

Effect of atorvastatin on pulmonary arterial hypertension in rats through PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effect of atorvastatin on pulmonary arterial hypertension (PAH) in rats and to observe its specific regulatory mechanism through the phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/AKT) signaling pathway.

MATERIALS AND METHODS: The model of PAH was successfully established in rats *via* hypoxia feeding. All rats were divided into three groups, including Control group (n=15), PAH model group (Model group, n=15) and atorvastatin treatment group (Ator group, n=15). Tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and nitric oxide (NO) were detected *via* enzyme-linked immunosorbent assay (ELISA). Right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index (RVHI) in each group were determined as well. Meanwhile, the pathological changes in lung tissues of rats were detected *via* hematoxylin-eosin (HE) staining. Furthermore, the apoptosis level of lung tissues in each group was detected *via* terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. In addition, the expression levels of PI3K/AKT signaling pathway and apoptotic genes in lung tissues were detected *via* quantitative Polymerase Chain Reaction (qPCR).

RESULTS: In Model group, the levels of TNF- α and IL-6 increased significantly, while the level of NO decreased. Both RVSP and RVHI in Model group were significantly higher than those of Control group and Ator group ($p<0.05$). The results of HE staining revealed that Model group showed significantly severe lung tissue injury ($p<0.05$). According to the results of TUNEL staining, the number of apoptotic cells in lung tissues in Model group was significantly smaller than that of Ator group ($p<0.05$). Meanwhile, the expression level of cysteinyl aspartate-specific proteinase-3 (Caspase-3) in Model group was markedly lower than that of Ator group ($p<0.05$). However, the expression level of B-cell lymphoma-2 (Bcl-2) in Model group was markedly

higher than that of Ator group ($p<0.05$). In Ator group, the expression levels of PI3K and AKT in lung tissues were remarkably higher than those of Model group ($p<0.05$). All the above results indicated that atorvastatin could effectively up-regulate the expressions of PI3K and AKT ($p<0.05$).

CONCLUSIONS: Atorvastatin regulates the symptoms of PAH in rats through activating the PI3K/AKT signaling pathway.

Key Words:

Atorvastatin, Pulmonary arterial hypertension (PAH), PI3K/AKT signaling pathway, Rats.

Introduction

Pulmonary arterial hypertension (PAH) is a common cardiovascular disease, seriously threatening the health and affecting the life quality of patients¹. However, the pathophysiological mechanism of PAH has not been fully elucidated yet. Currently, evidence has shown that after the occurrence of clinical symptoms, the vital activities of PAH patients are greatly restrained. Meanwhile, the physiological indexes are inhibited and the life cycle is shortened by about 2 years². The major pathological features of PAH include abnormal increase of pulmonary vascular resistance, vascular cell proliferation, and enhanced resistance to apoptosis. This may eventually lead to a persistent proliferation of pulmonary arteries, declined cross-sectional area of pulmonary arteries, pulmonary artery sclerosis, decreased pulmonary blood flow, as well as changes in blood supply. As a result, pulmonary vascular remodeling occurs and pulmonary arterial pressure increases significantly. This causes a series of adverse reactions, ulti-

mately inducing right heart failure^{3,4}. Currently, targeted therapy can reduce severe PAH and delay the referral of patients receiving lung transplantation. However, transplantation is still an important choice for advanced PAH patients. However, such choice results in the treatment abandoning of many patients, seriously harming the development of social welfare⁵. Nowadays, the pathogenesis of PAH remains unclear. Multiple factors can lead to increased pulmonary circulation blood flow and resistance, thus inducing PAH. Therefore, the pathological state of PAH patients cannot be effectively improved by most treatment means, leading to its poor prognosis^{6,7}. Furthermore, deeply analyzing the molecular mechanism of PAH pathogenesis and searching for new therapeutic targets are key issues in the effective treatment of PAH.

Phosphatidylinositol 3-hydroxy kinase/protein kinase B (PI3K/AKT) signaling pathway is involved in various biological functions throughout the whole life. The functions of the PI3K/AKT signaling pathway have been gradually studied. Scholars^{8,9} have found that PI3K/AKT can mediate cell proliferation and apoptosis. Moreover, PI3K/AKT exerts a survival effect and inhibits abnormal death, which is mainly mediated by inhibiting various pro-apoptotic proteins such as Caspase-9¹⁰. The involvement of PI3K/AKT pathway in the pathogenesis of PAH has been investigated as well¹¹. Several studies have indicated that the PI3K/AKT pathway may play a role in cell proliferation and apoptosis in PAH. However, the potential role of PI3K/AKT in the anti-apoptotic effect of atorvastatin is still unclear. Meanwhile, the downstream elements of the PI3K/AKT signal remain to be determined. Currently, few studies have focused on the regulatory effect of atorvastatin on PI3K/AKT and its influence on PAH.

In the present study, the model of PAH was successfully established in rats *via* hypoxia feeding. The inflammatory factors and morphological changes in lung tissues were detected *via* enzyme-linked immunosorbent assay (ELISA) and hematoxylin-eosin (HE) staining, respectively. Right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index (RVHI) were determined as well. The apoptosis level was detected *via* terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. Meanwhile, the changes in the PI3K/AKT pathway in lung tissues were determined using quantitative Reverse Transcription-Poly-

merase Chain Reaction (qRT-PCR). The effect of atorvastatin on PAH in rats was evaluated. Furthermore, the regulatory effect of atorvastatin on the PI3K/AKT signaling pathway was explored. Our findings might provide an experimental basis for subsequent researches and the development of new drugs.

Materials and Methods

Animal Grouping and Modeling

Male Sprague-Dawley (SD) rats weighing 250-280 g were purchased from the Shanghai Medical Laboratory Animal Center. All rats were adaptively fed in the specific pathogen-free house for 1 week. After that, rats were divided into Control group (n=15), PAH model group (Model group, n=15) and atorvastatin treatment group (Ator group, n=15). The model of PAH was successfully established in rats *via* hypoxia feeding. Rats in Control group were fed in a normal environment, while those in Model group and Ator group were fed in a hypoxia incubator. This investigation was approved by the Laboratory Animal Ethics Committee. All animal operations were performed in accordance with the NIH Laboratory Animal Guide.

Establishment of the PAH model: rats were fed in a hypoxia incubator with mixed gas of 90% nitrogen and 10% oxygen for 8 h every day in a hypoxic environment. After 3 weeks, if the model has been successfully established in rats was evaluated (the duration of hypoxic treatment could be prolonged appropriately to ensure successful modeling).

Detection of Right Ventricular Pressure and Hypertrophy in Rats

Rats were fixed on an experiment table and anesthetized. Subsequently, RVSP in each group was detected and recorded. Right ventricular free wall (RV) and left ventricle + interventricular septum (LV + S) were weighed. Finally, RVHI was calculated.

Detection of Serum Cytokines in Rats

Serum levels of tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and nitric oxide (NO) were detected according to the instructions of ELISA kit (R&D Systems, Minneapolis, MN, USA). Finally, the absorbance in each group was measured.

Pathological Changes in Lung Tissues of Rats

Lung tissues were first washed with running water for 24 h, dehydrated with gradient alcohol and routinely prepared into sections. After deparaffinization, the sections were hydrated with 95%, 90%, 80%, 75%, and 50% ethanol, respectively. Then, sections were stained with HE (Boster, Wuhan, China). Finally, pathological changes in lung tissues were observed under a light microscope.

TUNEL Apoptosis Assay

The sections were fixed with 4% paraformaldehyde, rinsed with phosphate-buffered saline (PBS) twice and infiltrated with 0.1% of Triton X-100. Then, apoptotic fragment DNAs were labeled with fluorescein isothiocyanate (FITC) according to the instructions of the TUNEL assay kit (Beyotime, Shanghai, China). Next, FITC-labeled TUNEL-positive cells were imaged at an excitation wavelength of 488 nm under a fluorescence microscope. Finally, the number of TUNEL-positive cells were counted in 10 fields of view.

Quantitative Reverse Transcription-Polymerase Chain Reaction (QRT-PCR)

An appropriate number of frozen lung tissues were taken, added to liquid nitrogen and homogenized under low temperature at 2000 rpm for 30 s. Total RNA was extracted from lung tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentration was determined using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). Subsequently, extracted RNA was reversely transcribed into complementary Deoxyribose Nucleic Acid (cDNA) according to the instructions of PrimeScript™ RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). The expression levels of target genes were detected *via* qRT-PCR. Primers of target genes and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed according to GenBank. The specific primer sequences used in this study were shown in Table I. QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative expression levels of genes

Table I. PCR primer sequences.

mRNA	Sequence
Caspase-3	F: 5'-CTACCGCACCCGGTTACTAT-3' R: 5'-TTCCGGTTAACACGAGTGAG-3'
Bcl-2	F: 5'-GGTGCTCTTGAGATCTCTGG-3' R: 5'-CCATCGATCTTCAGAAGTCTC-3'
PI3K	F: 5'-TGTTCTTGCGAAGTGAGATAG-3' R: 5'-CTGCTGCGTGAAGTCCTGTA-3'
AKT	F: 5'-TAGGCATCCCTTCCTTACAGC-3' R: 5'-CGCTCACGAGACAGGTGGA-3'
GAPDH	F: 5'-CAGTGCCAGCCTCGTCTCAT-3' R: 5'-AGGGCCATCCACAGTCTTC-3'

in lung tissues of each group were calculated by the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. The bar graph was plotted using GraphPad Prism 5.0. Experimental results were expressed as mean $\pm$ standard deviation ($\bar{x} \pm SD$). One-way ANOVA test was used to compare the differences among different groups, followed by post-hoc test (Least Significant Difference). $p < 0.05$ was considered statistically significant.

Results

General Data of Rats in Each Group

As shown in Table II, the body weight, activity, and food intake of rats were significantly reduced in Model group. Meanwhile, they were markedly improved in Ator group, but worse than those in Control group.

RVSP and RVHI in Each Group

After anesthesia, RVSP was determined *via* right cardiac catheterization. Meanwhile, the right ventricle was isolated to calculate RVHI. As shown in Figure 1, both RVSP and RVHI were significantly higher in Model group than those of Control group ($p < 0.05$); however, they were significantly lower in Ator group than Model group ($p < 0.05$).

Table II. General conditions of rats in each group.

Group	Case (n)	Manifestation
Control	15	Good mental state and normal food intake
Model	15	Decline in body weight loss, activity and food intake
Ator	15	Improvement in activity and food intake

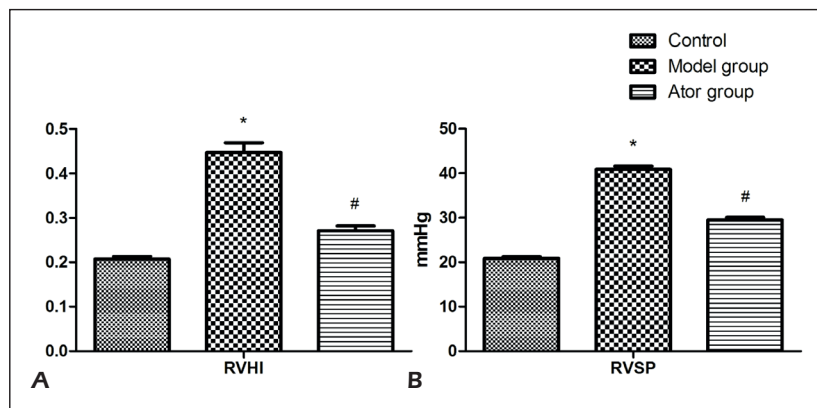


Figure 1. RVSP and RVHI of rats. Both RVSP and RVHI are remarkably higher in Model group than those of Control group. * $p < 0.05$ vs. Control group

Table III. ELISA results in each group.

Group	IL-6 (mg/L)	TNF- α (fmol/mL)	NO (mol/L)
Control	80.23 $\pm$ 8.72	39.23 $\pm$ 4.53	44.23 $\pm$ 8.56
Model	190.35 $\pm$ 15.05 ^a	83.21 $\pm$ 8.16 ^a	22.96 $\pm$ 6.57 ^a
Ator	100.29 $\pm$ 10.43 ^b	46.58 $\pm$ 6.23 ^b	36.29 $\pm$ 9.67 ^b

^a $p < 0.05$ vs. Control group, ^b $p < 0.05$ vs. Model group

ELISA Results in Each Group

As shown in Table III, the levels of IL-6 and TNF- α increased remarkably in Model group and Ator group ($p < 0.05$). The levels of IL-6 and TNF- α in Ator group were significantly lower than those of Model group ($p < 0.05$). However, the level of serum NO was significantly higher in Ator group when compared to Model group ($p < 0.05$).

Pathological Changes in Lung Tissues of Each Group

The pathological damage of lung tissues in each group was detected *via* HE staining. Results revealed that compared with Control group (A), pulmonary septal thickening, increased pulmonary shadow, unclear outline, and alveolar edema, fusion and hemorrhage were observed in Model group (B). In addition, lung tissue injury was significantly alleviated in Ator group (C) (Figure 2).

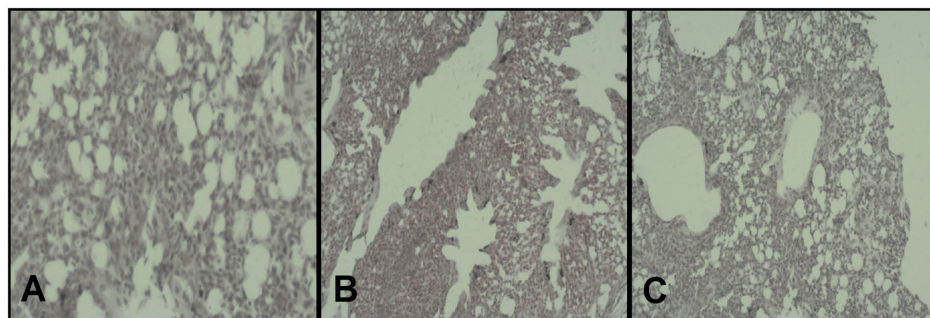


Figure 2. Pathological changes in lung tissues of each group detected *via* HE staining (magnification $\times 10$). Lung tissue injury is significantly alleviated in Ator group.

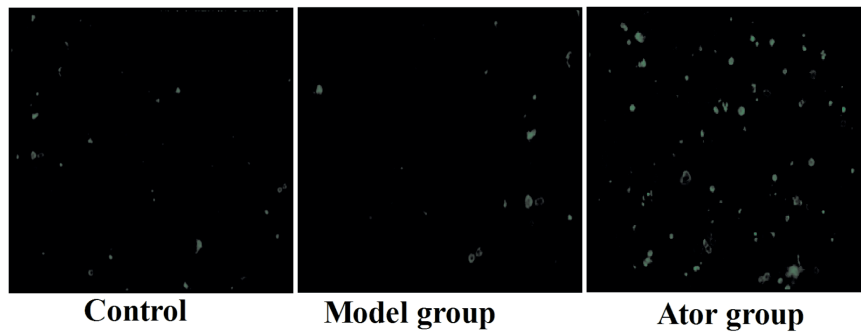


Figure 3. Apoptosis level of lung tissues in each group detected via TUNEL staining (magnification: 200×). The number of TUNEL-positive cells in lung tissues in Ator group is remarkably larger than that of Model group.

Apoptosis Level of Lung Tissues in Each Group

No evident TUNEL-positive cells were observed in lung tissues of Control group and Model group. However, the number of TUNEL-positive cells in lung tissues of Ator group was remarkably larger than that of Model group ($p < 0.05$) (Figure 3).

Expression Levels of Apoptosis Genes in Lung Tissues of Each Group

The expression levels of apoptotic genes in lung tissues of each group were detected via RT-PCR. Results showed that the expression level of Bcl-2 in lung tissues of Model group was significantly higher than that of Ator group ($p < 0.05$). However, the expression level of Caspase-3 in Model group was significantly lower than that of Ator group ($p < 0.05$) (Figure 4). The above results indicated that apoptosis was inhibited.

Regulatory Effect of Atorvastatin on PI3K/AKT Signaling Pathway-Related Genes

In Ator group, the expression levels of PI3K and AKT in lung tissues were remarkably higher than those of Control group and Model group ($p < 0.05$) (Figure 5).

Discussion

PAH can be induced by a variety of factors, including chronic exposure to moderate hypoxia¹². Meanwhile, PAH is a clinical syndrome characterized by pulmonary vascular occlusion lesions resulted from pulmonary vasoconstriction and structural changes in pulmonary arteries due to an increased pulmonary circulation pressure^{13,14}. In most PAH patients, the apoptosis of pulmonary vascular endothelial cells decreases, while cell pro-

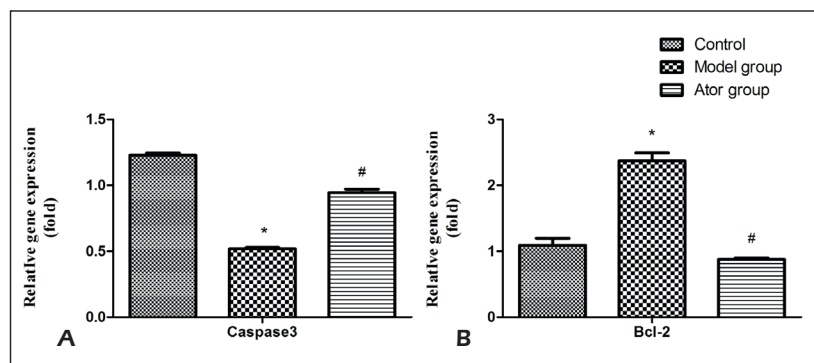


Figure 4. Expression levels of apoptotic genes. Compared to those in Model group, the expression level of Caspase-3 in lung tissues increases significantly, while the expression level of Bcl-2 decreases significantly in Ator group. * $p < 0.05$, # $p < 0.05$

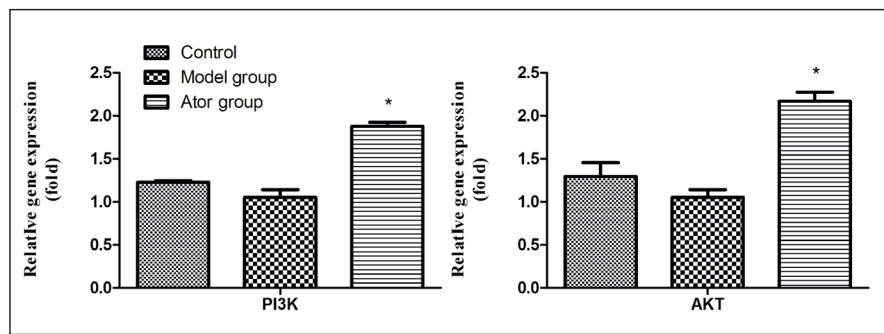


Figure 5. Expression levels of signaling pathway genes. The expression levels of PI3K and AKT in lung tissues are significantly up-regulated in Ator group. * $p < 0.05$ vs. Control group and Model group.

liferation markedly increases. Meanwhile, the imbalance between apoptosis and proliferation leads to the remodeling of pulmonary arterial smooth muscle cells and pulmonary vascular occlusion. Eventually, this may result in right heart overload and even right heart failure^{15,16}. Hypoxia feeding effectively simulates the pathogenesis of PAH in humans, which can be adopted to establish the rat model of PAH. Therefore, it is an important method to investigate the pathogenesis of PAH and to search for its therapeutic regimens¹⁷. In the present study, SD rats were fed in a hypoxic environment for 21 days. Compared to normal rats, the rats in Model group showed a significantly increased RVSP and RVHI after modeling. This indicated that the model of PAH was successfully established in rats. At the same time, the HE staining of lung tissues showed that lung tissue injury in Model group was severe. Sisniega et al¹⁸ have found that PAH leads to secondary pulmonary dysfunction and lung tissue injury. Inflammation plays an indispensable role in the occurrence and development of PAH as well, accompanied by increased inflammatory cells¹⁹. In the current study, the expression levels of IL-6 and TNF- α in Model group were significantly higher than those of Control group. However, the expression level of NO was significantly lower than that of Control group. After treatment with atorvastatin, the expression levels of IL-6 and TNF- α were both significantly declined, while the expression level of NO increased markedly. These results suggested that increased levels of IL-6 and TNF- α and decreased level of NO might further promote the development of PAH. However, the above condition was improved after treatment with atorvastatin, indicating that atorvastatin was effective for the PAH treatment. Our findings were consistent with the results of Pellicelli et al²⁰, Maki-Petaja et al²¹ and Gelosa et al²². To observe the

changes in pulmonary arterial pressure, RVSP and RVHI were further detected. Results manifested that RVSP and RVHI were markedly declined, and lung tissue injury was alleviated in Ator group. The above results strongly suggested that atorvastatin was involved in regulating PAH in rats and might affect the development of PAH.

Morty et al²³ have found that atorvastatin can significantly up-regulate the apoptosis level through mediating immune response and inflammatory response. In this work, the apoptosis level in lung tissues of PAH rats was detected *via* TUNEL staining in this study. Results revealed that the apoptosis level in lung tissues of Model group increased significantly, which was consistent with the results in the previous studies. In addition, RT-PCR results demonstrated that the expression levels of apoptosis-related genes (Caspase-3 and Bcl-2) in lung tissues were significantly changed in Ator group. Omar et al²⁴ applied statins in the intervention treatment of PAH patients. They have found that statins can inhibit the proliferation of pulmonary arterial smooth muscle cells in a concentration-dependent manner, consistent with the data in this work.

AKT is a serine/threonine protein kinase, which is activated by various growth factors and cytokines in a PI3K-dependent manner²⁵. The activation of the PI3K/AKT pathway exerts important influences on the differentiation, proliferation, and apoptosis of smooth muscle cells and vascular fibroblasts²⁶. This is mainly mediated by the inhibition of various pro-apoptotic proteins such as Caspase-9. The involvement of the PI3K/AKT pathway in the pathogenesis of PAH has been widely studied. Therefore, the PI3K/AKT pathway may play an important role in cell proliferation and apoptosis in PAH. However, the potential role of PI3K/AKT in the anti-apoptotic effect of atorvas-

tatin is still unclear. Meanwhile, the downstream elements of PI3K/AKT signal remain to be determined²⁷⁻²⁹. In the current years, few authors have explored the regulatory effect of atorvastatin on PI3K/AKT and its influence on PAH. To further verify the effect of atorvastatin on the PI3K/AKT signaling pathway in PAH, the expression levels of pathway genes were detected in this study. It was found that the expression levels of PI3K and AKT in lung tissues decreased markedly in Model group, while remarkably increased in Ator group. Atorvastatin alleviated the symptoms of PAH in rats. Moreover, it significantly increased the expressions of PI3K and AKT in lung tissues. The above findings suggested that the activation of the PI3K/AKT signaling pathway might be an important reason for the decline in PAH. Although such an effect was observed in the present study, there were still some limitations. Cells should be cultured *in vitro*, and multiple cell lines could be introduced. Furthermore, more genes and proteins in the PI3K/AKT signaling pathway should be detected to further verify the effect.

Conclusions

We found that atorvastatin regulates the symptoms of PAH in rats by activating the PI3K/AKT signaling pathway. Therefore, atorvastatin can be used as a therapeutic drug for PAH. Meanwhile, the therapeutic effect and prognosis of patients can be evaluated by the PI3K/AKT signaling pathway. In subsequent researches, more cell lines can be introduced *in vitro*, and other possible mechanisms of atorvastatin can be further verified *via* flow cytometry and protein assay.

Conflict of Interests

The authors declared that they have no conflict of interests.

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