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Original Research Article

Leflunomide inhibits inflammation and apoptosis of H9c2 cells induced by hydrogen peroxide

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Abstract

Purpose: *To investigate the effects of leflunomide (Lef) on inflammatory response and apoptosis after myocardial infarction, and to explore its molecular mechanisms of action.*
Methods: *H_2O_2 and H9c2 cells were used to establish myocardial cell injury model in vitro. H9c2 cells were divided into 3 groups: control group, H_2O_2 group, H_2O_2 + Lef group. The CCK-8 assay was used to determine the optimal concentration of H_2O_2 and Lef, while the expressions of TNF-α, IL-6, IL-1β, Bcl-2, Bax, Bad, TLR4, IκB-α, P65 and p-P65 were evaluated by Western blot. PCI was utilized to detect the expression of TNF-α, IL-6, IL-1β, Bcl-2, Bax and Bad mRNA. The levels of TNF-α, IL-6 and IL-1β in supernatant were assessed by ELISA, while apoptosis of the three groups was evaluated by TUNEL staining and flow cytometry.*
Results: *Compared with H_2O_2 group, TNF-α, IL-6, IL-1β, Bax and Bad expressions in H_2O_2+Lef group were significantly reduced ($p < 0.05$), but Bcl-2 expression significantly increased. The levels of TNF-α and IL-6 and IL-1β in supernatant of H_2O_2 + Lef group were also decreased compared to those in the H_2O_2 group ($p < 0.05$). In addition, TUNEL-positive cells and apoptotic rates were significantly reduced after treatment with Lef. Moreover, Lef inhibited expression of TLR4 and p-P65, but activated expression of IκB-α, indicating that Lef inhibited TLR4/NF-κB pathway ($p < 0.05$).*
Conclusion: *The results show that Lef inhibits H_2O_2-induced H9c2 cell apoptosis and inflammatory responses by inhibiting TLR4/NF-κB pathway. These findings may provide new targets for the treatment of myocardial infarction.*

Keywords: *Myocardial infarction, Leflunomide, Apoptosis, Inflammation, TLR4/NF-κB pathway*

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INTRODUCTION

At present, ischemic heart disease (IHD) has become one of the diseases with the highest morbidity and mortality rates in humans [1]. Most of the causes are related to age, obesity, diabetes, hypertension, hyperlipidemia and heredity [2]. The incidence of coronary heart disease (CHD) is rising year by year, and is affecting younger people [3]. Acute myocardial infarction (AMI) is the most dangerous type of CHD [4]. More than one million patients die from AMI every year in the world. Recovering the blood supply to the coronary artery as soon as possible (such as percutaneous coronary intervention, thrombolytic therapy) is the most

critical treatment for AMI [5]. However, after successful occlusion of the coronary artery, a considerable number of patients with myocardial infarction (MI) still have ventricular remodeling, which eventually leads to cardiac dysfunction or heart failure, resulting in decreased quality of life and increased long-term mortality [6]. A large number of studies have shown that inflammation and apoptosis play an important role in the development of MI [7]. The improvement of myocardial cell survival rate, reduction of apoptosis and myocardial inflammation, and the improvement of cardiac function after MI are the focus of the research into MI [8].

Leflunomide is a pyrimidine synthesis inhibitor that specifically inhibits the S phase of cell division and inhibits the synthesis of pyrimidine nucleotides by inhibiting the activity of dihydrolactate dehydrogenase which promotes pyrimidine synthesis in DNA synthesis [9]. At the same time, Lef also inhibits lymphocyte proliferation by inhibiting tyrosine kinase activity, thereby reducing antibody secretion and production [10].

Lef has been widely used in the treatment of rheumatoid arthritis and dermatological diseases [11]. In recent years, its clinical efficacy in anti-rejection of kidney transplantation and lupus nephritis has been more and more recognized, and some scholars have applied it to the treatment of immune-mediated glomerular diseases [12]. However, the effect of Lef on cardiomyocytes has not been studied.

In this study, H_2O_2-induced myocardial cell injury model was adopted to investigate whether Lef plays an anti-apoptotic and anti-inflammatory role, as well as its potential mechanism of action. The results showed that Lef may provide a potential new treatment for MI.

EXPERIMENTAL

Cell culture

H9c2 cells were cultured in Dulbecco's modified eagle medium (DMEM) (Procell, Wuhan, China), which contained 10% fetal bovine serum (FBS) (MCE, Nanjing, China) and 1% penicillin/streptomycin (MCE, Nanjing, China). The cell medium was changed once a day and cell passage was performed when the cell confluence reached about 80%. H9c2 cells were then treated with 100 μM of H_2O_2 for 4 hours and then treated with 50 μM of LeF or an equivalent amount of physiological saline. H9c2 cells were divided into three groups: control group, H_2O_2 group, and H_2O_2 + Lef group. Subsequent experiments were performed 24 h after the treatments.

Western blot assay

The cell lysate (purchased from Camilo Biological, Nanjing, China) was first prepared at 4°C in advance. The cell lysate was then added to each group of cell culture plates, washed with phosphate buffered saline (PBS) and allowed to stand on ice for 5 minutes. The lysed cell mixture was carefully scraped into a 1.5 mL eppendorf (EP) tube with a chopstick. Using a cryogenic refrigerated centrifuge set at a rotational speed of 15,000 rpm, a temperature of 4°C, and a time of 10 min, separating the resulting supernatant was the desired protein extract.

Protein concentration was measured using bicinchoninic acid (BCA) method. 0.5 μg/μL of BSA protein standard solution (ThermoFisher, Waltham, MA, USA) was dispensed into each well of the microtiter plate according to 0, 1, 2, 4, 8, 12, 16 and 20 μL volume, and was supplemented to 20 μL for standard protein curves. 1 μL of each group of protein samples was prepared and mixed thoroughly with 19 μL of PBS buffer. The mixture was prepared according to the ratio of liquid A: liquid B to liquid 50:1. And the mixture was added into each hole and beaten repeatedly. After the mixture was fully mixed, the solution reacted at 37°C for 20 - 30 min. The microplate reader was preheated for 15 minutes, and the reading wavelength was set to 570 nm.

After the protein was denatured, the sample was electrophoresed, and the membrane was transferred with a polyvinylidene fluoride (PVDF) membrane (EpiZyme, Shanghai, China), and then the membrane was blocked with 5% skim milk for 120 min, and the primary antibody (Bax, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; Bad, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; Bcl-2, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; TNF-α, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; IL-6, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; TLR4, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; IκB-α, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; p-P65, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; t-P65, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; glyceraldheyde 3-phosphate dehydrogenase (GAPDH), Abcam, Cambridge, MA, USA, Rabbit, 1:1000) was added for incubation at 4°C overnight, and the tris buffered saline-tween (TBST) solution was washed 3 times. Then, 1:5000 diluted secondary antibody was added for 120 min, washed with TBST 3 times, and developed with

electrochemiluminescence (ECL) Advance Western blot Detection Kit (Amersham Bioscience, Piscataway, NJ, USA).

Cell counting kit-8 (CCK-8) assay

The logarithmic growth phase of H9c2 cells were seeded in a 96-well culture plate with a cell suspension of 5 × 10^4 cells/mL. After 24 h of cell culture, the supernatant was discarded, washed with PBS 1 to 2 times, and then added per well with 90 μL of the sample dissolved in the serum-free medium (Life Technology, Wuhan, China) for 24 h. 10 μL of CCK-8 reagent (Dojindo, Kumamoto, Japan) was added, and then the culture was continued for the corresponding time to detect the optical density (OD) value at a suitable excitation wavelength.

Real-time polymerase chain reaction (PCR)

Total RNA from 3 groups of cells was extracted using TRIzol reagent (MCE, Nanjing, China). The reverse transcription kit (MCE, Nanjing, China) was used to reverse transcribe RNA into complementary deoxyribose nucleic acid (cDNA). SYBR Green qRCR Mix (MCE, Nanjing, China) was used to perform PCR. GAPDH was to normalize the expression of Bad, Bax, Bcl-2, TNF-α, IL-6. All the primers were listed in Table 1.

Determination of lactate dehydrogenase (LDH) levels

The content of LDH in the supernatant of the three groups of cells was detected by LDH enzyme-linked immunosorbent assay (ELISA) kit (Dojindo, Kumamoto, Japan).

Assessment of TNF-α and IL-6 and IL-1β

The supernatants of the three groups of cells were taken and the contents of TNF-α and IL-6 and IL-1β were detected by TNF-α and IL-6 and IL-1β ELISA kits (Dojindo, Kumamoto, Japan), respectively.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining

Three groups of cells were fixed with 4% paraformaldehyde and the cell membrane was disrupted with Triton. Then the prepared TUNEL reagent (Roche, Basel, Switzerland) was added and co-incubated with the cells; 4',6-diamidino-2-phenylindole (DAPI) (Roche, Basel, Switzerland) was added to stain the nucleus. The results were observed with a fluorescence microscope.

Flow cytometry

Three groups of cells in a 6-well plate were collected, centrifuged and washed with PBS a total of 3 times. Then, an appropriate amount of Binding Buffer (KeyGen, Shanghai, China) was added, and then 5 μL of Annexin V-FITC (fluorescein isothiocyanate) (KeyGen, Shanghai, China) and PI (KeyGen, Shanghai, China) were added to each. Finally, they were detected by flow cytometry.

Statistical analysis

Data are expressed as χ ± s, and were plotted using GraphPad Prism5 software (La Jolla, CA, USA). Differences between two groups were analyzed using the Student's t-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). Test level α = 0.05.

RESULTS

Lef alleviated H_2O_2-induced cardiomyocyte injury

First, in order to explore the optimal concentration of H_2O_2 for cell damage, we treated H9c2 cells with different concentrations (0, 50, 100, 150 and 200 μM) of H_2O_2.

Table 1: Real time PCR primers used in the study

Gene name	Forward (5' > 3')	Reverse (5' > 3')
Bax	CAGTTGAAGTTGCCATCAGC	CAGTTGAAGTTACCATCAGC
Bcl-2	GACTGAGTACCTGAACCGGCATC	CTGAGCAGCGTCTTCAGAGACA
Bad	TGAAGGGATGGAGGAGGA	TCTTTGGGCGAGGAAGTC
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
IL-6	ACAGAAGGAGTGGCTAAGGA	AGGCATAACGCACTAGGTTT
IL-1β	TTGAGTCTGCCCAGTTCC	TTTCTGCTTGAGAGGTGCT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

qRT-PCR + quantitative reverse-transcription polymerase chain reaction

The results of the CCK-8 assay showed that when the concentration of H_2O_2 was 100 μM, the cell viability was about 50% (Figure 1A). So we applied this concentration to induce H9c2 cell damage in subsequent experiments. Then, we treated H_2O_2-induced cardiomyocyte injury model with different concentrations of Lef (0, 10, 30, 50, 70 μM). The results of CCK-8 showed that when the concentration of Lef was 50 μM, the cell viability reached the highest (Figure 1B). So, 50 μM concentration was applied to subsequent experiments. Afterwards, we examined the LDH levels in the supernatants of the three groups of cells and found that Lef can markedly reduce the level of LDH (Figure 1C).

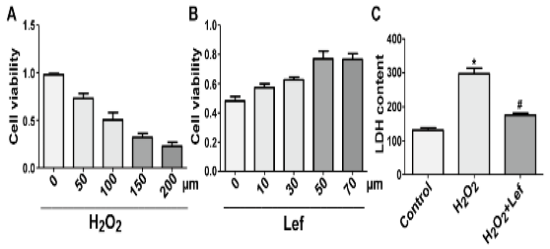

Figure 1: Lef alleviates H_2O_2-induced cardiomyocyte injury. (A) CCK-8 assay showed H9c2 cells viability at different concentrations of H_2O_2. (B) CCK-8 assay showed cell viability after addition of different concentrations of Lef in H_2O_2-treated H9c2 cells. (C) The LDH content increased significantly in the H_2O_2 group and decreased significantly in the H_2O_2 + Lef group ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3)

Lef inhibited H_2O_2-induced inflammation of cardiomyocytes

To observe the inflammation of the three groups of cells, we examined the expression of TNF-α and IL-6 and IL-1β (Figure 2A). Treatment of H_2O_2 greatly increased TNF-α, IL-6 and IL-1β expression, but Lef could inhibit their expression (Figure 2B~2D). TNF-α, IL-6 and IL-1β mRNA expression detected by PCR were consistent with protein levels (Figure 2E~2G). Elisa assay for TNF-α, IL-6 and IL-1β protein expression in three groups (Figure 2H~2J), and the results were same as before. So, Lef can inhibit cardiomyocyte inflammation.

Lef inhibited H_2O_2-induced apoptosis of cardiomyocytes

First, we examined Bax, Bad and Bcl-2 proteins in three groups of cells (Figure 3 A). The expression of Bax and Bad in H_2O_2 group was markedly increased, and Bcl-2 expression was greatly decreased. After treatment with Lef, the expression of three proteins was reversed (Figures 3 B - D). We also examined the expression of Bax, Bad and Bcl-2 mRNA in three groups of cells. The results were consistent with protein levels (Figure 3 E - G). In addition, we used TUNEL staining to measure the amount of TUNEL-positive cells in the three groups. As can be seen from the Figure 3 H, TUNEL-positive cells in the H_2O_2 + Lef group were greatly reduced compared with the H_2O_2 group. Similarly, from the results of flow cytometry to detect apoptosis, it was found that the apoptosis rate of Lef-treated cardiomyocytes was greatly reduced (Figure 3 I). Thus, Lef inhibits cardiomyocyte apoptosis.

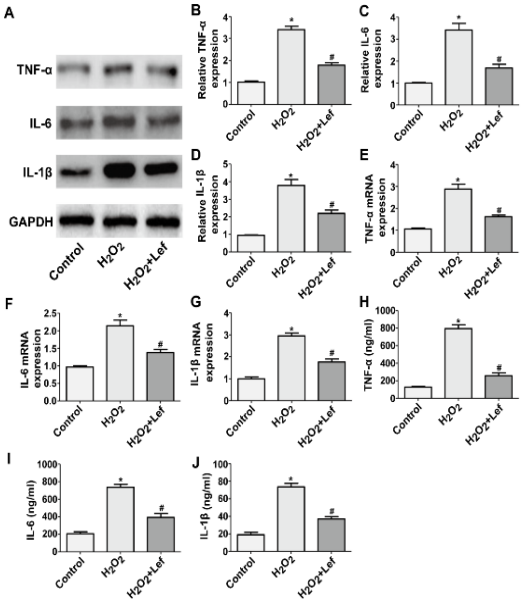

Figure 2: Lef inhibits H_2O_2-induced inflammation of cardiomyocytes. (A) The expression of IL-6 and TNF-α in H_2O_2 group increased significantly, but decreased in H_2O_2 + Lef group. (B) Statistical results of expression of TNF-α ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n=3). (C) Statistical results of expression of IL-6 ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3). (D) Statistical results of expression of IL-1β ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3). (E) TNF-α mRNA expression was similar to the results of Western blot ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3). (F) IL-6 mRNA expression was similar to the results of Western blot ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3). (G) IL-1β mRNA expression was similar to the results of Western blot ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3). (H) ELISA assay was used to detect the protein expression of TNF-α in the three groups ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3). (I) ELISA assay was used to detect the protein expression of IL-6 in the three groups ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3). (J) Elisa assay was used to detect the protein expression of IL-1β in the three groups ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3)

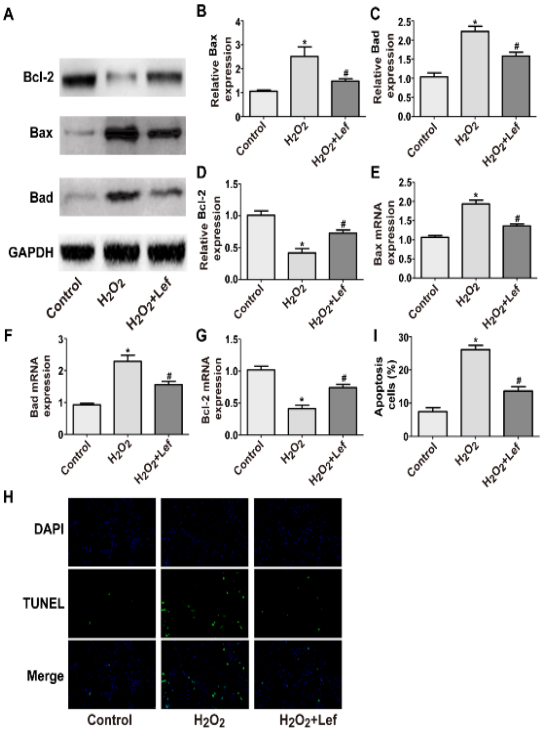

Figure 3: Lef inhibits H_2O_2-induced apoptosis of cardiomyocytes. (A) The expression of Bcl-2 in H_2O_2 group decreased significantly, but increased in the H_2O_2 + Lef group. Bax and Bad expression was opposite to Bcl-2. (B) Statistical results of protein level of Bax ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3). (C) Statistical results of expression of Bad ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3). (D) Statistical results of expression of Bcl-2 ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3). (E) The expression of Bax mRNA was consistent with the Bax protein ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3). (F) The expression of Bad mRNA was consistent with the Bad protein ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3). (G) The expression of Bcl-2 mRNA was consistent with the Bcl-2 protein ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3). (H) TUNEL staining showed that Lef can obviously reduce the increase of H9c2 cell apoptosis caused by H_2O_2. (Magnification: 400×) (I) The apoptotic rate of H_2O_2 group increased, and decreased in H_2O_2 + Lef group ("⋆" $p < 0.05$ *vs.* control, "#" $p < 0.05$ *vs.* H_2O_2, n = 3)

Lef inhibited TLR4/NF-κB pathway

To explore the mechanism by which Lef protects cardiomyocytes from inflammation and apoptosis, we examined the TLR4/NF-κB pathway using Western blot (Figure 4 A). We found that Lef can greatly inhibit the expression of TLR4 and also inhibit the phosphorylation level of P65, and also increase the expression of IκB-α (Figure 4 B ~ D). It was demonstrated that H_2O_2 activated the TLR4/NF-κB pathway, but Lef could greatly inhibit the pathway.

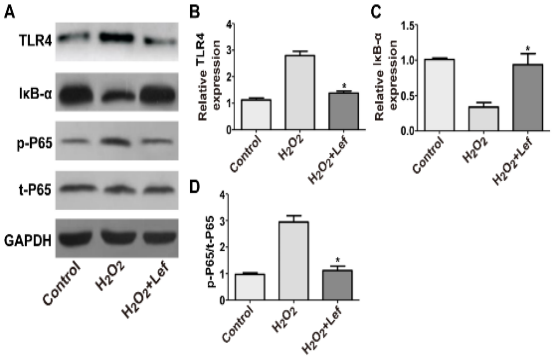

Figure 4: Lef inhibited the TLR4/NF-κB pathway. (A) The Western blot showed that Lef could increase the expression of IκB-α and inhibit the expression of TLR4 and p-P65. (B) Statistical results of protein level of TLR4 ("⋆" $p < 0.05$ *vs.* H_2O_2, n = 3). ***C,*** Statistical results of protein level of IκB-α ("⋆" $p < 0.05$ *vs.* H_2O_2, n = 3). ***D,*** Statistical results of protein level of p-P65 ("⋆" $p < 0.05$ *vs.* H_2O_2, n = 3)

DISCUSSION

After AMI, a strong inflammatory response begins, first with neutrophil infiltration, and followed by mononuclear-macrophage and lymphocyte infiltration [13]. Inflammatory factors from neutrophil infiltration play an important role in ventricular remodeling. Macrophages and lymphocytes are also involved in ventricular remodeling [14]. Various inflammatory cells can secrete a variety of cytokines, such as IL-1, IL-6, TNF-α, TGF-β, causing cardiac fibrosis and remodeling [15]. Necrotic cardiomyocytes are engulfed by inflammatory cells. Acute myocardial ischemia only causes a part of myocardial cell necrosis. With the infiltration of a large number of inflammatory cells into the infarcted area of myocardial tissue, the range of myocardial necrosis and apoptosis gradually expands, eventually leading to large-area MI [16]. It can be seen that the inflammatory response after myocardial infarction is an important cause of cardiac pathological damage.

Cardiomyocyte apoptosis is one of the forms of myocardial death after MI [17]. Cardiomyocyte loss due to apoptosis plays an important role in the development of heart failure after MI [18]. Apoptosis, also known as programmed cell death, is divided into two pathways: endogenous apoptosis and exogenous apoptosis [19]. Exogenous apoptosis is mediated through the activation of death receptors on the cell membrane, including Fas, tumor necrosis factor (TNF) and TNF-associated ligands. Endogenous apoptosis is mainly induced by regulating the Bcl-2 protein family, including signaling proteins such as Bax, Bad, Bcl-2 and Bcl-XL. Bcl-2 is a

key regulator of the endogenous apoptotic cascade [20]. Many apoptosis may be induced by inhibiting Bcl-2. It also plays an important regulatory role in cardiomyocyte injury.

Toll-like receptors (TLRs) are pattern recognition receptors in a natural immune system [21]. Among them, TLR4 is mainly distributed in macrophages, monocytes, dendritic cells, lymphocytes and epithelial cells [22]. TLR4 is a type I transmembrane receptor which contains extracellular, transmembrane and intracellular regions [23]. The extracellular domain is responsible for recognizing the receptor and consists of 22 leucine repeats of 20 to 30 amino acid residues in length. The transmembrane region is rich in cysteine and determines the cell membrane localization of TLR4. The intracellular domain contains a sequence-conserved domain, the Toll/IL-1 receptor domain, which recruits downstream adaptor proteins to initiate downstream pathways, resulting in the massive release of inflammatory factors such as TNF-α and IL-6 [24]. NF-κB is a key downstream nuclear transcription factor located at the pivotal position of the TLR4 signaling pathway, and forms an IκB kinase (IKK) complex with NF-κB inhibitory protein α (IκB-α) at rest, present in the cytosol [25]. When the cells are externally stimulated, IKK is activated, IκB phosphorylation is followed by ubiquitination, and finally NF-κB is activated, free NF-κB is rapidly translocated into the nucleus, transcription of the target gene is initiated, and gene expression is regulated and induced [26]. Inflammatory factors such as TNF-α and IL-6 are secreted, and participate in the regulation of inflammation and immune response in the body.

In this study, we found that H_2O_2 up-regulated the expression of Bad, Bax, TNF-α, IL-6 and IL-1β in H9c2 cells and inhibited Bcl-2 expression, indicating that H_2O_2 caused inflammation and apoptosis of cardiomyocytes. When the cells were treated with Lef, the condition was completely reversed. At the same time, the expression of TLR4 and p-P65 was also inhibited, while the expression of IκB-α was elevated. Thus, Lef inhibited the TLR4/NF-κB pathway, thereby inhibiting inflammation and apoptosis of cardiomyocytes.

CONCLUSION

Lef inhibits inflammation and apoptosis of H9c2 cells caused by H_2O_2 by inhibiting TLR4/NF-κB pathway. The results of the current study may provide new insights for the treatment of myocardial infarction.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jing Xie and Yeyu Qin contributed equally to this work.

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