LCZ696 Attenuates ROS/NLRP3 Mediated Pyroptosis in Trastuzumab-induced H9C2 Cell Model Via Ameliorating Sirt3 Expression

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Abstract

Trastuzumab-induced cardiomyopathy have been a kind of clinical crucial problems in the field of cardio-oncology. LCZ696, clinically named sacubitril/valsartan, was administered to treat the patients with heart failure, so that it may be a substantial prevention to attenuate chemotherapy-induced cardiotoxicity. We firstly confirmed that LCZ696 and trastuzumab can affect the content of Sirt3 and NLRP3 in H9C2 cell, which confirmed the concentration of LCZ696 (10μM) and trastuzumab (100nM). Then H9C2 cells were allocated into 3 groups: (1) Con group; (2) TRA group; (3) TRA+LCZ696 group. We investigated the change of mRNA expression and protein synthesis of cultured H9c2 cardiocardiomyocytes on exposure to trastuzumab alone or plus LCZ696. Meanwhile, it turns out that LCZ696 can ameliorate the mRNA expression and content of Sirt3, inhibit the level of ROS, NLRP3, ACS, Caspase-1 and IL-1β in trastuzumab-induced H9C2 cell model. In summary, LCZ696 reduces the oxidative stress caused by ROS and NLRP3-mediated pyroptosis by protecting the activity of Sirt3 in H9C2 cells.

Introduction

Oxidative stress and inflammation were substantial mechanism of myocardial injury when the patients with breast cancer received chemotherapy but developed cardiac insufficiency during treatment. The PERSEPHONE trial by Earl et al. [1] showed the percentage of decreased cardiac function is about 9%~11% after receiving trastuzumab treatment for 12 months. The reason for the above result may be attributed to trastuzumab affecting the expression of Akt and Erk1/2, resulting in mitochondrial dysfunction and cell death [2]. Meanwhile, trastuzumab altered the expression of genes essential for cardiac function in mice model and indirectly changed mitochondrial energy metabolism [3, 4]. A mice model had confirmed that Sirt3 protected mitochondrial DNA damage and blocked the development of chemotherapy-induced cardiomyopathy so that the expression of Sirt3 may affect the level of ROS and the markers of inflammation [5]. Metabolism, oxidative stress, and cell pyroptosis are a series of reactions, especially in inflammation-directed cardiomyopathy, and based on this hypothesis, intervening metabolism may prevent myocardial damage and even fibrosis [6, 7]. LCZ696 (Sacubitril Valsartan) was the first dual inhibitor of angiotensin receptor and neprilysin, recently confirmed by Ge et al. [8] to ameliorate diabetic cardiomyopathy by inhibiting inflammation, oxidative stress and apoptosis. In CKD models, LCZ696 administration improved renal function and histology, and attenuated most of the molecular markers of oxidative stress, inflammation and fibrosis via reduction in nuclear translocation of nuclear factor erythroid 2-related factor 2 and its key target products [9]. However, the underlying mechanism of LCZ696 on trastuzumab-induced cardiomyopathy is unclear. The present study was designed to confirm the effect of LCZ696 by measuring the expression of genes and proteins of the Sirt3/ROS/NLRP3 pathway in trastuzumab-induced H9C2 cell model.

Methods

H9C2 Cell Culture H9C2 cells were provided by Institute of Cardiovascular Disease, Harbin Medical University. After resuscitation, the cells were sub-cultured until they could be used for grouping experiments. The cultured H9C2 cells were divided into three groups as follows: (1) Con group: H9c2 cells were not intervened for 24 hours; (2)TRA group: H9c2 cells were exposed to trastuzumab (100 nM, GlpBio, Shanghai, China) for 24 hours; (3) TRA + LCZ696 group: LCZ696 (10 μM, Novartis AG, Switzerland) was pretreated to H9C2 cells for 30 minutes and then exposed to 200 nm trastuzumab (100 nM, GlpBio, Shanghai, China) for 24 hours.

Real-Time Polymerase Chain Reaction

The total RNA was extracted by TRIzol reagent Thermo Scientific™, MA, USA), and reverse transcribed into complementary DNA with GoScript Reverse Transcription System following the manufacturer's instruction. The mRNA expression levels were quantified from the total RNA using real-time polymerase chain reaction (RT-PCR) with SYBR Green. The mRNA expression of pyroptosis components (NLRP3, ACS, Caspase-1, IL-1β) and its upstream substantial target Sirt3 and SOD2 were analyzed with SYBR Green RT-PCR. GADPH was regarded as an internal control for the mRNAs. Primer sequences for RT-PCR amplification are shown in Table 1. Real-time PCR was performed with the CFX96 Touch Real-Time PCR Detection System (Thermo Scientific™, MA, USA). Based on 2 μL of the amount of each sample, the mRNA expression values are shown in folds by using the formula $2^{-\triangle\triangle ct}$.

Table 1
Primer sequences used for the study

Gene	Primers	Sequences
SOD2	Forward	5’-TGAGTGAGGTCAGTAGGGTG-3’
	Reverse	5’-GGAAGATGGTGAGACGAAAT-3’
ACS	Forward	5’-CTGTGGTTCCGAGACTGCTA-3’
	Reverse	5’-CTGTTGTTTCTGACGATGCC-3’
NLRP3	Forward	5’-AGCTTCAGCCACATGACTTTC-3’
	Reverse	5’-ATAGGACCTTCACGTCTCGGT-3’
Caspase-1	Forward	5’-GAAAGACAAGCCCAAGGTTA-3’
	Reverse	5’-GGTGTTGAAGAGCAGAAAGC-3’
IL-1β	Forward	5’-GGGATGATGACGACCTGCTA-3’
	Reverse	5’-CCACTTGTTGGCTTATGTTCTG-3’
SIRT3	Forward	5’-CATAGGTTAGGTGGCGAGTA-3’
	Reverse	5’-GTGTAGCAATCACAGAAGAG-3’
GAPDH	Forward	5’-TTCCTACCCCCAATGTATCCG-3’
	Reverse	5’-CCACCCTGTTGCTGTAGCCATA-3’

Western Blot Analysis

The H9C2 cells was homogenized and lysed with RIPA Lysis and Extraction buffer (Thermo Scientific™, MA, USA). The supernatant was collected after centrifugation at 18 000xg for 15 minutes at 4℃. The proteins were then quantified by the Bradford assay with a protein assay kit. Portions of protein (100 mg) per sample were separated through a 10% sodium deodecyl sulfate-polyacrylamide gel and transferred to Polyvinylidene difluoride (PVDF) membranes at 80℃ for 2 hours. Subsequently, the membrane was blocked with 5% bovine serum albumin (BSA) in 0.1% Tris-buffered saline Tween (TBST) and then probed with antibodies Sirt3 (1:1000; Abcam, Shanghai, China), NLRP3 (1:1000; Abcam, Shanghai, China), ACS (1:1000; Abcam, Shanghai, China), Caspase-1 (1:1000; Abcam, Shanghai, China), and β-actin (1:1000; Abcam, Shanghai, China) at 4℃ overnight. The membranes were washed 3 times in TBST (5 minute each wash), then incubated with secondary antibody rabbit anti-rat IgG (HþL)-Horseradish Peroxidase (HRP)-conjugated (1:10000; Abcam, Shanghai, China) for 2 hours at room temperature. After the last wash, signals were detected using Enhanced chemiluminescence (ECL) reagents. All the data were analyzed by Image J software (National Institute of Health, USA).

Enzyme linked immunosorbent assay

The H9C2 cell culture was centrifuged at 1000xg for 20 minutes, and then the supernatant was detected. Take out the strip from the aluminum foil bag after 60 minutes at room temperature, set up standard pore, blank pore and sample pore, add 50 μL standard of different concentrations into the standard pore, and add 50 μL sample to the sample pore. 50 μL of sample diluent was added to the blank pore, and then 100 μL of HRP labeled antibody was added to each pore. The reaction pore was sealed with plate membrane and incubated at 37 ℃ for 60 minutes. After repeated washing for 5 times, 50 μL substrate A and 50 μL substrate B were added and incubated at 37 ℃ for 15 minutes. The OD value was determined at the wavelength of 450nm. The concentration of each sample was calculated by the standard linear regression curve.

Measurement of oxidative stress

The changes of ROS level in H9C2 cells were detected using 2',7'-dichlorofluorescein-diacetate (DCFH-DA, Sigma-Aldrich, MO, USA) staining. Cells were seeded into 24-well plate with 3 × 103 cells per well and exposure to different treatment. Then adherent and floating cells in each group were harvested, washed with PBS for three times and stained by 10 μM DCFH-DA for 30 minutes at 37°C in the dark. The mean fluorescence intensity of each group was analyzed using flow cytometer, which represented the intracellular ROS level. And the level of MDA was measured by spectrophotometer using assay kit (Y-J Biological, Shanghai, China).

Statistical Analysis

All experiments in this paper were repeated at least three times. Data were presented as mean ± standard deviation (SD). Graphpad prism 7.0 was used for statistical analysis. Statistical comparison among three groups was made using one-way ANOVA, and Post Hoc Comparison was made using Bonferroni. $P < 0.05$ indicated difference was statistically significant.

Results

Real-Time PCR indicates LCZ696 activated the mRNA expression of Sirt3 and inhibited the mRNA expression of markers of oxidative stress and pyroptosis

As is showed in Fig. 1 in the trastuzumab -induced H9C2 cell model, the mRNA expression of Sirt3 and SOD2 were significantly reduced(P 0.05), meanwhile the mRNA expression of NLRP3, ACS, Caspace-1 and IL-1β were apparently increased compared with control group(P 0.05). Based on TRA group, treated by LCZ696, the biomarkers of pyroptosis and oxidative stress mRNA expression were reduced and Sirt3 mRNA expression was ameliorated(P 0.05).

LCZ696 upregulates the Sirt3 content and inhibits oxidative stress in trastuzumab-induced H9C2 cell model

As is showed in Fig. 2, we found that LCZ696 could upregulate the Sirt3 content and on the basis of trastuzumab-induced Sirt3 content decreasing (P 0.05). In the trastuzumab -induced H9C2 cell model, the

level ROS (Fig. 3) and MDA (Fig. 2) were higher than those in control group, but were inhibited by LCZ696.

LCZ696 downregulates the expression of pyroptosis in trastuzumab-induced H9C2 cell model

Emerging evidence in Fig. 4 has demonstrated that trastuzumab induced a higher level of biomarkers of NLRP3, ACS, Caspase-1 and IL-1β (P 0.05), a series of markers of pyroptosis, compared with the control group; but protein function of the above indicators will be significantly downregulated by LCZ696 in trastuzumab-induced H9C2 cell model (P 0.05).

Discussion

Chemotherapy is a kind of common treatment for cancer, but both traditional drugs, such as doxorubicin, cisplatin or paclitaxel, and targeted drugs, like trastuzumab or immune checkpoint inhibitors, can cause a certain degree of cardiotoxicity. Taking doxorubicin as an example, current studies have shown that doxorubicin can cause iron overload in cardiomyocytes, further aggravate oxidative stress [10, 11, 12], at the same time, cardiomyocytes metabolism disorder and structural change are contributed to myocardial cell apoptosis, necrosis and even increasing Gasdermin D sequentially leading to membrane dissolution [13, 14]. However, the specific mechanism of cardiotoxicity caused by targeted drugs has been on studying, but metabolism disorder, oxidative stress and inflammation are potential reasons. Intervening metabolism, reducing oxidative stress and delaying the development of inflammation may benefit the cardiomyocytes damage caused by chemotherapy.

LCZ696 is a new type of anti-heart failure drug, and it also benefits patients with hypertension. Its main components are transformed into LBQ657 and valsartan in vivo, which can inhibit natriuretic peptide system degradation by neprilysin and delay the activation of renin angiotensin aldosterone [15, 16]. From the mechanism, compared with valsartan alone, LCZ696 can significantly improve the active natriuretic peptide, which can be released through ventricular or atrial cardiomyocytes, which activate guanylate cyclase, play a series of positive roles, but the specific biological indicators need to be further verified [17]. In addition, LCZ696, which is clinically known as sacubitril/valsartan, has not been used as a preventive drug for cardiotoxicity caused by chemotherapy. It is necessary to further study its effects on metabolism, oxidative stress and inflammation, so as to clarify its possible effect.

Sirt-family are important regulators of mitochondrial function. Mitochondrial dysfunction plays a key role in cardiovascular diseases, including myocardial hypertrophy, heart failure, pulmonary hypertension, endothelial dysfunction, atherosclerosis, arrhythmia and so on [18, 19, 20]. In our study, the mRNA expression and the content of Sirt3 indicated that the level of metabolism was decreased by trastuzumab. Trastuzumab is a drug that targets a specific receptor common in some breast cancers, and the previous study indicated that knocking down Sirt6 increased the survival of a breast cancer cell exposed to trastuzumab [21], that is, trastuzumab may have some effect on Sirt-family. However, the

pretreatment of LCZ696 in our model ameliorated Sirt3 expression, thus, the myocardial injury caused by trastuzumab was effectively inhibited. In a pressure overload-induced heart failure model, LCZ696 could induced the upregulation of MnSOD through a Sirt3-dependent pathway [22]. We found that, in trastuzumab-induced H9C2 cell model, LCZ696 could increase the mRNA expression of SOD2 and reduce the content of MDA, and then, the fluorescence intensity of ROS was inhibited measured by flow cytometry. In mice with obesity-related metabolic heart disease, LCZ696 could improve myocardial energetics, and neprilysin inhibition exerted a positive effect on diastolic function [23]. Therefore, LCZ696 can improve the level of cell metabolism and downregulated oxidative stress induced by chemotherapeutic drugs.

Pyroptosis, a kind of programmed cell death, was thought to be closely related to inflammation, even pyroptosis was equal to inflammation in some research. In trastuzumab-induced H9C2 cell model, the mRNA and protein expression of NLRP3, ACS, Caspase-1 and IL-1β were significantly increased, but reversed by LCZ696-meaning that targeted chemotherapy was an important factor leading to the aggravation of inflammation and cell death, meanwhile LCZ696 could delay the progress of pyroptosis. A recent study revealed that sacubitril/valsartan (LCZ696) against pathological cardiac remodeling by inhibiting the NLRP3 inflammasome after relief of pressure overload in mice, but it's not pretreatment [24]. Thus, in our study, it also revealed that pretreatment via LCZ696 may reduce NLRP3/ACS inflammasome, substantially further delay myocardial fibrosis and prevent ventricular remodeling.

The therapeutic effect of LCZ696 on heart failure is clear, and some studies have found that LCZ696 can reduce doxorubicin-induced cardiotoxicity identified by Xia et al. [25] and Boutagy et al. [26] from a variety of pathways. However, in the field of onco-cardiology, there are few studies of LCZ696 on the prevention of myocardial injury caused by targeted chemotherapy drugs. Therefore, we found that LCZ696 can not only treat heart failure, but also prevent myocardial injury caused by trastuzumab via downregulating ROS and NLRP3-mediated pyroptosis by protecting the activity of Sirt3 in vitro. Next, we will verify whether LCZ696 can protect the heart function of trastuzumab chemotherapy in vivo.

Declarations

Ethical Approval

Not applicable. This study is H9c2 cell model, there is no ethical controversy.

Consent to Participate

All authors read and approved the manuscript.

Consent to Publish

All co-authors agree to publish the manuscript.

Author Contributions

Zang Yanxiang, Liu Guangzhong and Li Weimin conceived and designed research. Zang Yanxiang, Bai Nan, Lou Qi, Wang Hong, Duan Yuchen conducted experiments. Zang Yanxiang and Li Jianqiang wrote the manuscript. All authors read and approved the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Competing Interests

No application

Availability of data and materials

All data and materials of this work are included in this published article (and its supplementary information files).

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Figures

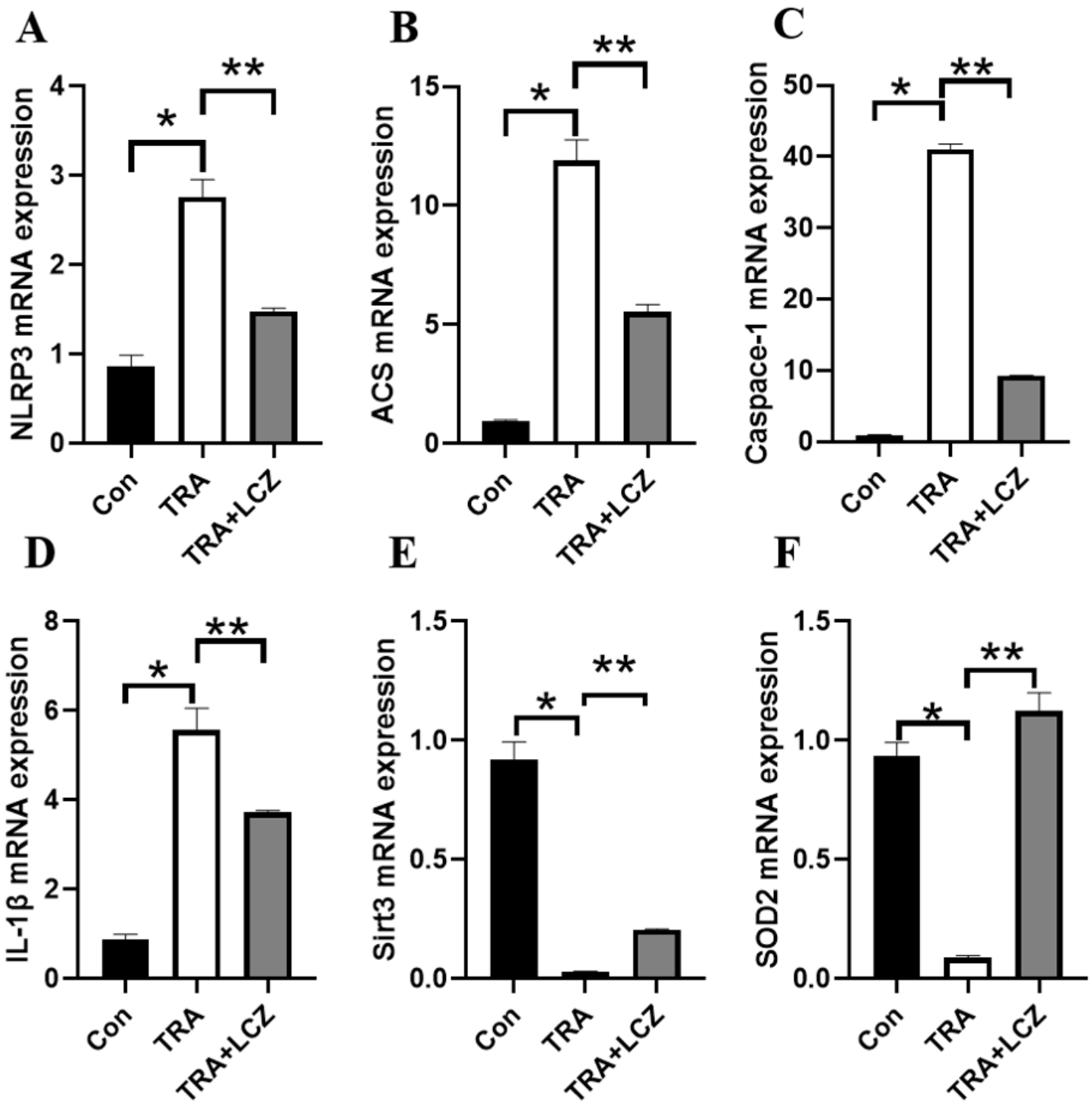

Figure 1

The mRNA expression by real-time PCR. (A) NLRP3 mRNA expression. (B) ACS mRNA expression. (C) caspase1 mRNA expression. (D) IL-1β mRNA expression. (E) Sirt3 mRNA expression. (F) SOD2 mRNA expression. * indicates P□0.05 between control group and TRA group. ** indicates P□0.05 between TRA group and TRA+LCZ group.

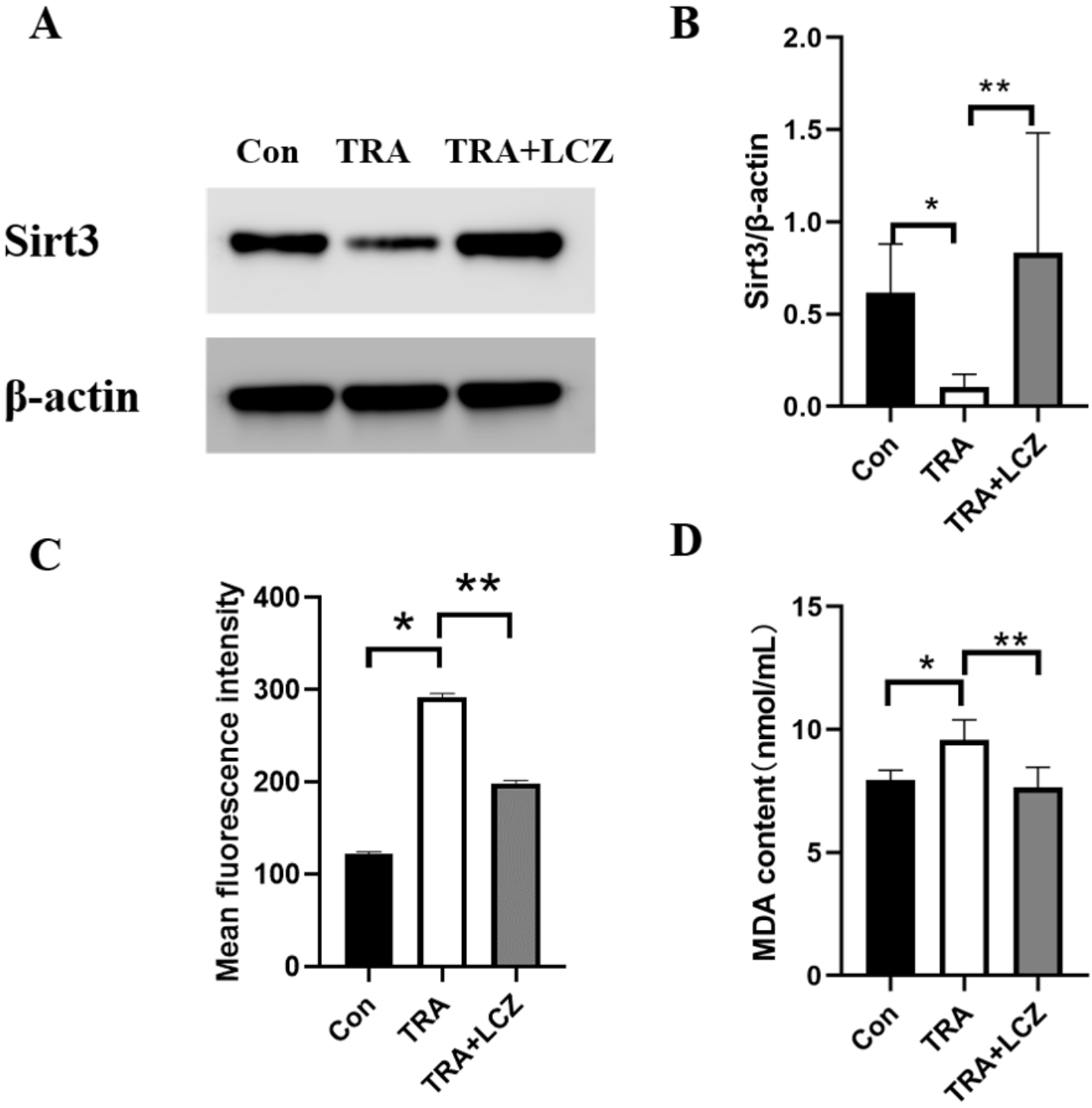

Figure 2

The level of Sirt3, ROS and MDA. (A) The western blot of Sirt3. (B) The relative expression of Sirt3. (C) The mean fluorescence intensity of ROS. (D) The level of MDA by ELISA. * indicates P□0.05 between control group and TRA group. ** indicates P□0.05 between TRA group and TRA+LCZ group.

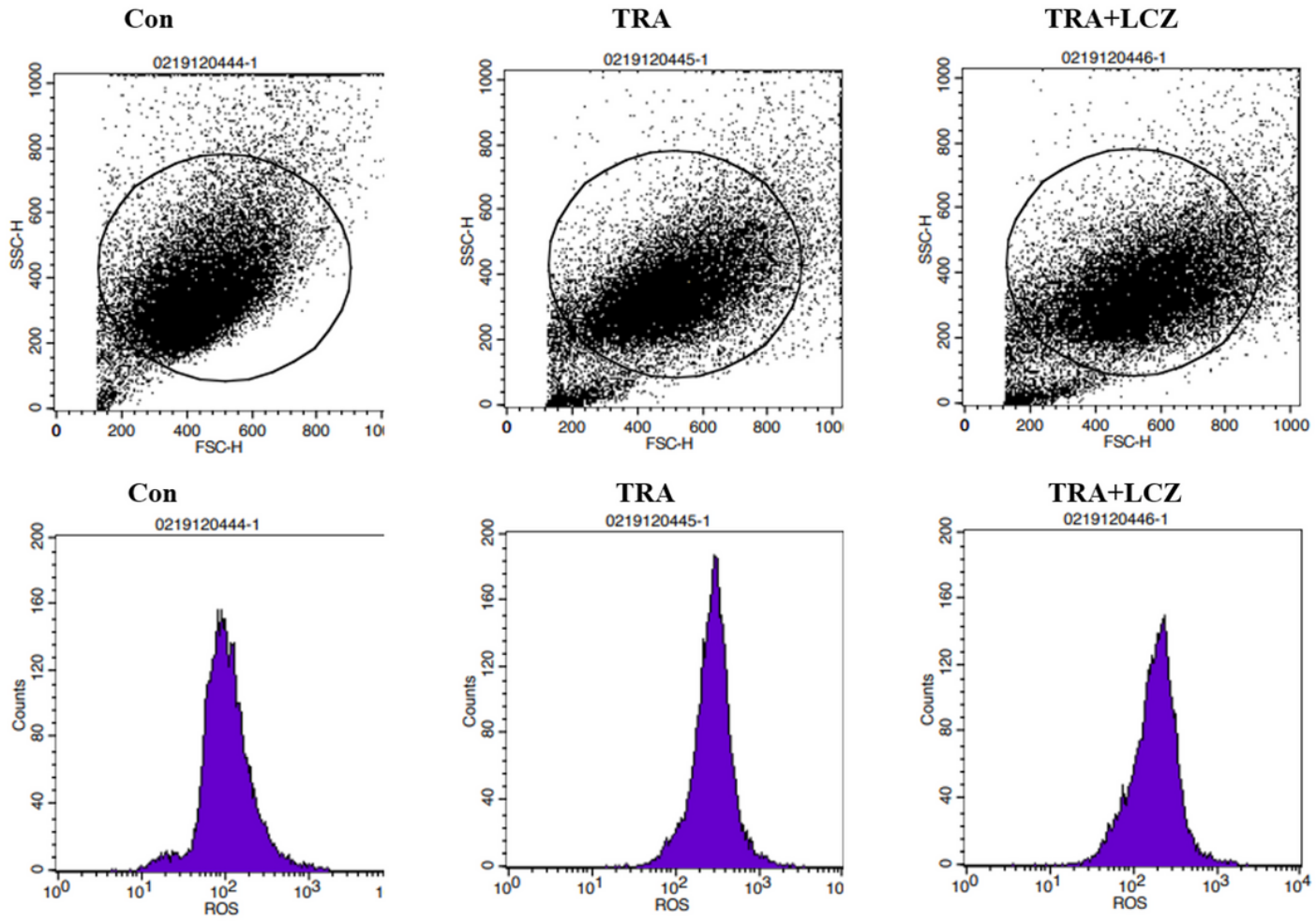

Figure 3

The level of ROS using flow cytometry. Upper figure indicates SSC / FSC scatter diagram. Lower figure shows fluorescence intensity of ROS.

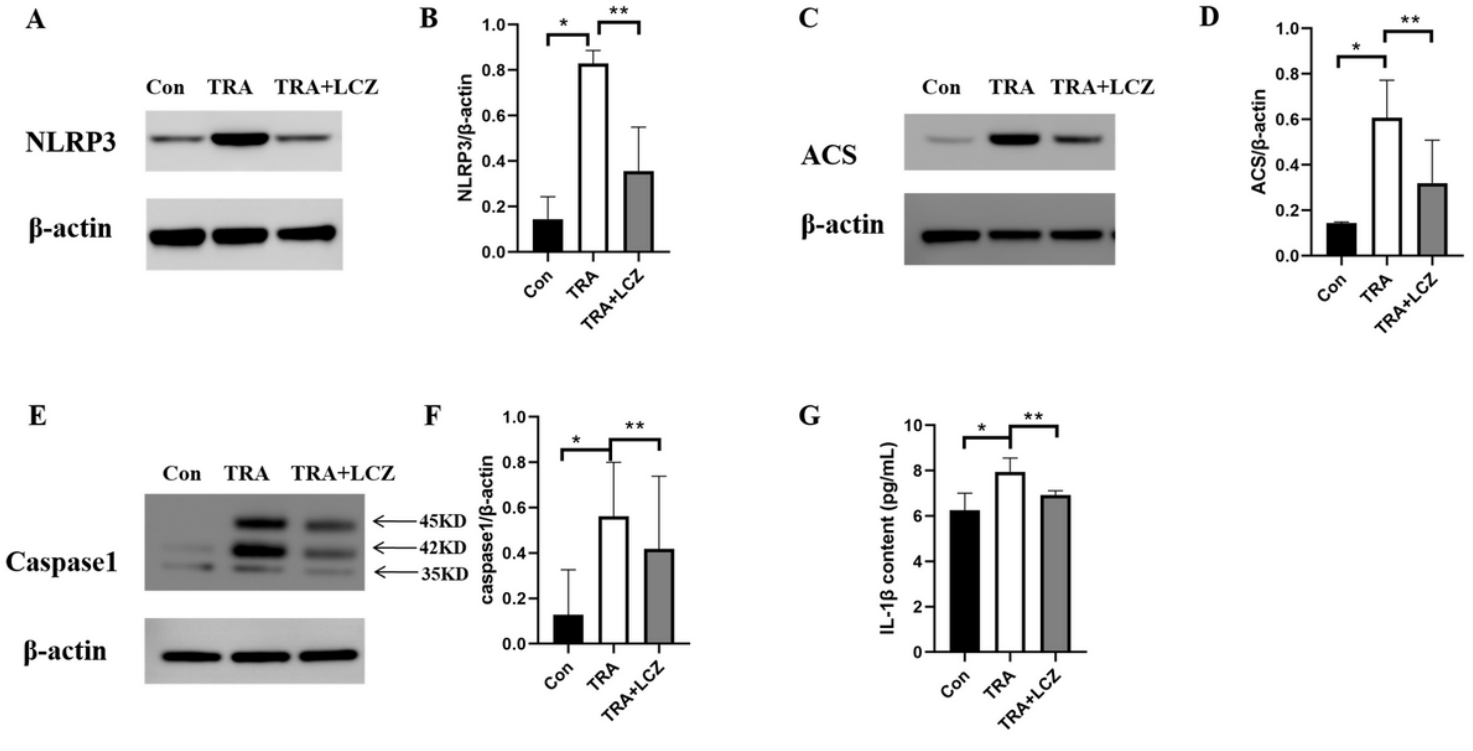

Figure 4

The level of pyroptosis and inflammation. (A) The western blot of NLRP3. (B) The relative content of NLRP3. (C) The western blot of ACS. (D) The relative content of ACS. (E) The western blot of Caspase-1. (F) The relative content of Caspase-1. (G) The level of IL-1β tested by ELISA. * indicates P□0.05 between control group and TRA group. ** indicates P□0.05 between TRA group and TRA+LCZ group.

Supplementary Files

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