Fluvastatin inhibits cardiomyocyte apoptosis after myocardial infarction through Toll pathway

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Abstract. The present study intended to investigate the effect of fluvastatin on cardiomyocyte apoptosis after myocardial infarction in rats. Eighty myocardial infarction rat models were established and randomly divided into 4 groups (n=20): experimental group (n=20) was given fluvastatin treatment; sham operation group (n=20) and normal control group (n=20) were given saline. The dose of fluvastatin was 20 mg/(kg·d), and irrigation gavage was given for 1 week. Western blot analysis and reverse transcription-quantitative PCR (RT-qPCR) were used to detect the expression of TLR4 mRNA and protein in cardiomyocytes. TUNEL method was used to detect the apoptosis of cardiomyocytes. After fluvastatin treatment for 1 week, RT-qPCR found that compared with myocardial infarction group, the TLR4 mRNA expression of fluvastatin treatment group and normal control group was significantly increased, and the differences between groups were a statistically significant difference (P<0.05). Western blot analysis showed that compared with the myocardial infarction group, the expression of TLR4 protein in normal control group, sham operation group and fluvastatin treatment group were significantly decreased, and they all were statistically significant (P<0.05). TUNEL method found that compared with the myocardial infarction group, the fluvastatin treatment group could significantly reduce the apoptosis of cardiomyocytes (19.2±3.8%), and the difference was statistically significant (P<0.05). Fluvastatin can inhibit myocardial infarction and decrease cardiomyocyte apoptosis by increasing the expression of TLR4-like receptor.

Introduction

Coronary atherosclerotic heart disease (CHD), also known as coronary heart disease, is caused by coronary atherosclerosis that leads to occlusion and stenosis of the coronary arteries leading to myocardial ischemia and hypoxia in patients (1,2). It is the most common cardiovascular disease for the elderly, and untimely treatment will lead to disability and death, and it is also a kind of disease with high mortality rate in the elderly (3). According to statistics, there are as high as 290 million people suffering from cardiovascular diseases in China, and mortality rate is higher than 30%, and the mortality and morbidity rates are now increasing year by year (4,5). Acute myocardial infarction (AMI) is the most serious type with the highest incidence among coronary heart disease in the elderly, mainly caused by coronary artery stenosis due to atherosclerosis, leading to myocardial ischemia, myocardial injury, and it is the main cause of sudden death clinically. There are as many as 8.5 million patients annually who die of AMI. At present, with the aging of the population, people pay more and more attention to the health problems of the elderly. This is also one of the problems to be solved immediately (6-8).

Fluvastatin is a reductase inhibitor, which mainly reduces the synthesis and storage of cholesterol in liver cells, and also has the effect of lowering blood cholesterol and low-density lipoprotein (9). Clinically, fluvastatin is the most widely used lipid-modifying drug, which has little side-effects and adverse reactions, playing an important role in atherosclerosis (10). Toll-like receptor 4 (TLR4), an innate immune receptor, is an important member of the Toll-like receptor family. It is a class of cytokines that recognizes the early invasion of pathogens and induces cell differentiation (11). TLR4 plays an important regulatory role in wound healing, anti-infective immunity and tumor formation. The relationship between fluvastatin and TLR4 signaling pathway in cardiomyocytes of myocardial infarction (MI) rats model has not been reported in domestic and foreign literature yet. In this study, we investigated the effect of fluvastatin on cardiomyocyte apoptosis and the effect of fluvastatin on TLR4 signaling pathway by constructing MI rats, and further explored the protective effect of fluvastatin on cardiomyocytes.

Materials and methods

Experimental animals. Eighty healthy Wistar rats, male, 6-9 weeks old, weighing 180-300 g, were purchased from Beijing Vital-Lihua Experimental Animal Technology Co., Ltd. (Beijing, China). The rats were fed in separated cages at
measured by the BCA method. A total of 30 µg of total protein
was extracted and subjected to a buffer. After denaturation at
94°C for 3 min, the protein was separated by 12% SDS-PAGE
electrophoresis and transferred to a PVDF membrane at a
constant current of 350 mA. The membrane was placed in
TBS buffer with 5% skim milk in room temperature and in the
dark for blocking for 1 h, and placed overnight at 4°C with the
corresponding antibody; rinsed 3 times with TBS buffer on the
next day for 5-10 min each, goat anti-rabbit and goat anti-rat
IgG were marked with HRP (1:2,000), then washed with TBS
buffer three times for 5-10 min each, fixed and developed by
using a color-developing system, and light-emitting imaging
technology was used for exposure imaging and grayscale
scanning.

Reverse transcription-qPCR (RT-qPCR) detection of tissue
TLR4. The spare tissue was added with TRIzol reagent, shook
and placed at room temperature for 30 min for complete lysis.
Total RNA was extracted from each group of cells according
to the kit manual, and the extraction process was performed
strictly according to the instructions. The extracted RNA was
analyzed by UV spectrophotometer (Hitachi, Tokyo, Japan)
and protein electrophoresis to determine its concentration
and purity. The extracted total RNA was reverse-transcribed
according to the instructions of the reverse transcription kit.
The extracted cDNA samples were stored at -20°C. TLR4
primers designed by Shanghai Sangon Biological Engineering
Corp., (Shanghai, Chian) are shown in Table I. PCR reaction
system was designed according to the instructions, and the
RT-qPCR reaction conditions: 94˚C pre-denaturation 10 min;
50 cycle of 94˚C 45 sec, 60˚C 45 sec, 72˚C 45 sec, a total of 40
cycles. The manufacturer’s software was used for amplification data
analysis, and β-actin was used as an internal reference gene.

Statistical analysis. SPSS 19.0 software (SPSS, Inc., Chicago,
IL, USA) was used for statistical analysis. The data were
expressed as mean ± SD, and the mean value of multiple groups
were compared by one-way ANOVA. Comparison between
each two groups were by Student-Newman-Keuls (SNK) q-test
or LSD-t. P<0.05 was considered statistically significant.

Results

Modeling results. In this experiment, among a total of 80 rats,
60 were performed with coronary artery ligation, and 38
survived 4 days after the operation; the other 20 rats were
normal control group.

RT-qPCR detection of the expression of TLR4 mRNA in the
cells. In this experiment, we detected the expression of TLR4
mRNA by RT-qPCR in rat cardiomyocytes of four groups,
and the results showed that TLR4 mRNA was expressed in all four groups. The expression of TLR4 in the normal control group was 0.902±0.140 and the TLR4 expression in the sham operation group was 1.214±0.274. TLR4 expression in IM group was 3.412±0.879 and TLR4 expression in fluvastatin treatment group was 1.864±0.467. Compared with IM group, TLR4 mRNA expression in sham operation group, fluvastatin treatment group and normal control group were significantly increased, and comparison in each group was statistically significant (P<0.05), indicating that fluvastatin can reduce the expression of TLR4 mRNA in cardiomyocytes. There was statistical significance between fluvastatin treatment group and sham operation group (P<0.05) (Table II and Fig. 1).

**Western blot analysis.** In this experiment, the expression of TLR4 protein in rat cardiomyocytes was detected by western blot analysis. It was observed that TLR4 protein was expressed in all four groups. Compared with IM group, the expression of TLR4 protein in normal control group, sham operation group and fluvastatin treatment group were all significantly decreased, and the differences were statistically significant (P<0.05). The difference between the normal control group and the fluvastatin group, sham operation group was statistically significant (P<0.05), and there was no significant difference between the normal control group and the sham operation group (P>0.05) (Table II).

**Cardiomyocyte apoptosis.** In this experiment, the apoptosis of rat cardiomyocytes was detected by TUNEL method. The nuclei of TUNEL-positive apoptotic cells were brownish yellow and the nuclei of TUNEL-negative was blue. It was observed that in normal control group, no apoptotic cardiomyocytes were found; a very small amount of cardiomyocytes in sham operation group occurred apoptosis; the amount of apoptosis cells in IM group increased significantly compared with normal control group and sham operation group (30.4±3.1%), and the difference was statistically significant; compared with the IM group, fluvastatin treatment could significantly reduce the apoptosis of cardiomyocytes (19.2±3.8%), and the difference was statistically significant (P<0.05); there was no difference between normal control group and sham operation group (P>0.05) (Fig. 2).

### Discussion

Apoptosis, also known as apoptosis, is a form of active death that occurs when genes are regulating cells. It plays a very important role in the growth and development of normal tissues and in the development of diseases (12,13). There are many factors that can lead to apoptosis of cells. There are studies which have shown that the main factors of apoptosis were hypoxia, persistent ischemia and reperfusion (14). It has been reported (15) that ischemia-induced perfusion injury in rabbit models leads to apoptosis in rabbit cardiomyocytes, and other studies (16) have shown that cardiomyocytes apoptosis was found in the myocardium of patients with acute IM and

![Figure 1. Expressions of TLR4 mRNA in each group. There was significant difference (P<0.05) in terms of the expression between each group according to reverse transcription-qPCR; there was difference between the fluvastatin treatment group and the sham operation group, the normal group (P<0.05); there was no difference between sham operation group and normal control group (P>0.05).](image1)

![Figure 2. Apoptosis of cardiomyocytes in each group by TUNEL method. There was significant difference in apoptosis of cardiomyocytes between the sham operation group and the normal group (P<0.05), there was difference between the IM group and the sham operation group (P<0.05), there was difference between the IM group and the normal group (P<0.05), there was difference between the fluvastatin treatment group and the sham operation group (P<0.05), there was difference between the IM group and the normal group (P<0.05), there was difference between the IM group and the normal group (P<0.05), there was difference between the fluvastatin treatment group and the sham operation group (P<0.05).](image2)

<table>
<thead>
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<th>Table II. Protein and mRNA expression.</th>
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<td><strong>Group</strong></td>
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<td>Normal control</td>
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<td>Sham operation</td>
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<td>Fluvastatin treatment</td>
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*P<0.05 compared with the IM group; *P<0.05 compared with the normal control group; *P<0.05 compared with the sham operation group.
end-stage heart failure. Cell apoptosis is directly controlled by genes that control apoptosis, and extracellular signals regulate cells and eventually cause cell death. There are many reasons for the apoptosis of myocardial cells after IM such as neurohormone system, inflammatory cytokines and CO (17). Because of the coexistence of multiple factors, it is very difficult to distinguish the role played by IM in a certain period of time and one stimulus. We found through animal experiments that after myocardial infarction, myocardial apoptosis occurs in rats at different time-points after IM, and cardiomyocyte apoptosis is mediated by multiple death signals and regulated by different genes. The mechanism of different genes on myocardial apoptosis is not yet fully understood.

TLR4, as a natural immune receptor, its mRNA expression was found for the first time in animal models of cardiomyocytes, and Kim et al (18) pointed out that activation of TLR4 signaling pathway can induce cardiomyocyte apoptosis, and the use of TLR4 blocking antibody to block TLR4 eventually leads to diminished cardiomyocyte apoptosis. It has been reported (19) that after TLR4 activation after acute IM, a large number of inflammatory factors are expressed. The release of inflammatory cytokines is related to cardiomyocyte apoptosis, and TLR4 signaling pathway-mediated apoptosis may be related to the release of inflammatory cytokines under TLR4-mediated conditions (20). Fluvastatin, as the first HMG-CoA reduction inhibitor, has obvious difference from other statins and its structure is a special open-ring type. Fluvastatin can produce better pharmacological activity without metabolic transformation; in addition to lipid-lowering function, it also has the functions of stabilizing plaque, suppressing inflammation and regulating coagulation (21).

In the present study, we explored the role of fluvastatin in myocardial cells of IM rats by treating rat IM model with fluvastatin and activating TLR4 signaling pathway to establish a model. The expression of TLR4 in cardiomyocytes of 4 groups was detected by TUNEL, western blot analysis and RT-qPCR. The results showed that the expression of TLR4 mRNA in all 4 groups of cardiomyocytes was detected by RT-qPCR, and was significantly expressed in IM group, which is consistent with the study of Yang et al (22): The expression of TLR4 mRNA in cardiomyocytes was significantly increased after IM. Liu et al (23) have found that after long-term IM, myocardial cells TLR4 and pro-inflammatory cytokines are upregulated to promote inflammation and thus increase heart failure. TLR4 expression in normal control and sham operation group was significantly different from that in IM group, while TLR4 expression in myocardial cells treated with fluvastatin decreased significantly compared with IM group. The expression of TLR4 protein in 4 groups of rats was detected by western blot, and the results showed that TLR4 protein expression in IM group was significantly higher than that in other groups, which was consistent with the conclusion of Sun et al (24). It is suggested that fluvastatin can inhibit the expression of TLR4 to reduce the apoptosis of cardiomyocytes, Thus we detected the apoptosis of cardiomyocytes by TUNEL method. It was observed that there was a significant difference in the apoptosis of rat cardiomyocytes between the fluvastatin treatment group and the IM group (19.2±3.8 vs. 30.4±3.1%, P<0.05), indicating that fluvastatin can regulate cardiomyocyte apoptosis by inhibiting the expression of TLR4.

This experiment showed that fluvastatin can inhibit the expression of TLR4, however, the specific mechanism of TLR4 inhibition is unclear, whether fluvastatin modulates TLR4 directly or indirectly through other proteins needs further study and experiments, and this experiment was based on the animal model, and whether different modeling methods and practices will affect the experimental results also needs to be studied in depth in the future.

In summary, fluvastatin can decrease the apoptosis of cardiomyocytes and inhibit IM by increasing the expression of TLR4-like receptors.

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Availability of data and materials

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

Authors' contributions

LJ drafted the manuscript. LJ and ZZ were mainly devoted to collecting and interpreting the data. WL, LW and GQ helped collecting and interpreting the data. WL, LW and GQ helped with RT-qPCR. XJ and ZZ were responsible for statistical analysis. All authors have read and approved the final study.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Qilu Hospital of Shandong University (Jinan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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