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The Role of GPER in Sepsis-Induced Myocardial Cell Damage and 28-Day Mortality Risk

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Abstract

Purpose: The role of GPER in sepsis-induced myocardial cell injury and its potential impact on the risk of death within 28 days in sepsis. Methods: An in vitro experiment was conducted to establish a sepsis-induced myocardial cell model. H9C2 myocardial cells were treated with 10 μg/ml lipopolysaccharide (LPS) for 24 hours. The effects of different concentrations of the GPER agonist G1 (1, 3, and 10 μmol/L) on cell viability, expression of inflammatory markers, cell apoptosis, and the NF-κB pathway were evaluated. A Mendelian randomization analysis was conducted using Single Nucleotide Polymorphism (SNPs) related to the GPER gene as instrumental variables to investigate the causal relationship between the GPER gene variations and sepsis (28-day death). Results: The results indicate that the group treated with LPS showed a significant decrease in myocardial cell viability, an increase in concentrations of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), higher apoptosis rates, and increased phosphorylation levels of NF-κB p65 (p-P65/P65) and IκB-α (p-IκB-α/IκB-α) compared to the control group ($P < 0.05$). These indicators were significantly reversed by low, medium, and high doses of G1 ($P < 0.05$), demonstrating a dose-dependent effect of G1. The protective effects of G1 were negated upon the addition of phorbol 12-myristate 13-acetate (PMA), indicating that its protective mechanism involves the inhibition of the NF-κB pathway. However, genetic evidence did not show a causal relationship between GPER gene variations and sepsis (28-day death) ($P < 0.05$). Conclusion: Activation of GPER mitigates sepsis-induced myocardial cell inflammation and apoptosis by inhibiting the NF-κB pathway. However, genetic evidence did not show a causal relationship between GPER gene variations and sepsis

(28-day death).

Keywords

G Protein-Coupled Estrogen Receptor, Sepsis-Induced Cardiomyopathy, Inflammation and Apoptosis, Sepsis (28-Day death), Mendelian Randomization

1. Introduction

Sepsis is a major global public health challenge due to its high incidence and mortality rates [1]. Sepsis-induced cardiomyopathy (SIC) is a myocardial dysfunction resulting from sepsis and is closely linked to increased short-term mortality from sepsis [2]. Studies have revealed a higher incidence of SIC in males compared to females, which may be related to estrogen and its receptor expressions. However, the biological mechanisms underlying these gender disparities remain unclear [3]. To explore the complex mechanisms of estrogen, it is necessary to understand the specific roles of different types of estrogen receptors. The G protein-coupled estrogen receptor (GPER) is a non-classical estrogen receptor that functions distinctly from the traditional estrogen receptors ERα and ERβ. Although the relationship between GPER activation and inflammatory and apoptotic processes is well-known, its specific role in SIC remains unclear. Furthermore, the abnormal activation of the NF-κB signaling pathway is a crucial factor in the advancement of myocardial inflammation [4] [5]. It remains to be investigated whether GPER can regulate this pathway and impact the progression of SIC.

Recent epidemiological studies have linked serum GPER levels with short-term survival rates in sepsis patients, suggesting that GPER may be a novel prognostic factor for sepsis [6]. However, it is important to note that observational studies can only demonstrate a correlation between GPER and short-term survival in sepsis, and the elucidation of underlying complex causal relationships is complicated by confounding factors and reverse causation. Therefore, further investigation is required to determine the causal relationship between GPER gene variations and sepsis (28-day death) using more rigorous methodologies. Mendelian randomization analysis, which uses genetic variants as instrumental variables to estimate the causal relationship between exposures and outcomes, aims to overcome the limitations of traditional observational studies and provide more rigorous causal inference [7]-[9].

This study aims to investigate the effect of GPER activation on inflammation and apoptosis of myocardial cells induced by lipopolysaccharide (LPS). Additionally, it aims to evaluate the causal relationship between GPER gene variations and sepsis (28-day death) using Mendelian randomization analysis. Through this dual approach, we aim to provide new insights into therapeutic strategies for sepsis-induced cardiomyopathy.

2. Materials and Methods

2.1. Fundamental Experiment Study

2.1.1. Materials

H9C2 cardiomyocytes (rat): American Type Culture Collection, USA; Fetal bovine serum, G1: Gibco, USA; Trypsin, LPS: Solarbio Science & Technology Co., Ltd., China; DMEM culture medium: Invitrogen, USA; TNF-α, IL-6 ELISA kits: Hengyuan Biotechnology Co., Ltd., China; Annexin V/PI apoptosis detection kit: TaKaRa, Japan; PVDF membrane: Millipore, USA; BCA protein assay kit, ECL chemiluminescence kit: Biyun Tian Biotechnology Research Institute, Shanghai, China; NF-κB activator Phorbol 12-myristate 13-acetate (PMA), NF-κB P65 antibody, NF-κB p-P65 antibody, p-IκB-α antibody, IκB-α antibody, Bcl-2 antibody, Bax antibody, β-actin: CST, USA; Horseradish peroxidase-conjugated secondary antibody: Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China.

2.1.2. Cell Culture

The H9C2 cardiomyocytes were cultured in DMEM supplemented with 10% fetal bovine serum. The cells were maintained in a humidified atmosphere of 5% CO_2 at 37˚C. Upon reaching 80% confluence, cells in good growth conditions were selected for subsequent experiments.

2.1.3. Cell Treatment

To prepare an LPS-induced myocardial inflammation model, H9C2 cardiomyocytes were treated with 10 μg/mL LPS for 24 hours, as described in reference [10]. The groups consisted of normal control, an LPS group, and three G1 pretreatment groups at concentrations of 1, 3, and 10 μmol/L. The LPS group was incubated with 10 μg/mL LPS for 24 hours. The G1 pretreatment groups were pre-incubated with respective G1 concentrations for 6 hours before adding 10 μg/mL LPS for another 24 hours. To explore G1's regulatory effects on sepsis-induced myocardial inflammation and apoptosis via the NF-κB pathway, 1 μmol/L PMA was added to the LPS + 3 μmol/L G1 group for 24 hours before assessment.

2.1.4. CCK-8 Cell Survival Test

Log-phase cardiomyocytes were adjusted to a concentration of 1×10^4 cells/mL. Next, 200 μL of the cell suspension was seeded into each well of a 96-well plate and cultured until adhesion. After treatment, as described in the 'Cell Treatment' section, 50 μL of CCK-8 solution was added to each well and incubated for 4 hours. The supernatants were removed, and 150 μL/well of DMSO was added, shaking for 10 minutes. The absorbance (OD values) were measured using a microplate reader to calculate the relative cell viability: Relative Cell Viability = (Experimental group OD value/Control group OD value) × 100%.

2.1.5. Protein Detection by Western Blot

Cardiomyocytes from each group were collected, lysed on ice for 30 minutes, and centrifuged to obtain supernatants. Protein concentration was quantified using

the BCA method. Samples were denatured by heating in the loading buffer. 40 μg of protein samples were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk for 2 hours, then incubated with primary antibodies overnight at 4˚C (dilutions: NF-κB p-P65 and P65 at 1:2000, p-IκB-α and IκB-α at 1:2000, Bax at 1:1000, Bcl-2 at 1:1000, β-actin at 1:20,000). Horseradish peroxidase-conjugated secondary antibody was applied at a 1:5000 dilution for 1 hour at room temperature. Detection was performed using ECL reagents, with images captured in a darkroom. Relative protein expression was analyzed using ImageJ software, and normalized to β-actin.

2.1.6. TNF-α and IL-6 Detection by ELISA

The cell density was adjusted to 1×10^4 cells/ml, and 200 μL of cell suspension was seeded into each well of a 96-well plate. After treatment as described in the 'Cell Treatment' section and collection of supernatants, TNF-α and IL-6 levels were measured using ELISA kits, following the manufacturer's instructions. The absorbance was measured at 450 nm.

2.1.7. Flow Cytometry

After treatment as described in the 'Cell Treatment' section, cells were digested with trypsin and washed twice with PBS. Approximately 5×10^5 cells were collected, resuspended in binding buffer, and stained with 5 μL Annexin V-FITC and 5 μL PI for 15 minutes in the dark at room temperature. Apoptosis was analyzed within 1 hour using flow cytometry.

2.1.8. Statistical Analysis

Each experiment was repeated three times, and the data are presented as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism software version 6.0 (GraphPad, San Diego, CA, USA). Differences between two groups were assessed using the Student's t-test, while differences among more than two groups were assessed using one-way ANOVA followed by Tukey's post hoc test. A p-value of <0.05 was considered significantly different.

2.2. Mendelian Randomization Analysis

2.2.1. Study Design

The study data were obtained from publicly available GWAS summary datasets, which included eQTL (expression quantitative trait locus) data for the GPER gene variations and GWAS data for sepsis (28-day death) [11]. The original studies obtained ethical approvals and informed consent. A two-sample Mendelian randomization approach was used to investigate the causal association between GPER gene variations and sepsis (28-day death).

2.2.2. Instrumental Variables for GPER

The eQTL data of GPER genes were derived from the IEU Open GWAS database (https://gwas.mrcieu.ac.uk/), and the GWAS ID of the dataset was eqtl-a-ENSG00000164850. The criteria for selecting instrumental variables were as

follows: Instrumental variables achieving genome-wide significance ($P < 5 \times 10^{-8}$), and ensured minimal linkage disequilibrium ($r^2 < 0.001$) within a clump window exceeding 10,000 kb to ensure independence. The LD level was based on the European population-based 1000 Genomes Scheme estimation [12]. To ensure a strong association between instrumental variables and the exposure, the F-statistic of SNPs was used to assess the strength of association, with an F-statistic >10 indicating no bias from weak instrumental variables, calculated as F-statistic = (β/SE).

2.2.3. Study Outcome

The data on sepsis (28-day death) was obtained from the UK Biobank (UKB) and IEU Open GWAS database (https://gwas.mrcieu.ac.uk/), with the GWAS ID for the dataset being ieu-b-5086. The dataset included genotype data for 1896 sepsis patients and 484588 controls of European descent.

2.2.4. Statistical Analysis

MR analysis was conducted using R software (version 4.1.2, http://www.R-project.org) and the Two-Sample MR package (version 0.5.6) [13]. The causal relationship between the GPER gene variations and sepsis (28-day death) was assessed using the inverse variance weighted (IVW) method, The IVW method assumes all instrumental variables are valid; any SNP not meeting this assumption introduces bias [14]. Additional analyses were performed using the Weighted median and MR-Egger methods. The Weighted median approach requires at least 50% of the SNPs to be valid [15], while MR-Egger regression provides an unbiased estimate even in the presence of pleiotropic instrumental variables [16]. The MR-Egger intercept test was used to assess the presence of pleiotropic associations among genetic variants and potential confounders. Significant causal relationship between GPER gene variations and sepsis (28-day death) was considered if: (1) the IVW method showed a significant difference ($P < 0.05$). (2) Estimates from IVW, Weighted median, and MR-Egger methods were directionally consistent. (3) The MR-Egger intercept test was not significant ($P > 0.05$) [17]. Cochran's Q test and funnel plots were used to assess heterogeneity among SNPs [18]. The leave-one-out method, akin to classical statistical iteration, was applied to determine if the exclusion of any SNP significantly altered the results [19].

3. Results

3.1. G1 on the Relative Survival Rate

The CCK-8 assays did not reveal any significant differences among the 0, 1, 3, and 10 μmol/L G1 groups ($P > 0.05$). However, the 30 μmol/L G1 group exhibited a reduced cell survival rate compared to the 0 μmol/L G1 group ($P < 0.05$) (**Figure 1(A)**). Additionally, the LPS group showed a decreased cell survival rate compared to the 0 μmol/L G1 group ($P < 0.05$). However, the LPS+G1 (1, 3, 10 μmol/L) groups exhibited increased cell survival rates compared to the LPS group ($P < 0.05$) (**Figure 1(B)**).

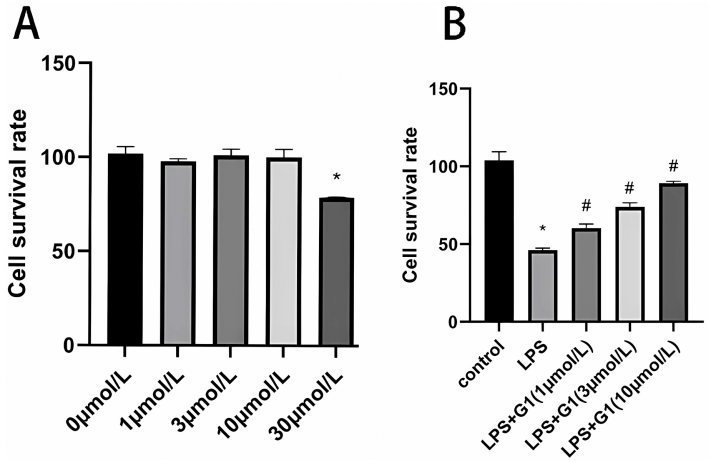
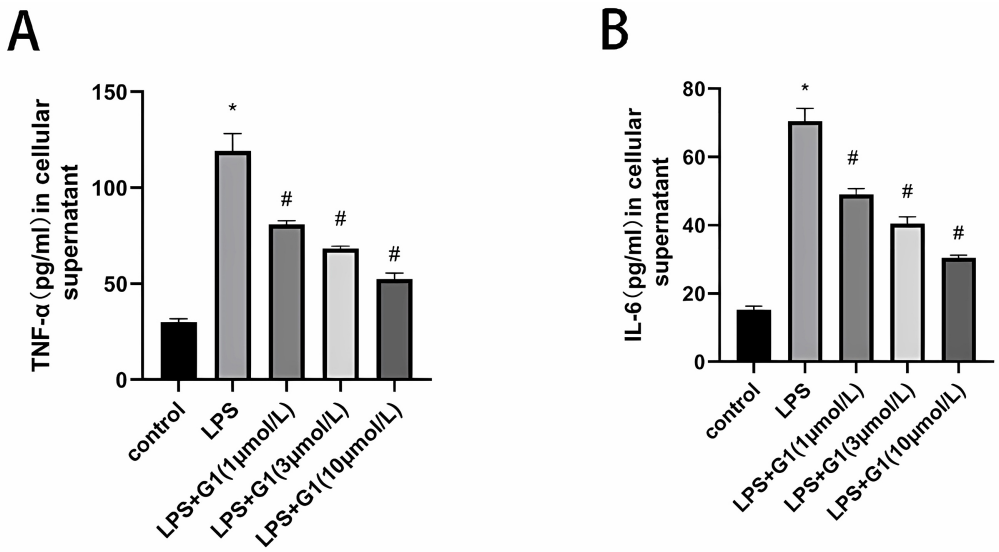


Figure 1. Effects of Different Concentrations of G1 on the Relative Survival Rate of sepsis-induced myocardial cell model. (A) H9c2 cells were exposed to various concentrations of G1 (0, 1, 3, 10, and 30 μM) for 24 hours, and cell viability was measured using a CCK8 assay. (B) H9c2 cells were pre-treated with different concentrations of G1 (1, 3, and 10 μM) for 6 hours, then exposed to 10 μg/mL LPS for 24 hours, and cell viability was measured using a CCK8 assay. * P < .05 versus Control; #p < .05 versus LPS.

3.2. G1 on Inflammatory Markers and Apoptosis

The levels of TNF-α and IL-6 in the supernatant of the LPS group were increased compared with the control group (P < 0.05). The levels of TNF-α and IL-6 in the supernatant of the LPS+G1 (1, 3, and 10 μmol/L) group were decreased compared with those of the LPS group (P < 0.05) (Figure 2(A) & Figure 2(B)).

Compared with the control group, Bcl-2 protein levels were decreased (P < 0.05) and Bax protein levels were increased (P < 0.05) in cells of the LPS group. Compared with the LPS group, Bcl-2 protein levels were increased in cardiomyocytes in the LPS+G1 (1, 3, and 10 μmol/L) group (P < 0.05). And the level of Bax protein was decreased (P < 0.05) (Figure 2(C) & Figure 2(D)).



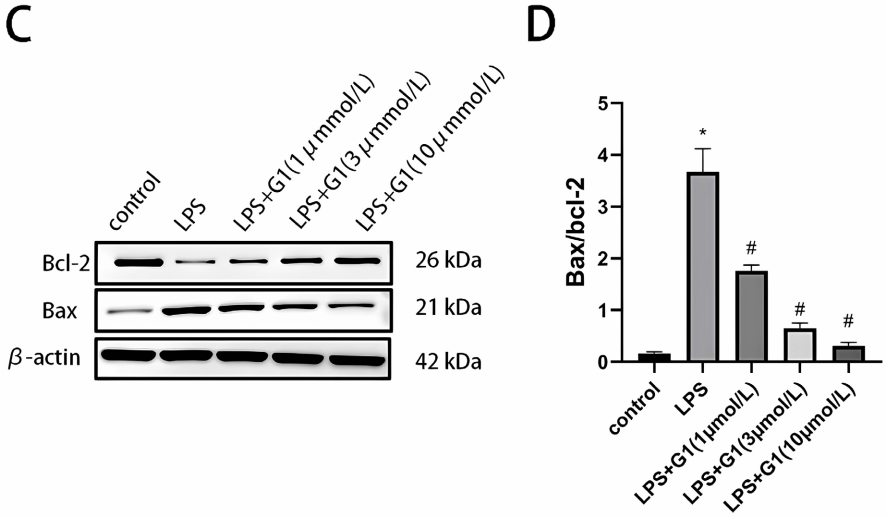


Figure 2. G1 Inhibits Inflammatory Cytokines and Apoptosis in sepsis-induced myocardial cell model. (A) Expression levels of the inflammatory mediator TNF-α in H9c2 myocardial cells. (B) Expression levels of the inflammatory mediator IL-6 in H9c2 myocardial cells. (C) Western blot showing the expression changes of Bcl-2 and Bax proteins. (D) Statistical analysis of Bax/Bcl-2 ratio in H9c2 myocardial cells. *p < .05 versus Control; #p < .05 versus LPS.

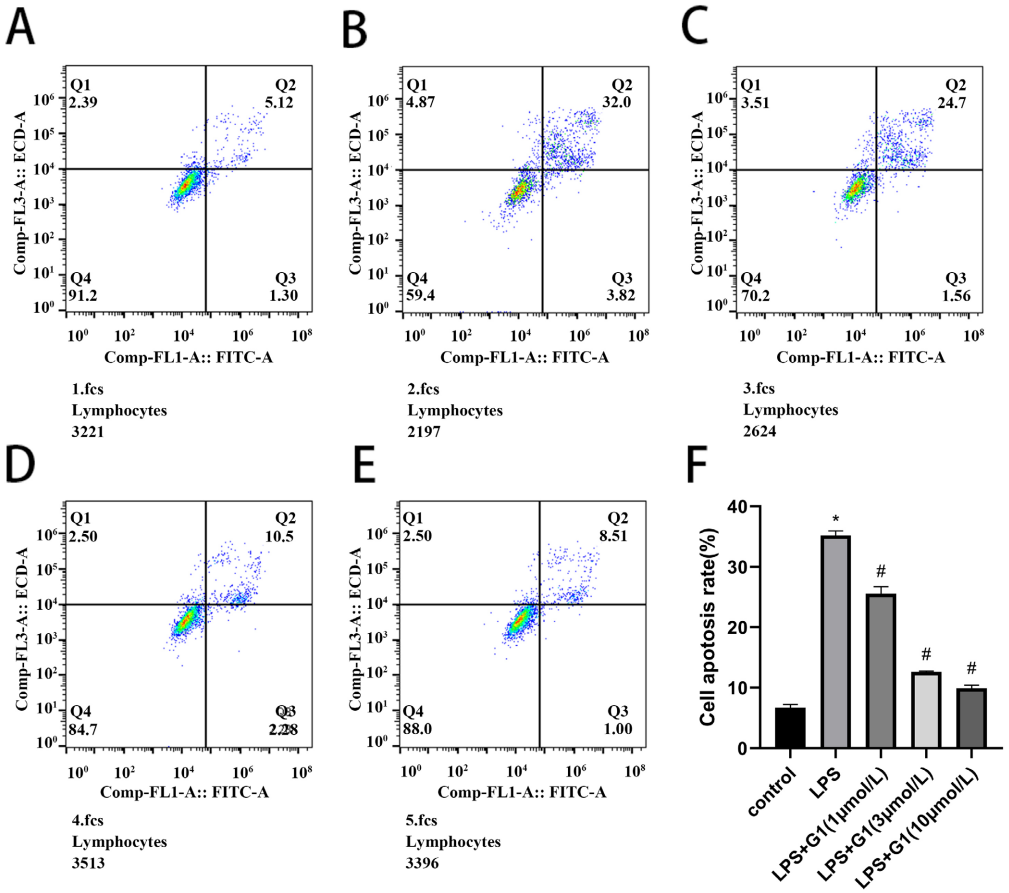


Figure 3. Effect of G1 on the Apoptosis Rate of sepsis-induced myocardial cell model. (A) Control group; (B) LPS group (10 μg/mL). (C) LPS + G1 (1 μmol/L). (D) LPS + G1 (3 μmol/L). (E) LPS + G1 (10 μmol/L). (F) Statistical analysis of apoptosis rate in H9c2 myocardial cells. *p < .05 versus Control; #p < .05 versus LPS.

The apoptosis rate was elevated in the LPS group compared with the control group ($P < 0.05$). The apoptosis rate was decreased in the LPS+G1 (1, 3, and 10 μmol/) group compared with the LPS group ($P < 0.05$) (Figure 3(A)-(F)).

3.3. G1 on NF-κB Pathway

Compared to the control group, NF-κB p-P65/P65 and p-IκB-α/IκB-α protein levels in the LPS group were elevated ($P < 0.05$). Compared to the LPS group, LPS+G1 (1, 3, 10 μmol/L) groups showed reduced NF-κB p-P65/P65 and p-IκB-α/IκB-α protein levels ($P < 0.05$) (Figure 4(A)-(D)).

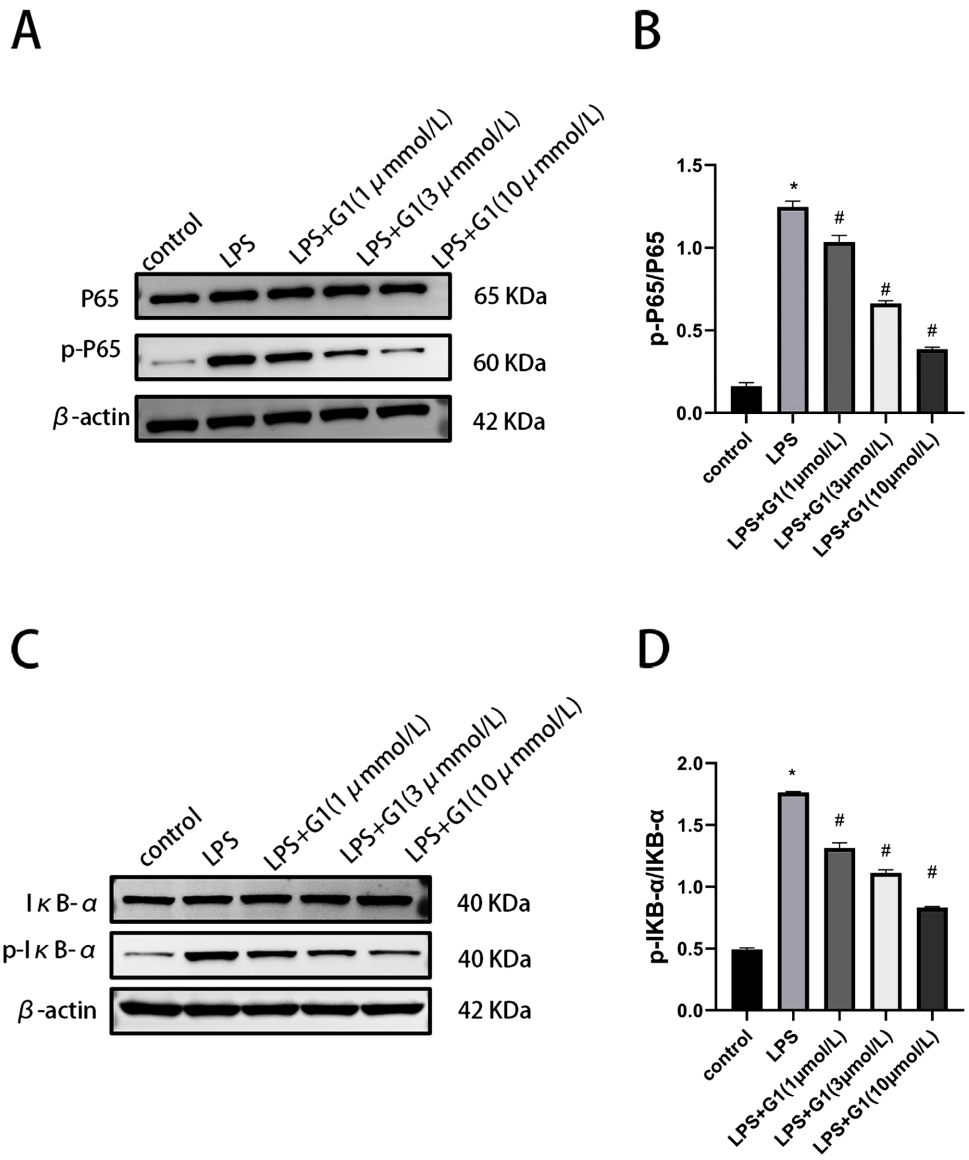


Figure 4. G1 Upregulates Phosphorylation of P65 and IκB-α in sepsis-induced myocardial cell model. (A) Western blot showing the expression changes of p-P65 and P65 proteins. (B) Statistical analysis of p-P65/P65 protein expression changes. (C) Western blot showing the expression changes of p-IκB-α and IκB-α proteins. (D) Statistical analysis of p-IκB-α/IκB-α protein expression changes. *p < .05 versus Control; #p < .05 versus LPS.

3.4. PMA on Inflammatory Markers and Apoptosis

The PMA group showed reduced levels of TNF-α and IL-6, and the apoptosis rate in the PMA group increased, compared to the LPS + 3 μmol/L G1 group (Figure 5(A)-(E)).

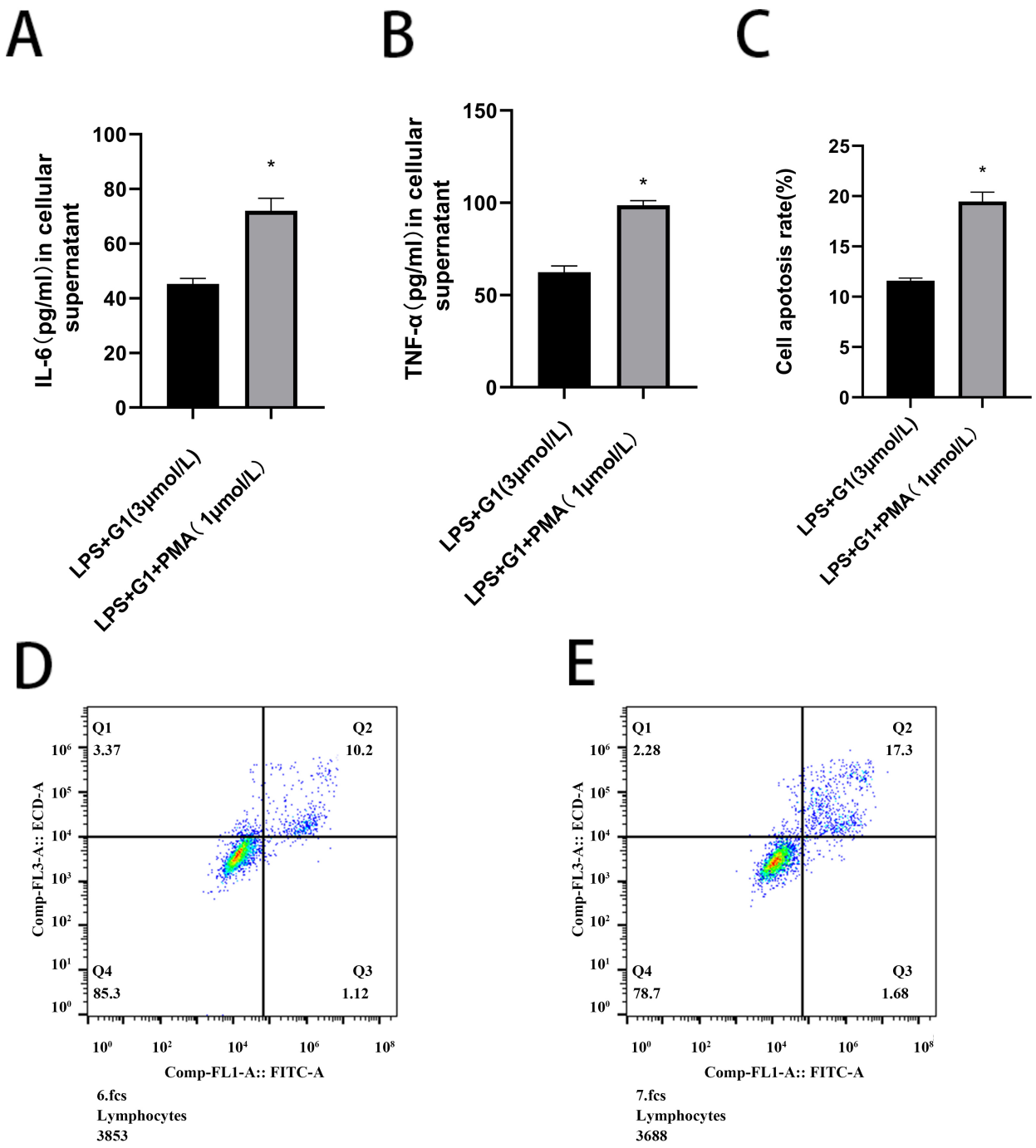


Figure 5. PMA Reverses the Cardioprotective Effects of G1. (A) Expression levels of the inflammatory mediator IL-6 in H9c2 myocardial cells. (B) Expression levels of the inflammatory mediator TNF-α in H9c2 myocardial cells. (C) Statistical analysis of apoptosis rate in H9c2 myocardial cells. (D) LPS + PMA (1 μmol/L) group. (E) LPS +PMA (1 μmol/L) + G1 (3 μmol/L) group. *p < .05 versus Control; #p < .05 versus LPS.

3.5. PMA on NF-κB Pathway

The PMA group showed increased NF-κB p-P65/P65 and p-IκB-α/IκB-α protein expression levels, compared to the LPS + 3 μmol/L G1 group (Figure 6(A)-(D)).

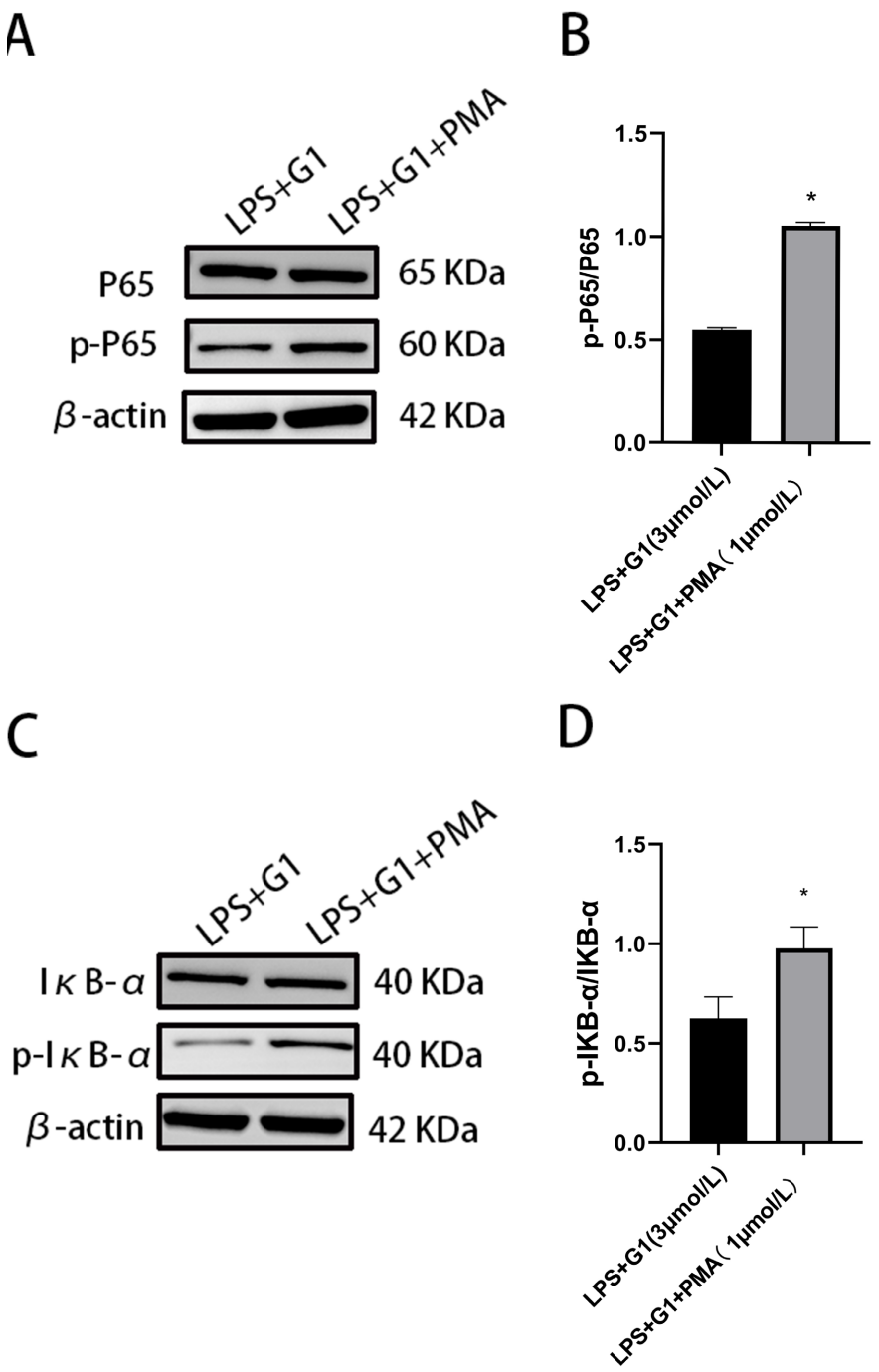


Figure 6. PMA Reverses the Cardioprotective Effects of G1. (A) Expression levels of the inflammatory mediator IL-6 in H9c2 myocardial cells. (B) Expression levels of the inflammatory mediator TNF-α in H9c2 myocardial cells. (C) Statistical analysis of apoptosis rate in H9c2 myocardial cells. (D) LPS + PMA (1 μmol/L) group. (E) LPS + PMA (1 μmol/L) + G1 (3 μmol/L) group. *$p < .05$ versus Control; #$p < .05$ versus LPS.

3.6. Mendelian Randomization Analysis

The IVW, weighted median, and MR-Egger method all showed no causal association between the GPER gene variations and the sepsis (28-day death) (P > 0.05). There was no heterogeneity based on individual variants (Cochran Q test value for IVW = 1.348, P value for heterogeneity = 0.853). We found no evidence of horizontal multivariate validity based on the intercept term of the MR-Egger regression (MR-Egger intercept = -0.026, P = 0.460) (Table 1).

Table 1. MR estimates of the effect of GPER on Sepsis (28-day death).

Outcome	Method	OR (95% CI)	P	Q statistic	P-heterogeneity	Egger intercept	P-intercept
Sepsis of 28-day death	IVW	1.05 (0.88-1,26)	0.517	1.348	0.853		
	MR-Egger	1.18 (0.86-1.63)	0.366	0.637	0.887	-0.026	0.460
	Weighted median	1.07 (0.88-1.29)	0.474				
	Simple mode	1.00 (0.72-1.38)	0.981				
	Weighted mode	1.08 (0.87-1.34)	0.520				

Abbreviations: OR: odds ratio.

4. Discussion

The findings of our study highlight the significant role of GPER in modulating sepsis-induced myocardial cell injury. Our in vitro experiments demonstrated that LPS treatment markedly reduced myocardial cell viability, increased the production of inflammatory markers such as TNF-α and IL-6, and elevated apoptosis rates. Furthermore, the phosphorylation levels of NF-κB p65 and IκB-α were significantly upregulated, indicating activation of the NF-κB pathway. However, the lack of a causal relationship between GPER gene variations and 28-day mortality in sepsis.

We observed that G1 significantly reduced TNF-α and IL-6 levels in sepsis-stimulated cardiomyocytes and modulated the expression of apoptosis-related proteins Bax and Bcl-2, confirming G1's efficacy in alleviating sepsis-induced myocardial cell damage. Further analysis revealed a dose-dependent protective effect of G1, aligning with findings by Yaqin Zhang *et al.*, who reported similar dose-dependent effects of GPER agonists in other cell types [20]. Notably, G1 inhibited the NF-κB signaling pathway, reducing the ratios of NF-κB p-P65/P65 and p-IκB-α/IκB-α, thereby diminishing inflammation and apoptosis. This mechanism is consistent with J-L Wei *et al.*'s study on the role of the NF-κB pathway in sepsis-induced myocardial cell damage [21]. Previous research has explored GPER's role in modulating cardiovascular functions, showcasing the clinical potential of

GPER agonists or antagonists in treating cardiovascular diseases [22]. A study highlighted the improvement of coronary and cardiac functions, worsened by aging, through GPER activation, significant for reducing cardiovascular disease risk in postmenopausal women [23]. Joseph Adu-Amankwaah *et al.* investigated the mitigation of stress-induced cardiac damage and inflammation by estrogen via the GPER/PI3K pathway, downregulating ADAM17 [24]. Our study further extends the value of GPER activation in sepsis-induced myocardial cell damage, particularly highlighting the dose-dependent response of G1 and its role in inhibiting the NF-κB signaling pathway, providing crucial dose references for theoretical research.

Recent studies have correlated GPER expression levels positively with prognosis in triple-negative breast cancer (TNBC) [25], while low levels of GPER mRNA expression were associated with poor prognosis in gastric cancer patients [26]. In high-grade serous carcinoma (HGSC) patients, GPER was an independent prognostic factor for worse progression-free survival [27]. However, despite discovering GPER activation's protective effects on septic cardiomyocytes, genetic evidence did not demonstrate a direct causal relationship between GPER gene variations and sepsis (28-day death), conflicting with previous observational studies [6]. These varying results may stem from the complex pathophysiology of sepsis and differences in experimental study designs.

Our study confirms the protective role of GPER activation against sepsis-induced myocardial cell damage, crucial for understanding GPER's complex role in SIC. However, this study has some limitations: Firstly, in vitro models may not fully replicate the complex in vivo environment, necessitating further validation in animal models or clinical trials. Secondly, genetic analysis failed to establish a direct causal link between GPER gene variations and sepsis mortality risk, suggesting the need for larger or more diverse genetic studies. Lastly, while G1 is considered a specific agonist for exploring GPER's biological functions, its role should also be investigated in more complex experiments, such as transgenic animal studies. This suggests that while GPER activation can provide significant cellular protection against sepsis-induced damage, the genetic variations in GPER alone may not be a determinant of clinical outcomes in sepsis. Further research is necessary to explore the underlying mechanisms and potential therapeutic applications of GPER in the clinical management of sepsis.

5. Conclusion

In conclusion, our study underscores the protective role of GPER activation in sepsis-induced myocardial cell injury. The GPER agonist G1 effectively enhanced cell viability, reduced inflammatory marker expression, decreased apoptosis rates, and inhibited the NF-κB pathway in a dose-dependent manner, highlighting its potential as a therapeutic agent. Despite these promising in vitro findings, our Mendelian randomization analysis did not reveal a causal link between GPER gene variations and sepsis-related 28-day mortality.

Data Sharing Statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical Statement

Ethical approval for this study was not required as these analyses were based on summary statistics from published GWAS or the data were publicly accessible, and no individual-level data were used.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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