

Original Article

Serum apelin-angiotensin II expression and Aktphosphorylation pathway in children with congenital heart disease complicated with pulmonary hypertension

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Abstract: Objective: This study is to investigate the relationship between serum Apelin-angiotensin II (AgII) expression in children with congenital heart disease (CHD) complicated with pulmonary hypertension (PAH), the severity of the disease and the therapeutic effect, and to further explore its possible mechanism relating to Akt phosphorylation pathway. Methods: A total of 86 children with CHD were enrolled in our hospital from May 2017 to May 2018 and further subdivided into no PAH, mild, moderate and severe groups. The mean pulmonary artery pressure (PAP) was calculated according to echocardiography. Serum Apelin and AgII levels were measured by radioimmunoassay before and 7 days after treatment, and p-Akt/Akt was detected by Western blot. Results: The PAP and AgII levels were significantly increased after 7 days of treatment compared to those before treatment ($P < 0.05$). There was biggest decrease of PAP level in severe group, followed by moderate and mild group after the treatment ($P < 0.05$). Apelin and p-Akt/Akt levels in PAH group were significantly lower than that in no PAH group ($P < 0.05$). The treatment significantly increased Apelin and p-Akt/Akt levels, while there was largest elevation of these levels in severe group compared to mild or moderate group ($P < 0.05$). Conclusions: The PAP and AgII levels were found elevated in PAH groups, along with significant decrease of Apelin and p-Akt/Akt levels. The treatment can retard the changes of these levels while in severe group, the level was altered markedly compared to mild or moderate group, which provides new insight for the diagnosis and evaluation of PAH progression.

Keywords: Congenital heart disease, pulmonary hypertension, apelin, angiotensin II, Aktphosphorylation pathway, children

Introduction

Children with congenital heart disease (CHD) are prone to secondary pulmonary hypertension (PAH) [1]. The disease is characterized by early lack of typical clinical symptoms, mainly due to dyspnea, decreased activity endurance, arrhythmia, which contribute to the major cause of death [2]. The diagnostic method includes echocardiography, which presents non-invasive and repeatable measurement, but the accuracy of the result is overly dependent on the clinical experience of the examining physician, the degree of cooperation of the subject and the influence of cardiac blood flow. In addition, electrocardiogram, chest X-ray, CT, etc. can also be used for the diagnosis. Intraoperative Swan-Ganz catheter can directly measure pulmonary arterial pressure

(PAP), which is considered as "gold standard" for PAH diagnosis, but the operation is complicated and traumatic.

The mechanism of PAH is mainly pulmonary vasoconstriction and pulmonary vascular remodeling while the development of PAH is associated with the multiple signaling pathways, such as NADPH/ROS/p38, RhoA/ROCK [3, 4]. Pulmonary microvascular endothelial cells (PMVECs) function as active inflammatory cells and effector cells and become the first target cells to be damaged. The stress regulators, hemodynamics, vasoactive substances, inflammatory mediators, etc., participate in the occurrence and progression of PAH [5]. Apelin and its receptor APJ are expressed in the central nervous system and peripheral tissues, and are found highly

expressed in the lung and heart. They play an important role in regulating various pathophysiological processes such as vasodilation, inhibition of bacterial toxins, and reduction of inflammatory response [6]. The study revealed [7] significantly lower serum Apelin level in PAH patients, suggesting that Apelin may play a protective role in the occurrence of PAH. Angiotensin II (AngII) is recognized as a potent vasoactive mediator and involves in hypertension and cardiac hypertrophy [8]. It has been shown that the fetal and the maternal cardiovascular compartment undergo dramatic functional changes during pregnancy and the highly vasoactive peptide apelin decreases due to a faster elimination as a consequence of an increase in placental angiotensin-converting-enzyme 2 [9]. However, whether there is a relationship between Apelin and AngII levels in the occurrence of PAH in children, and the significance of both in the severity of the disease and the therapeutic effect, remains to be determined. In this regard, this study explored the expression of Apelin and AngII in children with PAH, investigate its role in early diagnosis of PAH and assessment of prognosis, and further analyzed the mechanism of Apelin and AngII in the pathogenesis of PAH.

Objects and methods

Object information

A total of 86 children with CHD were enrolled in our hospital from May 2017 to May 2018. The diagnostic criteria: 1. Aged 3 months to 18 years old; 2. Clinical data was complete and analysis was possible; 3. Intervention was accepted. Or after surgical treatment, no obvious complications were found; 4. Informed consent was given. Exclusion criteria: 1. Recurrent CHD, combined with myocarditis, cardiomyopathy, and concurrent infection; 2. Cardiopulmonary, liver and kidney dysfunction, coagulation abnormalities, autoimmune diseases, metabolic diseases; 3. Recent angiotensin Converting enzyme inhibitors/angiotensin receptor antagonists, vasodilators, beta-blockers. The study was approved by the Ethics Committee of Jiangxi Provincial Children's Hospital. All patients and their families voluntarily participated in the study had written informed consents after a detailed explanation.

General information

Admitted echocardiography was performed by two experienced sonographers, and the PAP was calculated using the simplified Bernoulli equation and the maximum reflux velocity of the tricuspid valve. According to the AHA guidelines recommended: PAP \geq 25 mmHg indicates PAH. 26-35 mmHg is mild, 36-55 mmHg is moderate, and \geq 56 mmHg is severe. Among the 86 patients, they were aged of 3 months to 14, with median age of 7.2 years old, while weighed of 3.5 to 52.6 kg, with median weight of 20.8 kg. There were 16 patients without PAH as no PAH group, 25 patients were set as mild group, 30 patients as moderate group, and 15 patients as severe group accordingly. Specifically, in no PAH group, there were 7 boys and 9 girls, with age ranged from 10 months to 10 years (median: 6.5 years), including 8 patients with VSD, 5 patients with ASD and 3 patients with PDA. In mild group, there were 12 and 13 girls, with age ranged from 8.5 months to 12 years (median: 7.5 years), including 11 patients with VSD, 9 patients with ASD, and 5 patients with PDA. In moderate group, there were 14 boys and 16 girls, with the age ranged from 8.0 months to 13 years (median: 7.3 years), including 13 patients with VSD, 10 patients with ASD, and 7 patients with PDA. In severe group, there were 7 males and 8, with the age ranged from 7.0 months to 14 years (median: 7.7 years), including 8 patients with VSD, 5 patients with ASD, and 2 patients with PDA. No differences were found in gender, age and disease type among these groups ($P > 0.05$).

Research methods

Complete preoperative examination was performed, according to the guidelines recommended reasonable intervention or surgical treatment. There were 6 patients out of 10 from no PAH group receiving surgery, 9 out of 16, 11 out of 18, 7 out of 12 in mild, moderate or severe group, respectively, were given operation. The surgery types were comparable ($P > 0.05$). PAP was detected by echocardiography 7 days after operation. Serum Apelin and AngII levels were measured by radioimmunoassay before and 7 days after treatment, and p-Akt/Akt values were detected by Western blot. 2 mL and 5 mL of fasting venous

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Table 1. PAP levels before and after treatment (mmHg)

Groups	n	Before	7 d	Decrease	t	P
No PAH group	16	17.5±4.6	15.6±4.3	1.6±0.4	0.532	0.524
Mild group	25	30.3±4.9	24.3±3.6 ^a	5.8±1.2	5.006	0.018
Moderate group	30	47.8±5.2	29.8±4.2 ^a	19.2±3.3 ^b	5.659	0.006
Severe group	15	65.3±5.8	35.6±4.9 ^a	28.7±4.6 ^{b,c}	6.524	0.000
F		12.635	8.625	25.263		
P		0.000	0.000	0.000		

Note: a, compared with PAH before treatment, $P < 0.05$; b, compared with the mild group, $P < 0.05$; c, compared with the moderate group, $P < 0.05$.

blood were collected from patients. After centrifuged at 3000 g for 10 min, the serum was obtained and stored at -80°C for testing.

Western blot

Cells were treated with RIPA cell lysate (Hyclone, Logan, Utah, USA), while protein was extracted and quantified by BCA kit (sigma, St. Louis, MO, USA). Western blot was performed starting with 60 V electrophoresis for 30 min, followed by 120 V electrophoresis for 120 min. After electrophoresis, proteins were transferred to NC membrane (R&D, Minneapolis, MN, USA) under 300 mA for 30 min. The membrane was then blocked with 5% defatted milk powder for 60 min at room temperature. Human monoclonal antibody Akt and p-Akt (1:3000, Invitrogen, Carlsbad, California, USA) was added for 4°C room temperature incubation overnight. The membrane was then washed with phosphate buffered solution tween (TBST) (Jiangsu Biyuntian Technology Co., Ltd.) for 30 min, followed with incubation with rabbit anti-mouse polyclonal antibody secondary antibody was added (1:500, Jiangsu Biyuntian Technology Co., Ltd. for 60 min. After washed three times with PBST, chemiluminescence detection reagent was used to develop and fix. Gel image system was used to analyzed the band density (Bio-rad, Hercules, CA, USA). The Odyssey 9120 two-color infrared laser imaging system (Media Cybernetics, Rockville, MD, USA) was used for scanning.

Statistical method

SPSS20.0 software was applied for analyzing data. Measurement data were displayed as mean ± SD and assessed by t-test or one-way ANOVA. The count data were displayed as the number of cases or (%), and assessed by chi-square test. $P < 0.05$ indicates a significance.

Results

PAP levels before and after treatment in each group

Except for the no PAH group, the PAP levels after 7 days of treatment were significantly reduced compared to that before treatment ($P < 0.05$).

The PAP reduction was the highest in severe group, fol-

lowed by moderate and mild group ($P < 0.05$) (Table 1).

Serum Apelin levels before and after treatment in each group

Serum Apelin level was gradually decreased as the severity of PAH elevated. The treatment significantly increased the levels of Apelin in PAH groups, while the biggest up regulation of Apelin level was found in severe PAH group compared to that in mild or moderate group ($P < 0.05$) (Table 2).

Serum AgII levels before and after treatment in each group

On the contrary to the changes of Apelin level, the level of AgII was gradually increased as the severity of PAH elevated. The treatment can significantly limit the AgII level ($P < 0.05$). There was biggest elevation of AgII level in severe group compared to that in mild or moderate group ($P < 0.05$) (Table 3).

p-Akt/Akt values before and after treatment in each group

The Western blotting result indicated that p-Akt/Akt value were gradually decreased as the PAH aggravated. The value was significantly elevated after the treatment in in PAH groups ($P < 0.05$). Of note, there was largest reduction of p-Akt/Akt value in severe group compared to that in mild or moderate group ($P < 0.05$) (Table 4; Figure 1).

Discussion

CHD-related PAH formation is mainly due to left-to-right shunt, resulting in increased pulmonary circulation, vascular permeability and tissue shear, decreased oxygen content, function-

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Table 2. Serum Apelin levels before and after treatment (pg/ml)

Groups	n	Before	7 d	Increase	t	P
No PAH group	16	113.5±12.6	115.6±14.3	1.9±0.4	0.332	0.624
Mild group	25	96.3±7.9 ^a	104.3±9.6 ^{a,*}	7.8±1.3	4.806	0.025
Moderate group	30	77.8±6.2 ^{a,b}	89.8±6.9 ^{a,b,*}	11.2±3.5	5.259	0.016
Severe group	15	62.3±5.4 ^{a,b,c}	83.6±5.9 ^{a,b,c,*}	19.7±4.4	5.624	0.010
F		10.545	7.725	20.563		
P		0.000	0.000	0.000		

Note: a, compared with no PAH group, $P < 0.05$; b, compared with the mild group, $P < 0.05$; c, compared with the moderate group, $P < 0.05$. *, compared with the value before treatment.

Table 3. Serum AgII levels before and after treatment in each group (pg/ml)

Groups	n	Before	7 d	Decrease	t	P
No PAH group	16	33.5±5.6	31.6±4.3	1.8±0.4	0.232	0.724
Mild group	25	36.3±5.9 ^a	33.3±4.6 ^{a,*}	2.7±0.8	4.956	0.021
Moderate group	30	47.8±6.2 ^{a,b}	39.8±5.9 ^{a,b,*}	7.8±2.5	5.149	0.017
Severe group	15	62.3±6.4 ^{a,b,c}	48.6±6.3 ^{a,b,c,*}	12.7±4.6	5.454	0.012
F		9.645	7.965	14.563		
P		0.000	0.000	0.000		

Note: a, compared with no PAH group, $P < 0.05$; b, compared with the mild group, $P < 0.05$; c, compared with the moderate group, $P < 0.05$. *, compared with the value before treatment.

Table 4. p-Akt/Akt values before and after treatment in each group (%)

Groups	n	Before	7 d	Increase	t	P
No PAH group	16	83.5±15.6	84.6±14.3	1.1±0.2	0.132	0.824
Mild group	25	76.3±12.9 ^a	81.3±13.6 ^{a,*}	4.7±0.6	4.756	0.029
Moderate group	30	57.8±8.2 ^{a,b}	68.8±10.9 ^{a,b,*}	10.8±2.5	5.049	0.018
Severe group	15	42.3±6.4 ^{a,b,c}	60.6±8.3 ^{a,b,c,*}	18.7±3.6	5.354	0.009
F		8.645	6.965	11.563		
P		0.000	0.000	0.000		

Note: a, compared with no PAH group, $P < 0.05$; b, compared with the mild group, $P < 0.05$; c, compared with the moderate group, $P < 0.05$. *, compared with the value before treatment.

al impairment of PMVECs as well as local thrombosis [10]. Intimal fibrosis decreases vascular elasticity, thickens wall and leads to infiltration of inflammatory cells around the blood vessels, along with progressive occlusion of the pulmonary vascular bed. Among them, PMVECs release increased vasoactive substances (such as renin-AgII, catecholamines, endothelin). The reduction of vasoactive substances (such as prostacyclin, NO) eventually causes pulmonary vascular structural remodeling and PAH formation [11-13].

According to the study, it is noted that the PAP levels were significantly increased. In no PAH group, factors such as shorter course of disease, type of CHD, size of defect, and the level of PAP after intervention was not obvious. PAP levels were significantly decreased after intervention in children with PAH. After surgery, the patients were reexamined by ultrasound. The changes tend to be stable after 7 days. Therefore, the preoperative and PAP levels were measured at 7 day after surgery. Because of the intervention or surgery, the defect-specific abnormal shunt channel was directly repaired, and the normal and pulmonary circulation pathways were restored. The PAP level was significantly reduced. The normal level of PAP in mild and moderate groups could be basically restored. Early pulmonary vascular reconstruction was reversible, but advanced PAH is frequently irreversible. Some patients with severe group still have higher PAP after treatment [14, 15].

The levels of Apelin in the no PAH group before treatment and 7 days after treatment were significantly elevated compared to mild group, followed by moderate and severe group. It is suggested that the decrease in Apelin level may be one of the important reasons for the formation of PAH. Studies have confirmed [16, 17] that Apelin/APJ system is involved in LPS-induced PMVECs injury in rats, and exogenous Apelin can reverse some PMVECs injury, which may be related to activation of Akt phosphorylation pathway. Apelin is a vasoactive polypeptide with multiple molecular subtypes. Serum concentration is lower than tissue concentration. It

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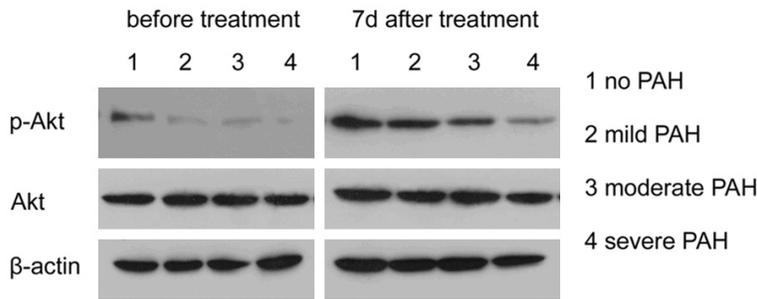


Figure 1. Western blot was used to detect the p-Akt/Akt values before and after treatment (1: non-PAH group; 2: mild-PAH group; 3: moderate-PAH group; 4: severe-PAH group).

plays a role in autocrine and paracrine forms, promotes neovascularization, relaxes blood vessels, regulates inflammatory responses, etc. [18-21]. Zeng et al [22] found that Apelin can promote the apoptosis of pulmonary artery smooth muscle cells, the decrease of Apelin level leads to increased cell proliferation, decreased apoptosis, pulmonary vascular middle layer hypertrophy, positive vascular remodeling, and PAH formation. The result from no PAH group showed no change of Apelin level after treatment compared to before treatment. The reason was that the Apelin level in the PAH group was not stable, and the Apelin level changed for a period of time. After intervention or surgery, the pulmonary circulation blood flow gradually stabilized, the stress damage to PMVECs was reduced, the cell hypoxia was improved, and the ability to express Apelin was gradually increased [23, 24].

The levels of AgII in the severe group before and 7 d after treatment were higher than moderate group, followed by moderate, mild group and no PAH group. The role of AgII is closely related to Apelin, and increased expression of AgII promotes PAH formation. AgII is mainly produced in the lungs and binds to angiotensin receptors on the cell membrane to exert biological effects, such as stimulating vascular smooth muscle cell division and proliferation, participating in vascular remodeling; participating in vascular inflammatory and injurious stress responses. Indirect changes in blood vessel wall tension, redistribution of blood flow, result in PAH [25, 26].

The p-Akt/Akt values in the PAH-free group before treatment and 7 days after treatment

were higher than that in mild group, followed by moderate and severe group. It is suggested that a decrease in Akt phosphorylation activity may be involved in PAH formation. The PI3K/Akt signaling pathway can decrease the activity of pro-apoptotic genes and up-regulate the expression of anti-apoptotic genes. Akt is a direct target protein downstream of PI3K, and its phosphorylation is involved in various

regulation of cellular activities [27, 28]. Previous studies suggested the development of PAH was associated with the multiple signaling pathways, such as NADPH/ROS/p38, RhoA/ROCK [3, 4]. Recent findings indicated that Akt/mTOR signalling pathway was involved in pulmonary vascular remodeling in rat PAH model by inhibiting cell proliferation, which was in line with our study [29]. However, the limitation in the study still exists that there were only 86 children with CHD in the study. The reliability of the research needs to be improved within a large number of samples. Also, based on that, the correlation between serum Apelin-AgII level and PAP should be determined and whether there is a direct regulation of Apelin, AgII on Akt phosphorylation signaling remains to be identified.

In summary, serum Apelin levels are decreased, and elevated levels of AgII may mediate the activity of the Akt phosphorylation pathway to promote the development and progression of CHD with PAH, which can be retarded by the treatment. Our preliminary data demonstrate significant changes of serum apelin-angiotensin II expressions and Akt phosphorylation pathway in children with congenital heart disease complicated with pulmonary hypertension, which provides new leads for the diagnosis in the future.

Disclosure of conflict of interest

None.

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