pulmonary vascular endothelial cells may introduce differences. Given that BPD-PH results from alterations in multiple cell states, we cannot exclude the possibility that arginine may play similar roles in other cell types. Since this study was designed to explore the underlying mechanism, the sample size was determined based on the practical feasibility and actual circumstances of clinical recruitment.

Conclusion

In summary, we found that in BPD-PH, arginine supplementation may act through RARS1 to regulate vascular endothelial cell function via arginylation. To some extent, this study elucidated the possible mechanism by which HES1 arginylation modification regulates vascular endothelial cell function, and pioneered the exploration of the potential role of aminoacylation modification in physiological and pathological processes. Furthermore, this research holds potential for exploring the intervention efficacy of targeting arginine metabolism to regulate HES1 in BPD-PH. Arginine was associated with changes in endothelial-cell proliferation, migration, and selected molecular markers in vitro, and with changes in selected lung-tissue transcripts in a neonatal hyperoxia model. The findings suggest possible involvement of RARS1–HES1 signaling but do not establish direct HES1 arginylation or therapeutic efficacy in BPD-associated pulmonary hypertension. Independent mechanistic studies and clinically relevant animal outcomes are required.

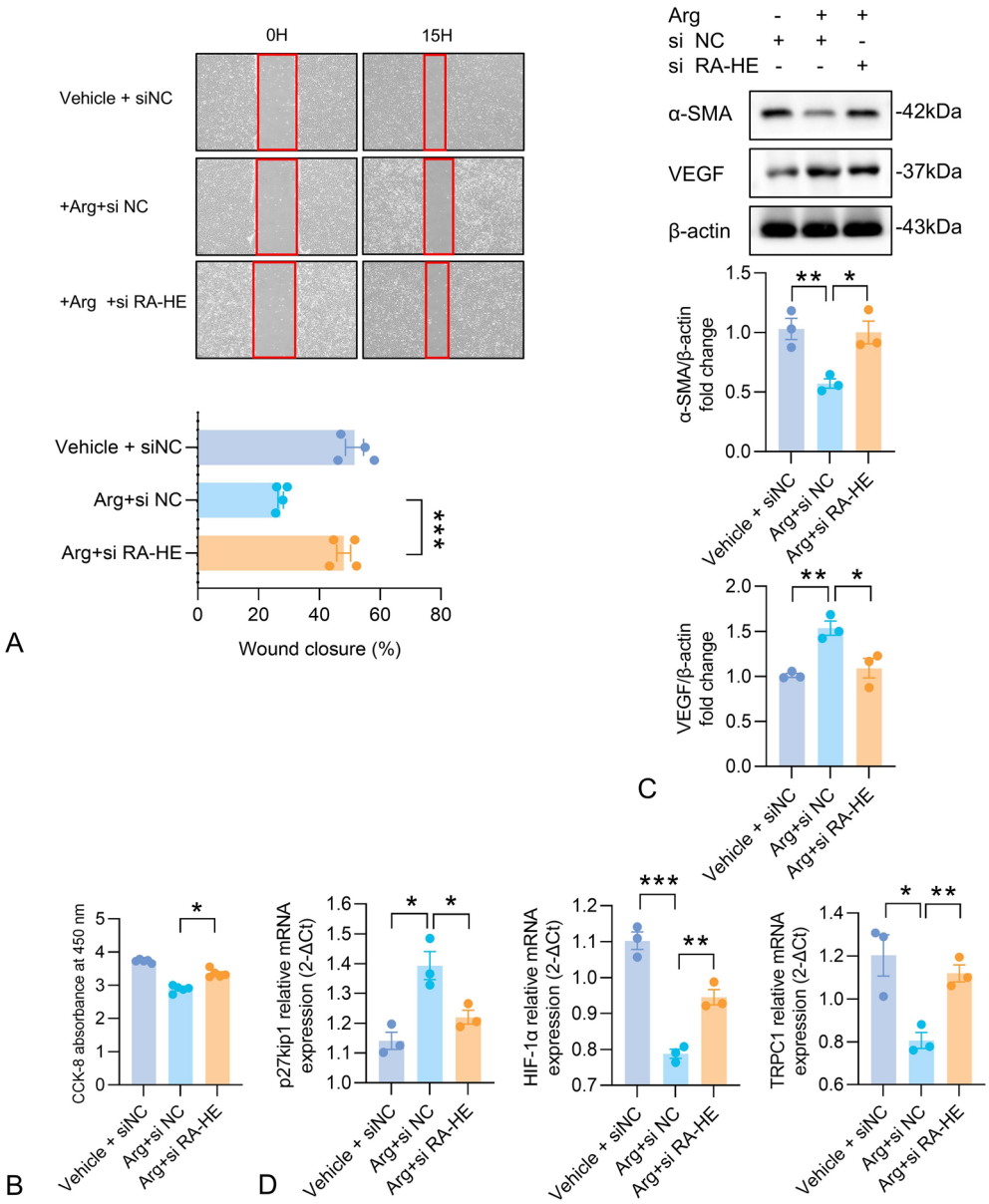


Figure 5. Si RARS1-HES1 reverse arginine effects. (A) hCMEC/D3 cells were treated with si-RNA for 48 hours. Wound healing assays were performed at 0 and 15 hours (×10 magnification), n = 4 in each group. n represents the number of independent biological experiments. (B) hCMEC/D3 cells were treated in the same way as in (A), followed by treatment with CCK-8 solution and measured at 450 nm, n = 5 in each group. n represents the number of independent biological experiments. (C) Protein content of α-SMA and VEGF in hCMEC/D3 cells treated as in (A), n = 3 in each group. n represents the number of independent biological experiments. (D) Analysis of TRPC1, HIF-1α, and P27Kip mRNA levels in hCMEC/D3 cells treated as in (A), n = 3 in each group. n represents the number of independent biological experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. One-way ANOVA followed by Dunnett's multiple comparison test versus the arginine-only control group. Graph displays mean ± standard deviation. HES1, hairy and enhancer of split homologue-1; HIF-1 α, hypoxia-inducible factor-1α; RARS1, arginyl-tRNA synthetase-1; α-SMA, α-smooth muscle actin; TRPC1, transient receptor potential channel 1; VEGF, vascular endothelial growth factor. Si RA-HE refers to the simultaneous small interfering RNA (siRNA)-mediated knockdown of both RARS1 and HES1.

Conflict of interest statement

The authors declare no conflict of interest.

Author contributions

Li Y and Zhou D designed and performed most experiments, analyzed the data, and wrote the manuscript. Dai X participated in the mouse experiments. Li Yunxia and Han Y participated in the collection and analysis of clinical samples. Kang L and Li X conceptualized, supervised, and interpreted the experiments.

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Ethical approval of studies and informed consent

The human-sample component was approved by the Ethics Committee of Children's Hospital Affiliated to Shandong University (approval no. QLET-IRB/P-2021055, approved on

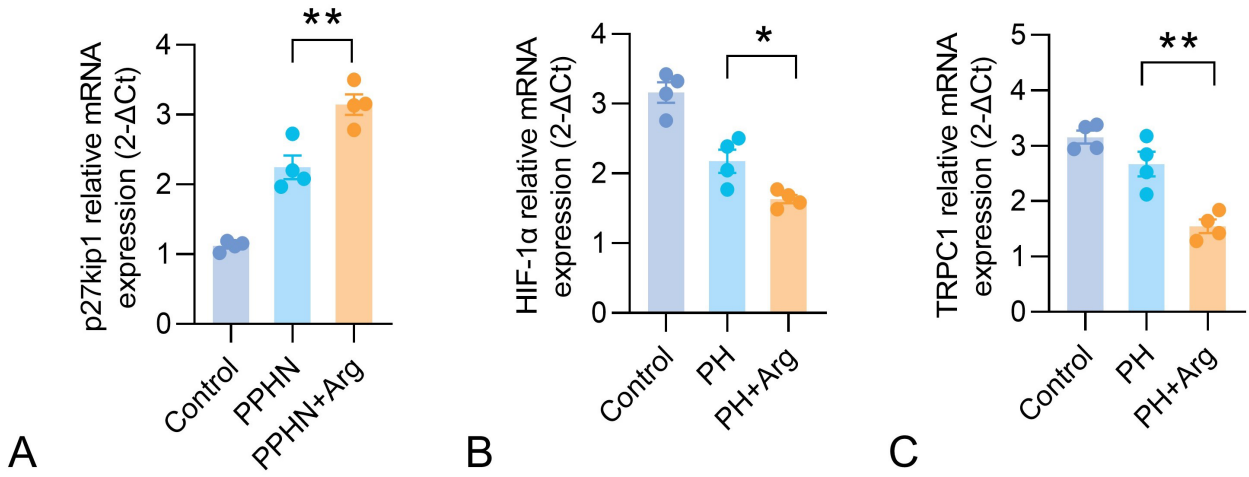


Figure 6. Effects of arginine on selected lung-tissue transcripts in neonatal rats exposed to hyperoxia. Animal experiments were divided into 3 groups: control, PH, and PH+Arg. (A) The results showed P27Kip1 mRNA in lung tissue. (B) The results showed HIF-1α mRNA in lung tissue. (C) The results showed TRPC1 mRNA in lung tissue, n = 4 in each group, n represents different animals. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. One-way ANOVA followed by Dunnett's multiple comparison test versus the arginine-only control group. Graph displays mean ± standard deviation. Arg, arginine; HIF-1 α, hypoxia-inducible factor-1α; PH, pulmonary hypertension; TRPC1, transient receptor potential channel 1.

October 10, 2021), covering samples collected from January 2023 to May 2025. Written informed consent was obtained from the parents or legal guardians. The neonatal-rat experiments were approved separately by the Ethics Committee of Children's Hospital Affiliated to Shandong University (approval no. QLET-IRB/P-2021055, approved on October 10, 2021).

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