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Original Research Article

Effect of Panaxadiol on gene expression in rat cardiovascular endothelial cells based on RNA sequencing

Dongyang Guo, Xiufang Hong, Lingzhi Shen, Genxiang Mao, Zhouxin Yang[*]

Department of Intensive Medicine Laboratory, Zhejiang Hospital, Hangzhou 310030, China
* Corresponding author: Zhouxin Yang, yangzhouxin@hotmail.com

ABSTRACT

Objective: To systematically analyze the effect of Panaxadiol on gene expression of cardiac microvascular endothelial cells (cmecs) during lipopolysaccharide (LPS) infection. **Methods:** By isolating primary cmecs from rats, the cells were divided into control group and experimental group. The control group was stimulated by LPS alone, and the experimental group was stimulated by LPS and Panaxadiol together. After that, the differentially expressed genes and related signal pathways were analyzed by transcriptome sequencing, and some differentially expressed genes were verified by quantitative PCR. **Results:** Panaxadiol could significantly inhibit the expression of some genes, which were mainly concentrated in inflammatory signal related pathways, metabolic related pathways and epigenetic regulation. **Conclusion:** Panaxadiol may affect the expression of inflammation related genes through epigenetic regulation, and then regulate the inflammatory response of cmecs.

Keywords: lipopolysaccharide; cardiac microvascular endothelial cells; Panaxadiol; transcriptome sequencing

1. Introduction

Lipopolysaccharide (LPS) is the main component of the cell wall of gram-negative bacteria. As an important pathogen associated molecular pattern (pamps), LPS is recognized by pattern recognition receptors (PRRS) on the surface of immune cells, which will activate downstream signal pathways and cause the production and release of inflammatory factors in host cells, Excessive release of inflammatory factors can lead to a series of inflammatory diseases, even sepsis or septic shock[1,2]. Cardiac microvascular endothelial cells (cmecs) are similar to the endothelial cells of other tissues in the body. They have the functions of barrier, substance transport, anticoagulation, procoagulation and regulation of vasoconstriction. The change of vascular permeability caused by their dysfunction is recognized as one of the key links of myocardial injury caused by LPS infection[3,4]. Ginseng belongs to Araliaceae of Umbelliferae. As a traditional Chinese medicine, it has been known as the "king of herbs" since ancient times. Studies have proved that the fleshy roots of ginseng can be used to adjust blood pressure, restore heart function, neurasthenia and physical weakness. Panaxadiol (20 r-panaxadiol, PD) is the main aglycone of saponins in ginseng, Panax notoginseng, Panax quinquefolium, Gynostemma pentaphyllum and other medicinal plants. Its molecular weight is very small,

which is more conducive to the absorption and pharmacological activity of the body[5]. Studies have shown that PD can effectively inhibit the proliferation of tumor cells and is considered as a promising anticancer drug[6,7]. However, there are few reports on whether it plays a role in the injury of cmecs induced by endotoxins and its related molecular mechanisms. In this study, LPS and LPS combined with PD were used to treat rat cmecs. Transcriptome sequencing (RNA SEQ) was used to detect the changes of cell transcriptome, and quantitative PCR was used to verify some differential genes. The purpose was to study the effect of PD on cmecs gene expression under LPS infection, screen the differentially expressed genes and related signal pathways, and explore the role of PD in LPS induced cardiovascular endothelial cell injury. The report is as follows.

2. Materials and methods

2.1. Laboratory animals and reagents

SD rats were purchased and raised in the experimental animal center of Zhejiang Academy of Medical Sciences. They were 1~2 months old and weighed 100~150 g. DMEM culture medium and fetal bovine serum were purchased from GIBCO company of the United States; type II collagenase and 0.25% trypsin were purchased from sigma of the United States; endothelial growth factor was purchased from American peprotech company. The experimental operation was approved by the ethics committee of Zhejiang Academy of Medical Sciences.

2.2. Method

Cell isolation and culture:

After anesthesia, SD rats were given intraperitoneal injection of heparin for 20 min. After disinfection, the heart tissue was taken out and washed with d-hanks solution for several times; the left and right atrium, interventricular septum and right ventricle were removed, immersed in 75% ethanol for 20~30 s, and then about 1/4 of the outside of the left ventricular free wall and the endocardium were cut off. The remaining myocardial tissue was fully reduced to about 1 mm3 small pieces. After washing twice with d-hanks, about 1 mL of 0.2% type II collagenase was added and incubated in a 37 °C water bath for 30 min; then add equal volume of 0.02% trypsin, gently blow and incubate at 37 °C for 20 min until most tissue blocks are completely digested. Finally, add the culture medium containing 20% serum to terminate digestion, filter with 200 mesh sieve, centrifuge at 1000 r/min for 5 min at 4 °C, and collect cells after washing with d-hanks. The collected cmecs were resuspended in complete medium (containing 20% fetal bovine serum and growth factor) and inoculated in 10 cm petri dishes. The non adherent cells were discarded after 4 h. The adherent cmecs were used for subsequent experiments.

RNA extraction and sequencing:

The primary cmecs were inoculated into 6-well plates and treated with LPS (100 ng/mL) (LPS group) or LPS (100 ng/mL) + pd (10 µG/mL) (lps + pd group), 3 wells in each group, cells were collected after 6 h, total RNA was extracted by Trizol method, and quality control was carried out. The cdna library was constructed according to the instructions of the RNA SEQ sample preparation kit of Illumina biotechnology. Comparing the genomic information and complete transcriptome information of rats, the sequenced reads were compared to the reference genome, and then the genes or transcripts were annotated and quantified. After the reads were compared to the genome, stringtie (2016) was used to annotate and quantify the expression. Stringtie uses

network flow algorithm and optional denovo assembly to assemble complex data sets into transcripts. Fpkm values of genes and transcripts (gene expression obtained by base mapping per million exon fragments) were further calculated.

Differential transcript analysis:

Balltown was used to compare the two different treatment groups, and the p value, Q value and fold change between transcripts were obtained; in this study, the two groups were compared and screened for differential genes. The screening conditions were $p < 0.05$ and fold change >1.5 times and <0.66667 times.

Go function category and channel enrichment analysis:

Go database describes gene and protein functions in detail in a tree hierarchical manner, and clarifies the hierarchical relationship between gene functions. Gene functions are divided into three categories: molecular function, biological pathway and cellular component. KEGG (Kyoto Encyclopedia of genes and genes) is a database for systematic analysis of the relationship between genes and their coding products, gene function and genome information. Signal pathway analysis is based on KEGG database, using Fisher exact test and $\chi 2$ test: the pathways involved in the target gene were statistically analyzed and screened according to $p < 0.05$ to screen the signal pathways with significant differences.

Quantitative PCR validation:

The sequenced RNA samples were reverse transcribed into cdna. Five genes, chemokine ligand (CCL)-11, interleukin (IL)-22, IL-17, matrix metalloproteinase (MMP)-9 and epidermal growth factor (EGF), were verified by quantitative PCR. The quantitative PCR was analyzed by SYBR®Premix extaq. The mrna expression of the target gene was β-Actin was used as internal parameter and calculated by 2-△△CT least square method. Repeat 3 multiple holes for each sample, and repeat more than 3 times for each experiment to ensure the stability of the results.

2.3. Statistical processing

The graphpad7.0 statistical software was used for statistical processing. The measurement data were expressed in. The paired t-test was used for comparison between groups. $P < 0.05$ was statistically significant.

3. Results

3.1. The results of RNA SEQ cluster analysis are shown in Figure 1 in the insert

It can be seen from **Figure 1** in the inset that different treatment groups can be completely separated, and the expression of their corresponding transcripts varies greatly. Red represents the high expression value of differential transcripts in the grouped samples, and green represents the low expression value of differential transcripts in the grouped samples. By comparing the two treatment groups, 2256 differentially expressed genes were obtained, including 845 up-regulated genes and 1411 down regulated genes.
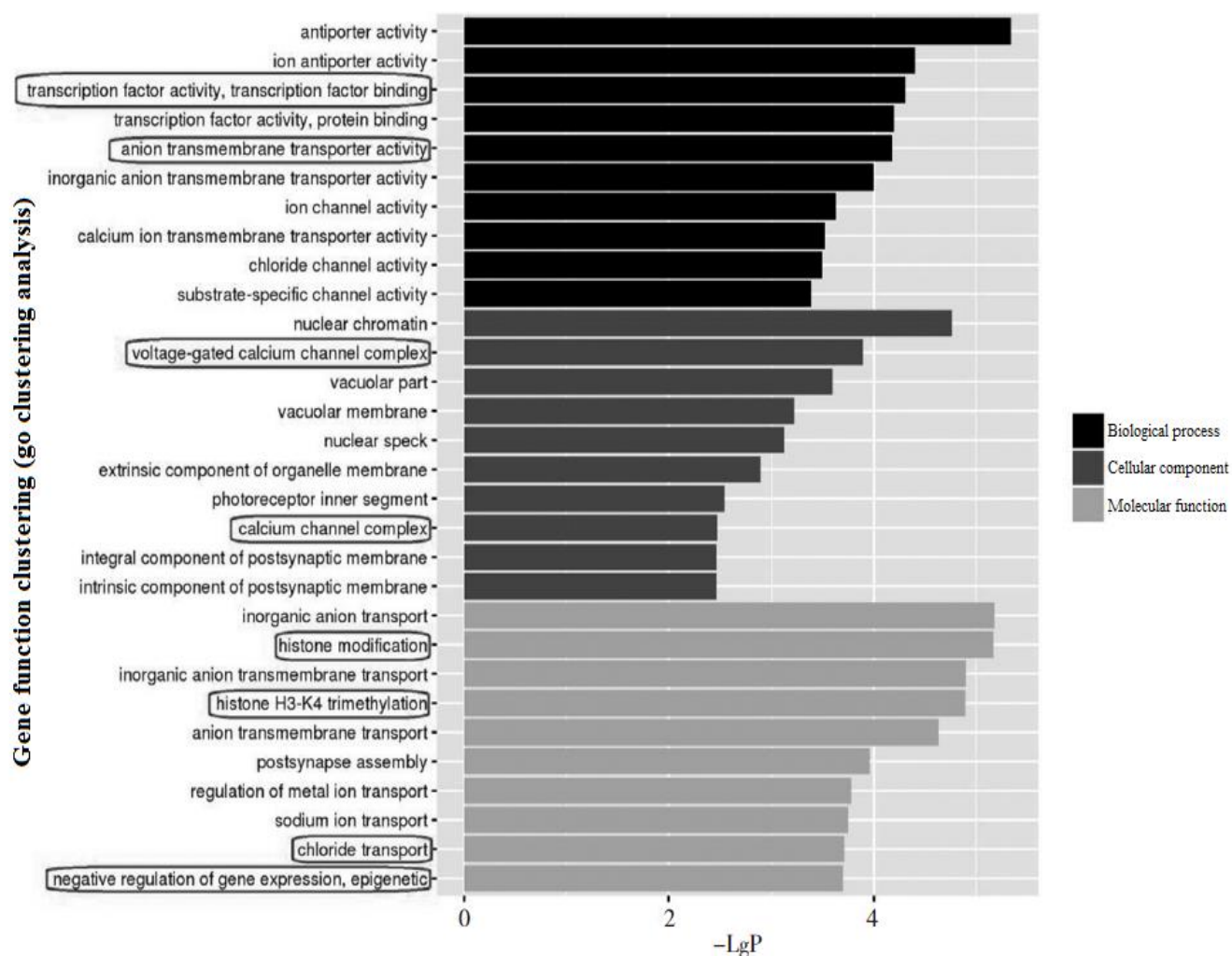
Figure 1. Go analysis results of significant enrichment of differential genes (the ordinate is the functional name of the differential gene, and the abscissa is the negative logarithm (−lgp) of the p value. The larger the value, the smaller the *p* value, that is, the higher the functional significance level of the differential gene).

3.2. Go analysis results of significant enrichment of differential genes are shown in Figure 2

According to the significance level of down-regulated differential genes in the analysis results in 2.1, the distribution map can be seen in the three categories of genes: molecular function, biological process and cell composition, The significantly down regulated functional genes are mainly concentrated in: cell transport and ion channel related proteins (ion transporter, calcium ion, chloride ion, metal ion transmembrane transport, etc.), transcriptional activity regulation and epigenetic modification (histone H3-K4 trimethylation modification, epigenetic negative regulation), etc.
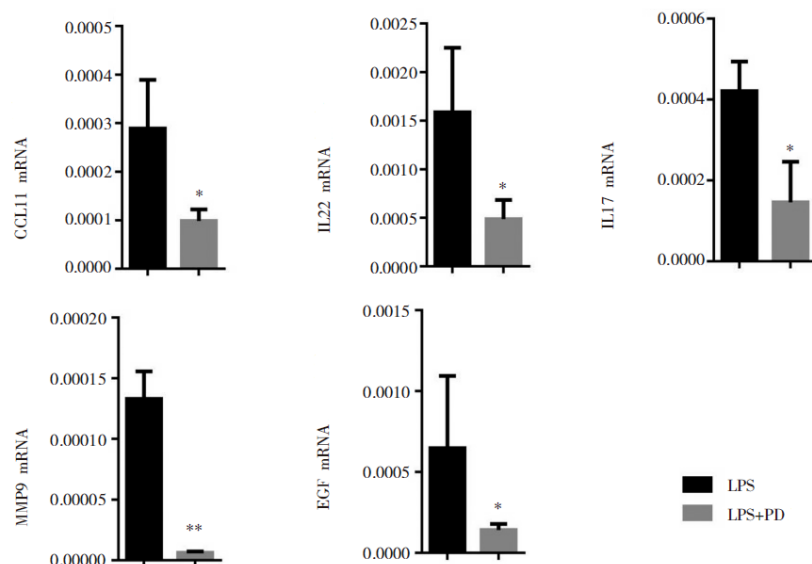
Figure 2. Expression difference of relevant genes verified by fluorescence quantitative PCR (compared with LPS group, $*p < 0.05$, $**p < 0.01$).

3.3. The analysis results of pathway significance enrichment of differentially expressed genes are shown in Figure 3

It can be seen from **Figure 3** in the inset that through the analysis of KEGG database, the down-regulated genes are obviously enriched in the signal pathways related to inflammation: PI3K Akt signal pathway, MAPK signal pathway, IL-17 signal pathway, chemokine signal pathway, camp signal pathway, complement related pathway, etc. In addition, there are some metabolic related signal pathways (glycerophosphingolipid metabolism and choline metabolism, etc.). It indicates that PD mainly affects the expression of inflammatory signaling pathway. 2.4 quantitative PCR verification results are shown in **Figure 4**.
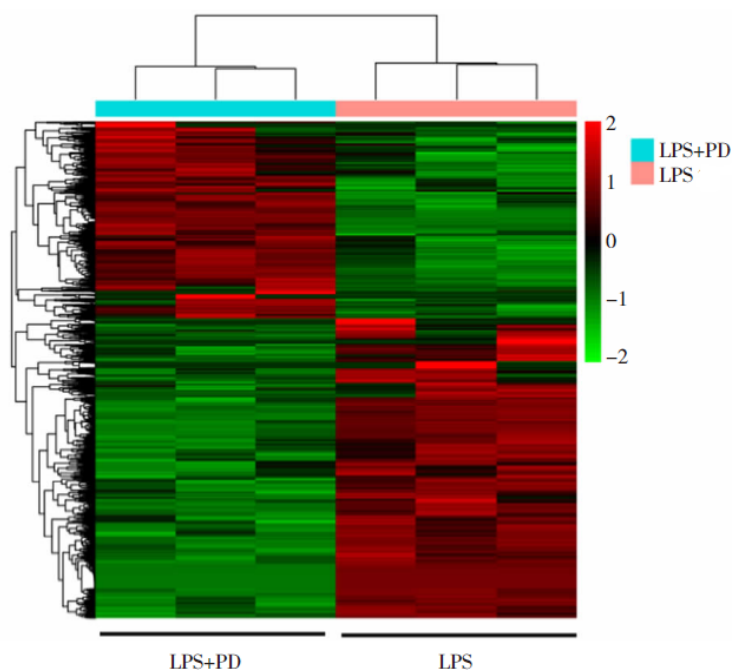
Figure 3. RNA SEQ cluster analysis diagram (abscissa represents samples of different treatment groups, and ordinate represents differential transcripts).
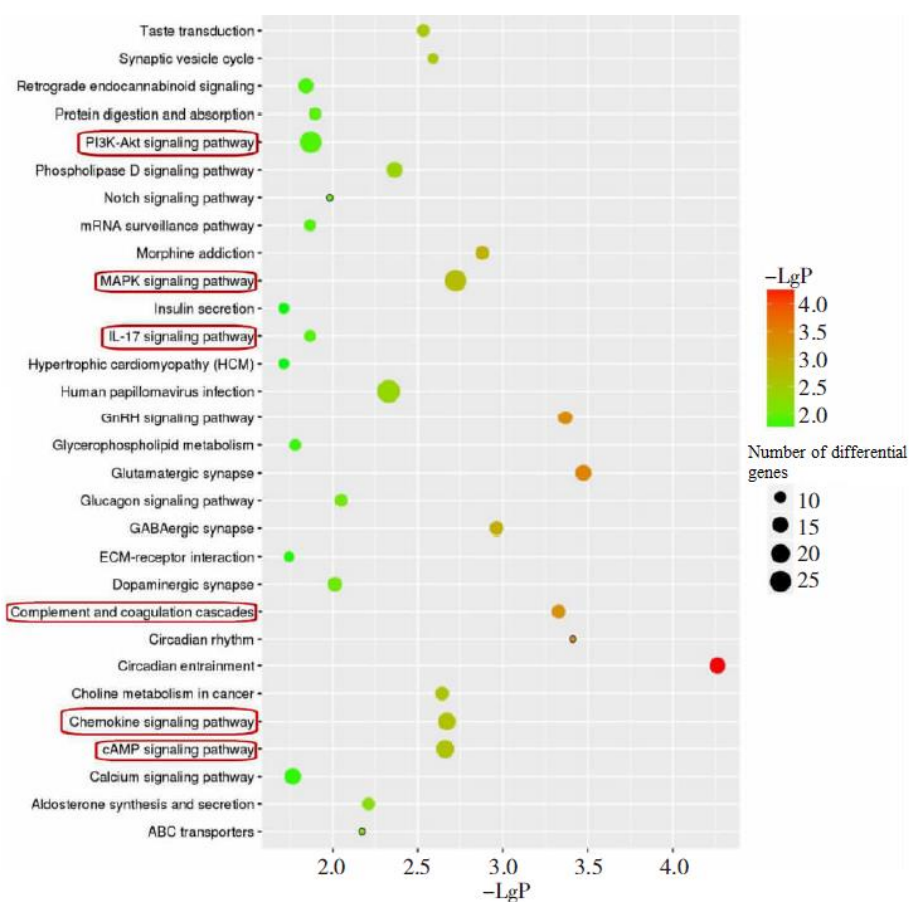
Figure 4. Pathway significance enrichment analysis of differentially expressed genes [the ordinate is the name of the differentially expressed gene pathway, and the abscissa is the negative logarithm (−lgp) of the p value. The larger the value, the smaller the *p* value, that is, the difference.

It can be seen from **Figure 4** that the quantitative PCR verification of the five differentially expressed genes (ccl-11, IL-22, IL-17, MMP-9 and EGF) in the sequencing results shows that the expression trend of these five genes is consistent with the sequencing results. PD treatment can significantly inhibit the expression of ccl-11, IL-22, IL-17, MMP-9 and EGF genes, and the differences are statistically significant (all $p < 0.05$). MMP-9 gene has a significant difference after PD treatment ($p < 0.01$).

4. Discussion

In the process of gram-negative bacteria infection, the body will produce a series of immune responses to eliminate pathogens, and the uncontrolled immune response will lead to the occurrence of diseases, even life-threatening severe diseases such as sepsis or septic shock[8,9]. In the inflammatory reaction caused by LPS infection, even in the process of sepsis and septic shock, the damage of heart tissue is inevitable, and endothelial cells play an important role[10]. Many studies have revealed the relationship between LPS infection and myocardial injury: LPS infection is first recognized by immune cells and triggers inflammatory reaction, leading to endothelial cytoskeleton remodeling and ve cadherin changes, leading to the disintegration of adhesion junction structure between cmecs, structural changes, increased vascular permeability, a large amount of fluid infiltrating into cardiac tissue space, aggravating tissue edema and cell hypoxia; A large number of inflammatory cells adsorb and infiltrate the myocardium, causing myocardial cell damage[11,12]. This study investigated the effect of PD, a Chinese herbal monomer, on the gene expression changes of cmecs cells induced by LPS, in order to explore the effect of PD on the gene expression changes of cmecs induced by LPS and its molecular mechanism.

Ginseng is a traditional Chinese medicinal material. Extensive studies have been conducted on the pharmacological effects of its effective components ginsenosides (Rb1, Rb2, RB3, RC, RD, Rh2, Rg3, etc.). They have been proved to play a certain protective role in cardiovascular diseases, and can antagonize calcium channels to play a protective role in myocardial ischemia and reperfusion; it can also regulate the energy metabolism of myocardial cells and resist myocarditis. RB1 can improve inflammation induced atherosclerosis[13] and inhibit tumor necrosis factor α (tumour necrosis factor-α, TNF-α), expression of inflammatory factors such as IL-6; in addition, in myocardial ischemia-reperfusion injury, Rb1, RB3 and RD can reduce the infarct area of the heart, reduce the levels of creatine kinase (CK), lactic dehydrogenase (LDH) and cardiac troponin I (ctni) in the serum, and improve the myocardial function after ischemia-reperfusion[14–16]. The molecular weight of PD is very small, which is more conducive to the absorption and pharmacological activity of the body. However, there are few studies on whether it plays a role in LPS induced cmecs damage and its related molecular mechanisms. Our research systematically detected the effect of PD on gene expression changes caused by LPS in cmecs through RNA SEQ high-throughput sequencing technology. According to the results of RNA SEQ, it can be found that PD treatment can significantly reduce some gene expression changes caused by LPS infection, including some transcription factor activity genes, ion channel related genes, epigenetic regulatory genes, etc, The genes with significantly reduced expression are mainly concentrated in immune related signal pathways (IL-17, PI3K Akt, MAPK signal pathways, etc.), epigenetic pathways and cmecs cell metabolism related pathways (choline, glycerophosphatidyl metabolism, etc.), indicating that PD can inhibit the inflammatory response, which may play an inhibitory effect on the inflammatory response of cmecs caused by LPS. In addition, PD can significantly change the expression of epigenetic related genes, such as histone h3-k4 trimethylation modifying enzyme. We speculate that PD may affect the expression of inflammation related genes by affecting epigenetic enzymes and regulating transcriptional activity.

The results of this study provide a systematic experimental basis for revealing the mechanism and target of PD and its effect on gene expression in cardiovascular endothelial cells, and may provide a theoretical basis for the clinical treatment of gram-negative bacterial infection.

Conflict of interest

The authors declare no conflict of interest.

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