

EXPLORING THE PROTECTIVE ROLE AND THE MECHANISM OF SPHINGOSINE 1 PHOSPHATE IN ENDOTOXIC CARDIOMYOCYTES

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ABSTRACT—FTY720 is a sphingosine 1 phosphate (S1P) receptor agonist approved for the treatment of multiple sclerosis, which is a chronic inflammatory autoimmune disorder. Sepsis is a complex syndrome associated with progressive endotoxemic developments, which finally leads to damage of multiple organs, including the heart. In critical patients, cardiovascular dysfunction due to sepsis is a major cause of death. Previous studies have shown an association between S1P and cardioprotection in the situation of ischemia reperfusion and myocardial infarction. Therefore, we will study the role of S1P towards endotoxic cardiomyocytes. Different doses of FTY720 were applied or not to endotoxic cardiomyocytes. The concentration of inflammatory cytokines, including tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, and IL-10 was measured by enzyme-linked immunosorbent assay. Western blotting was used to analyze the downstream signaling pathways. We discovered that FTY720 reduced the levels of TNF- α and IL-6 through the NF- κ B pathway, inhibited the expression of caspase-3, and activated both protein kinase B and extracellular signal-regulated kinase 1/2. Additionally, the activation of protein kinase B and extracellular signal-regulated kinase 1/2 could be inhibited by the S1P1 and S1P3 receptor antagonist vulcanized polyethylene23019. Therefore, we infer that S1P exerts a protective effect towards endotoxic cardiomyocytes by decreasing the levels of TNF- α and IL-6, regulating apoptotic and survival signaling pathway. The S1P1 and S1P3 receptors are involved in the prosurvival signal activation.

KEYWORDS—Apoptosis, endotoxic cardiomyocytes, FTY720, inflammatory cytokines, NF- κ B, sphingosine 1 phosphate, survival

INTRODUCTION

Sepsis is characterized by an imbalance between pro- and anti-inflammatory responses and ultimately leads to the patients' own organ failure (1). Bacterial lipopolysaccharide (LPS) or other microbial components may induce systemic inflammatory response. Cardiovascular dysfunction due to sepsis has a high morbidity and mortality in critical patients. Studies have shown that approximately 50% of septic patients have heart dysfunction and approximately 7% have heart failure (2). Exploring the potential interaction between cardiac dysfunction and endotoxemia has important clinical significance. It is now widely thought that the host response to sepsis is a dysregulated inflammation, which damages the patients' own organs (2). Inflammatory cytokines play a crucial role during this pathological progress.

Sphingosine 1 phosphate (S1P) is an important lipid signaling molecule that is widely present in the plasma and is long believed to act as an intracellular second messenger. S1P receptors, which are expressed on the cell membrane, are members of the G protein coupled receptor (GPCR) family. Extracellular S1P interacts with five GPCRs, including S1P1-S1P5, and transduces intracellular signals to regulate many biological processes, such as cell migration, adhesion, survival, and proliferation (3). The distribution of the S1P subtypes varies, which produces diverse effects. The S1P1, S1P2, and S1P3 receptors are found in larger numbers in the heart and blood vessels (4). Through downstream S1P receptor signaling, S1P significantly alleviated vascular leakage in LPS-induced lung injury in mice (5). Theilmeier et al. (6) demonstrated that S1P associated high-density lipoprotein protected the heart of mice against ischemia/reperfusion injury through S1P3-mediated and NO-dependent pathways. After myocardial infarction (MI), the level of S1P1 receptor protein decreased (7). Administration of the S1P1-selective agonist SEW2871 could reduce apoptosis of the uninfarcted myocardium and alleviate cardiac function during the first 2 weeks after MI (8). FTY720 is a structural analogue of S1P, which has been approved for the treatment of MS. The research has shown that FTY720 could interfere with the pathological processes of several diseases, such as sepsis, MI, atherosclerosis, and inflammatory bowel disease (9). In the LPS-induced lung injury model, vascular leakage and inflammatory cell infiltrates were significantly restricted by FTY720 (10). Wang et al. (11) presented a model in which FTY720 had a protective effect against hypoxia/reoxygenation injury of cardiomyocytes. This study tested the hypothesis that FTY720, a structural analogue of S1P, would attenuate the sequelae to endotoxic cardiomyocytes.

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MATERIALS AND METHODS

Materials

FTY720 was obtained from Cayman Chemical. The primary antibodies including caspase-3, P-I κ B α , extracellular signal-regulated kinase (ERK)1/2, and protein kinase B (AKT) were purchased from Cell Signaling Technology. Vulcanized polyethylene (VPC)23019, polyether diols (PD)98059, and Wortmannin were obtained from Gibco Company. The ELISA kits were purchased from R&D Company (Minneapolis, Minn).

Cell culture

A cardiomyocyte cell line called H9C2 derived from embryonic rat heart tissue. The cells were purchased from the Chinese Academy of Science Cell Bank (Shanghai), cultured in high glucose Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, and placed in an incubator of a humidified atmosphere consisting of 5% CO₂ and 95% air at 37°C.

Endotoxic cardiomyocytes protocol

H9C2 cells were stimulated with different concentrations of LPS (0 μ g/mL, 1 μ g/mL, 2 μ g/mL, 5 μ g/mL, and 10 μ g/mL) for 8, 12, 24, 36, and 48 h, respectively. The supernatant of the cell cultures was collected. Different concentrations of FTY720 were added to the cells.

Western blotting analysis

For whole cell protein extraction, the cells were lysed for 5 min on ice. The bicinchoninic acid method was used to determine the protein concentration. Equal amounts of protein were resuspended in $\times 6$ Laemmli sample buffer and boiled for 5 min. The protein samples were resolved by sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking in 5% nonfat milk dissolved in tris buffered saline tween (TBST) for 2 h at 4°C, the membranes were probed with primary antibodies overnight, washed three times with TBST for 10 min, and then probed with secondary antibody for 2 h at 4°C. The membranes were washed again in TBST three more times, and the signal was detected by chemiluminescence.

ELISA method

After treating the cells with LPS and different doses of FTY720, the supernatants were collected and centrifuged. The standards and supernatant samples

were added to the ELISA wells and incubated for 2 h at room temperature. Each well was washed with the washing buffer five times. Specific conjugate of cytokines was added to each well and incubated for another 2 h at room temperature. After washing five times, the substrate solution and stop solution were added in turn. The standards and samples were read on a microplate reader set at 450 nm. Finally, the concentrations of cytokines were calculated.

Cell proliferation assay

The cells were seeded into 96-well plates at a density of 4,000 cells/well and cultured for 24 h. A volume of 20 μ L of methyl thiazolyl tetrazolium (MTT) was added into each well and incubated for another 4 h. The supernatant was discarded, and 150 μ L of dimethylsulfoxide was added to each well. The optical density was measured at 490 nm.

Statistical analysis

Data were expressed as mean \pm SD, which were compared using the method of one-way or two-way analysis of variance (ANOVA). To assess the secretion of cytokines in the different concentrations of LPS over time, we compared the levels of cytokines using two-way ANOVA. Analysis of one-way ANOVA with the *post hoc* Turkey–Kramer multiple comparison test was used for comparison between multiple groups. In the article, all the use of higher, lower, and inhibit in the results and discussion below mean $P < 0.05$ and the method of pairwise comparison in one-way ANOVA was used. Analyses were performed using Graphpad Prism 5 (Graphpad Software, La Jolla, Calif). $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

LPS induces cardiomyocytes cultured in vitro to secrete inflammatory cytokines

Various concentrations of LPS (0 μ g/mL, 1 μ g/mL, 2 μ g/mL, 5 μ g/mL, and 10 μ g/mL) stimulated cardiomyocytes for 8, 12, 24, 36, and 48 h, respectively. The supernatant was collected, and the levels of inflammatory cytokines including tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, and IL-10 were measured by ELISA. As shown in Table 1, the levels of cytokines increased after LPS stimulation. The

TABLE 1. Inflammatory cytokine secretion of cardiomyocytes cultured with LPS

	Control	1 μ g/mL	2 μ g/mL	5 μ g/mL	10 μ g/mL
TNF- α (pg/mL)					
8 h	13.4 \pm 1.8	13.6 \pm 1.1	14.7 \pm 2.2	24.5 \pm 4.5*	30.3 \pm 5.3*
12 h	16.7 \pm 1.1	15.6 \pm 1.3	19.8 \pm 1.8	19.1 \pm 2.0	26.1 \pm 3.5*
24 h	15.5 \pm 1.8	21.0 \pm 2.1*	26.4 \pm 2.3*	33.3 \pm 2.4*	39.1 \pm 3.2*
36 h	13.8 \pm 0.9	17.9 \pm 1.1*	18.9 \pm 1.9*	29.7 \pm 3.0*	31.9 \pm 4.2*
48 h	15.5 \pm 1.4	17.2 \pm 2.4	17.7 \pm 1.3	30.9 \pm 2.9*	32.7 \pm 2.0*
IL-1 β (pg/mL)					
8 h	7.8 \pm 0.7	19.5 \pm 2.3*	19.3 \pm 1.3*	19.8 \pm 1.6*	42.3 \pm 2.5*
12 h	9.7 \pm 0.6	16.2 \pm 1.2*	37.3 \pm 1.5*	49.8 \pm 4.4*	45.4 \pm 3.4*
24 h	17.1 \pm 1.9	20.0 \pm 0.5	38.4 \pm 2.5*	44.7 \pm 2.3*	48.5 \pm 2.3*
36 h	7.9 \pm 0.7	16.7 \pm 1.3*	16.6 \pm 1.6*	28.5 \pm 2.0*	39.2 \pm 2.5*
48 h	9.5 \pm 1.8	17.6 \pm 1.7*	33.5 \pm 3.2*	25.6 \pm 3.3*	34.1 \pm 3.1*
IL-6 (pg/mL)					
8 h	7.7 \pm 0.9	19.3 \pm 2.5*	27.5 \pm 2.8*	43.9 \pm 6.2*	98.7 \pm 11.2*
12 h	8.9 \pm 0.8	15.8 \pm 1.9	60.4 \pm 12.5*	112.1 \pm 22.3*	132 \pm 25.9*
24 h	9.7 \pm 1.1	40.3 \pm 10.5*	49.8 \pm 12.8*	119.5 \pm 18.9*	187.5 \pm 29*
36 h	8.1 \pm 1.2	8.5 \pm 0.8	18.9 \pm 2.2*	107.1 \pm 20.7*	192.9 \pm 32.1*
48 h	9.1 \pm 1.3	12.9 \pm 0.9	40.2 \pm 10.5*	43.9 \pm 5.7*	168.1 \pm 29.7*
IL-10 (pg/mL)					
8 h	11.1 \pm 1.2	12.0 \pm 0.6	17.5 \pm 1.9*	22.2 \pm 1.6*	27.9 \pm 2.0*
12 h	11.7 \pm 1.5	12.4 \pm 1.4	14.2 \pm 2.6	26.2 \pm 2.3*	30.4 \pm 2.1*
24 h	10.5 \pm 1.2	17.5 \pm 2.2*	20.7 \pm 1.8*	17.1 \pm 4.7*	28.5 \pm 3.6**
36 h	11.1 \pm 1.9	11.7 \pm 1.8	19.3 \pm 1.4*	23.1 \pm 2.9*	29.9 \pm 5.6*
48 h	14.0 \pm 1.9	16.1 \pm 1.4	17.3 \pm 0.7	19.5 \pm 3.5*	27.3 \pm 5.2*

LPS with different concentrations promoted the secretion of inflammatory cytokines, including TNF- α , IL-1 β , IL-6, and IL-10. The data are expressed as the means \pm SD of three independent experiments. The method of one-way ANOVA was used to analyze the data.

* $P < 0.05$ compared with the control group.

LPS indicates lipopolysaccharide.

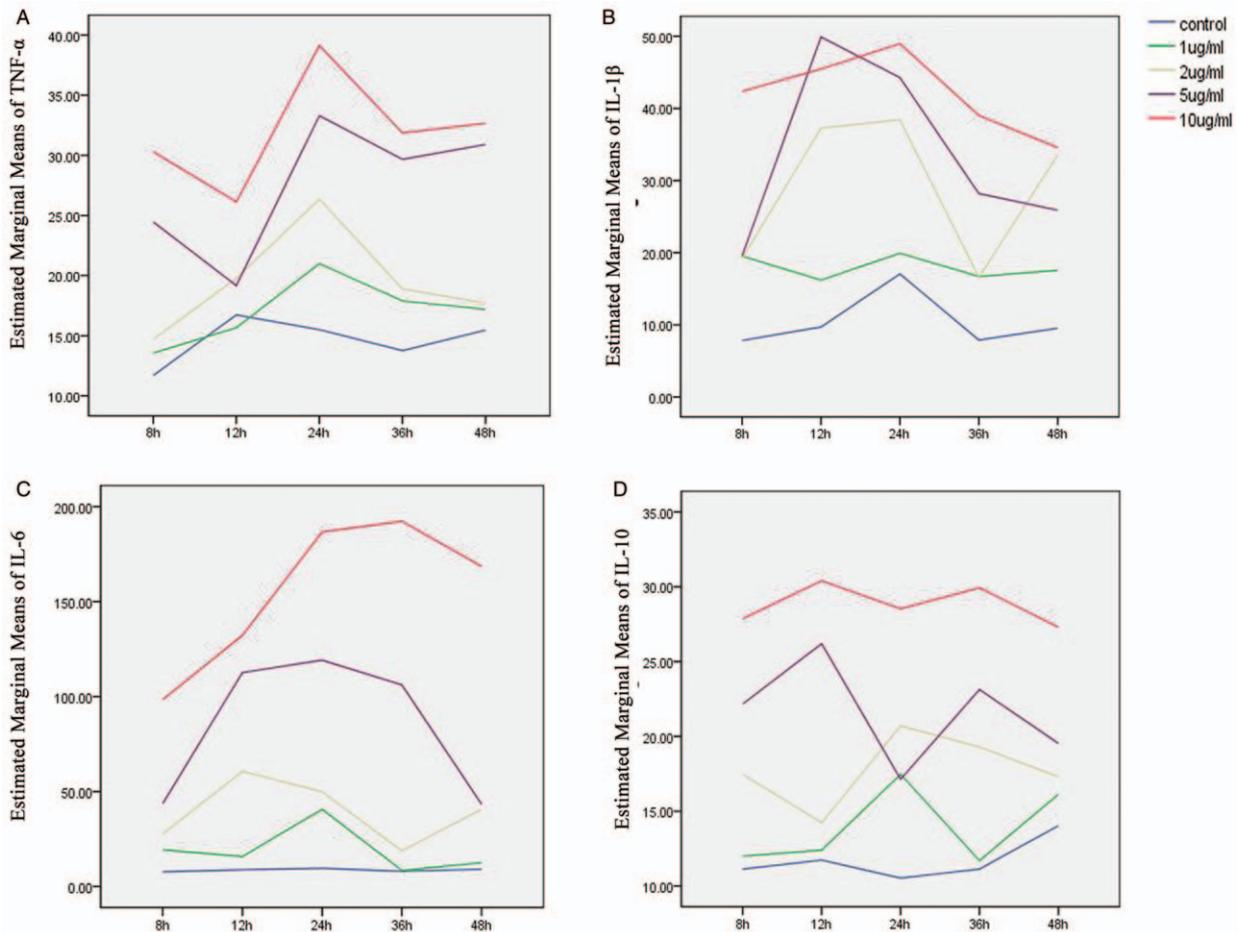


Fig. 1. The secretion of cytokines induced by LPS is in approximately in a dose- and time-dependent manner. The method of two-way ANOVA further analyzes the levels of cytokines. The increasing of cytokines was approximately in a dose- and time-dependent manner. LPS with 5 ug/mL and 10 ug/mL significantly stimulated the secretion of TNF- α , IL-1 β , and IL-6, with the highest increase at 12 to 24 h. We used the method of two-way ANOVA to analyze the data. LPS indicates lipopolysaccharide.

data were compared using one-way ANOVA. The method of two-way ANOVA further analyzes the data. As shown in Figure 1, the increasing of cytokines was approximately in a dose- and time-dependent manner. IL-10 is a pleiotropic cytokine, which can inhibit the inflammatory response. The level of IL-10 at the point of 24 h was at its minimum level. The concentrations of LPS of 5 ug/mL and 10 ug/mL significantly stimulated the secretion of TNF- α , IL-1 β , and IL-6 compared with the control group ($P < 0.05$), with the highest increase at 12 to 24 h. Therefore, LPS (5 ug/mL) treating for 24 h was used to establish the experiment model.

LPS activates the apoptosis and inflammatory pathway, while inhibits the prosurvival signals of H9C2 cardiomyocytes

The caspase-3 plays an important role in the apoptotic pathological process. Western blot was used to determinate the levels of caspase-3, AKT, and ERK1/2 after LPS treating. As shown in Figure 2, the expression of procaspase-3 and cleaved caspase-3 increased from 12 h, while ERK1/2 decreased with the corresponding lowest level at 12 h ($P < 0.05$). AKT was inhibited by LPS ($P < 0.05$) and

reached the minimum expression at 8 h. Furthermore, the expression of P-I κ B α in the LPS group was higher than that in the control group, reaching a maximum at 24 h ($P < 0.05$). The mean values were compared using one-way ANOVA. LPS not only activated the inflammatory and apoptosis pathways, but also inhibited the prosurvival signals of cardiomyocytes.

FTY720 reduces the secretion of TNF- α and IL-6 via the NF- κ B pathway

The changes of cytokine secretion after FTY720 intervening were measured by ELISA. The results showed that the levels of TNF- α and IL-6 in the LPS group were higher compared with those in the control group ($P < 0.05$). FTY720 at the concentrations of 50 nM, 100 nM, and 200 nM decreased the LPS-induced secretion of TNF- α (29.8 ± 2.4 , 27.4 ± 2.9 , 28.1 ± 1.8 vs. 40.8 ± 3.5) and IL-6 (96.5 ± 17.7 , 71.0 ± 8.3 , 56.1 ± 7.2 vs. 146.0 ± 13.7) ($P < 0.05$). However, the changes were not so obvious of IL-1 β and IL-10. In addition, FTY720 (50 nM, 100 nM, 200 nM) attenuated the expression of P-I κ B α by approximately 50%, 125%, and 91% respectively ($P < 0.05$). All data were compared using one-way ANOVA.

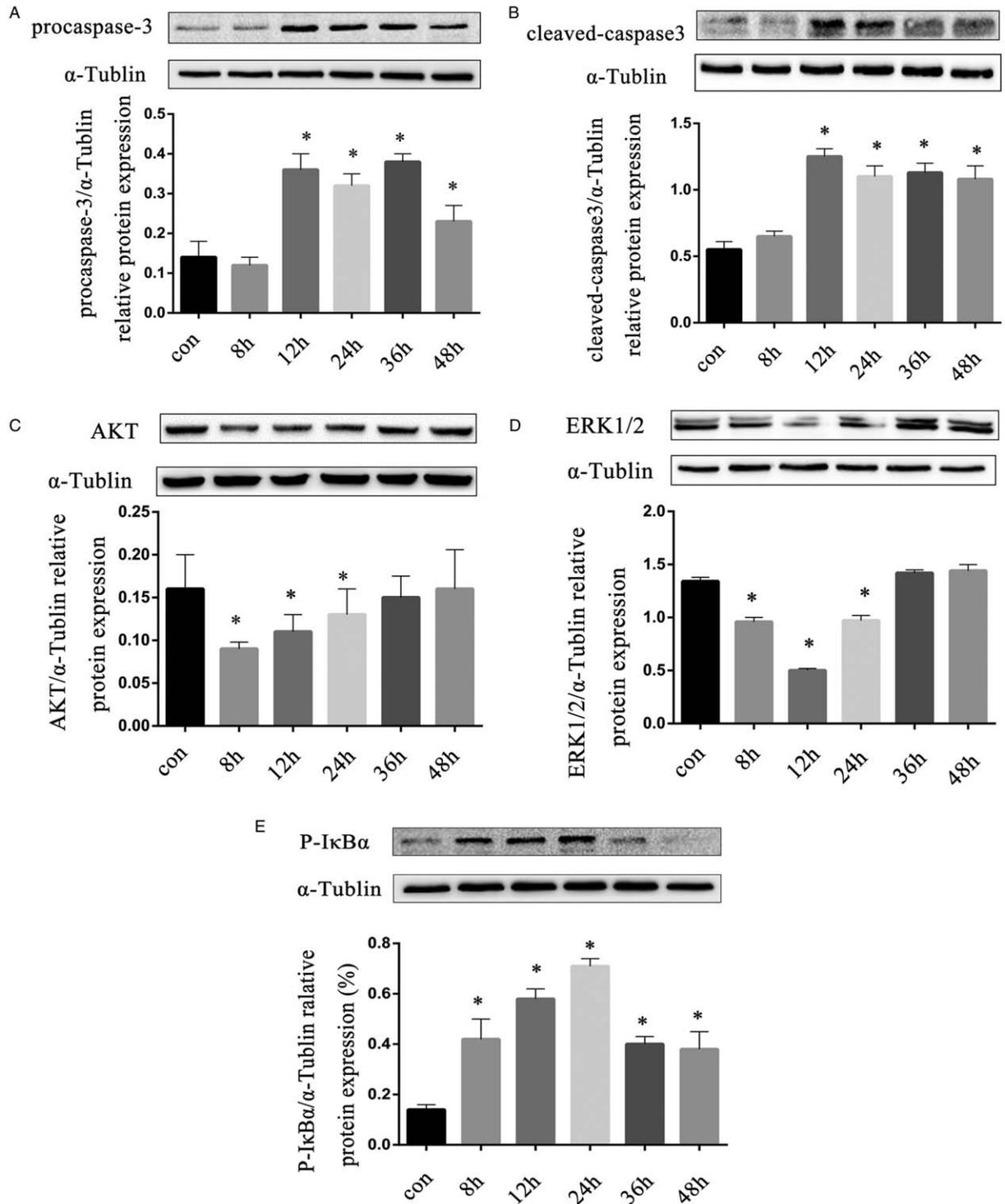


FIG. 2. LPS activates the apoptosis and inflammatory pathways, while it inhibits the prosurvival signal of H9C2 cardiomyocytes. Cardiomyocytes were treated with LPS (5 ug/mL) for different time intervals. Western blot analysis of the expression of caspase-3, AKT, and ERK 1/2 proteins. A and B, LPS-induced caspase-3 activation. C and D, The expression of AKT and ERK1/2 was inhibited by LPS stimulation. E, The expression of P-IκBα was significantly increased. Equal gel loading was confirmed using an antibody against α-tubulin. The data are expressed as the means ± SD of three independent experiments. One-way ANOVA was used to analyze the data. **P* < 0.05 compared with the con group. LPS indicates lipopolysaccharide.

FTY720 inactivates caspase-3, while it oppositely activates the AKT and ERK1/2 pathways of endotoxic cardiomyocytes

The levels of caspase-3, ERK1/2, and AKT in the cells exposed to LPS changed significantly compared with those in the control group (*P* < 0.05). FTY720 at the concentration of

100 nM maximally reduced expression of the procaspase-3 protein by approximately 2.2-fold and cleaved caspase-3 by approximately 1.6-fold, while it increased the expression of AKT and ERK1/2 both in a dose-dependent manner. The mean values were compared using one-way ANOVA.

FTY720 activates AKT and ERK 1/2 through S1P1 and S1P3 receptors in H9C2 cardiomyocytes

The results showed that PI3-kinase inhibitor Wortmannin restricted FTY720-mediated AKT expression by approximately 82% ($P < 0.05$). ERK 1/2 was inhibited by PD98059, an ERK kinase inhibitor by approximately 30% ($P < 0.05$). Furthermore, VPC23019, which was a selective S1P1 and S1P3 receptor antagonist, inhibited the protein expressions of both AKT and ERK1/2 ($P < 0.05$). The data were all compared using one-way ANOVA. The results further suggested that FTY720 stimulated AKT and ERK activation through the S1P1 and S1P3 receptors.

Cell proliferation

The MTT method was used to evaluate cell proliferation in each group. The result revealed that FTY720 significantly increased cell proliferation by approximately 4.3% ($P < 0.05$). The antagonists VPC23019, PD98059, and Wortmannin could

inhibit cell proliferation by approximately 5%, 3.7%, 3.7%, respectively ($P < 0.05$). The method of one-way ANOVA was used to analyze the data.

DISCUSSION

The major findings of this study are that the levels of TNF- α and IL-6 decrease via the NF- κ B pathway of endotoxic cardiomyocytes, which are treated with FTY720 (Fig. 3). FTY720 can inhibit the protein expression of caspase-3, while activate both AKT and ERK 1/2 of endotoxic cardiomyocytes (Fig. 4). Furthermore, we find that the prosurvival signals can be inhibited by the selective S1P1 and S1P3 receptor antagonist VPC23019 as shown in Figure 5. Thus, we conclude that FTY720 exerts a protective effect on endotoxic cardiomyocytes, involving the S1P1 and S1P3 receptors in this process.

Sepsis, which is a dysregulated host response to infection, is mainly caused by gram-negative bacteria. Its pathophysiologic

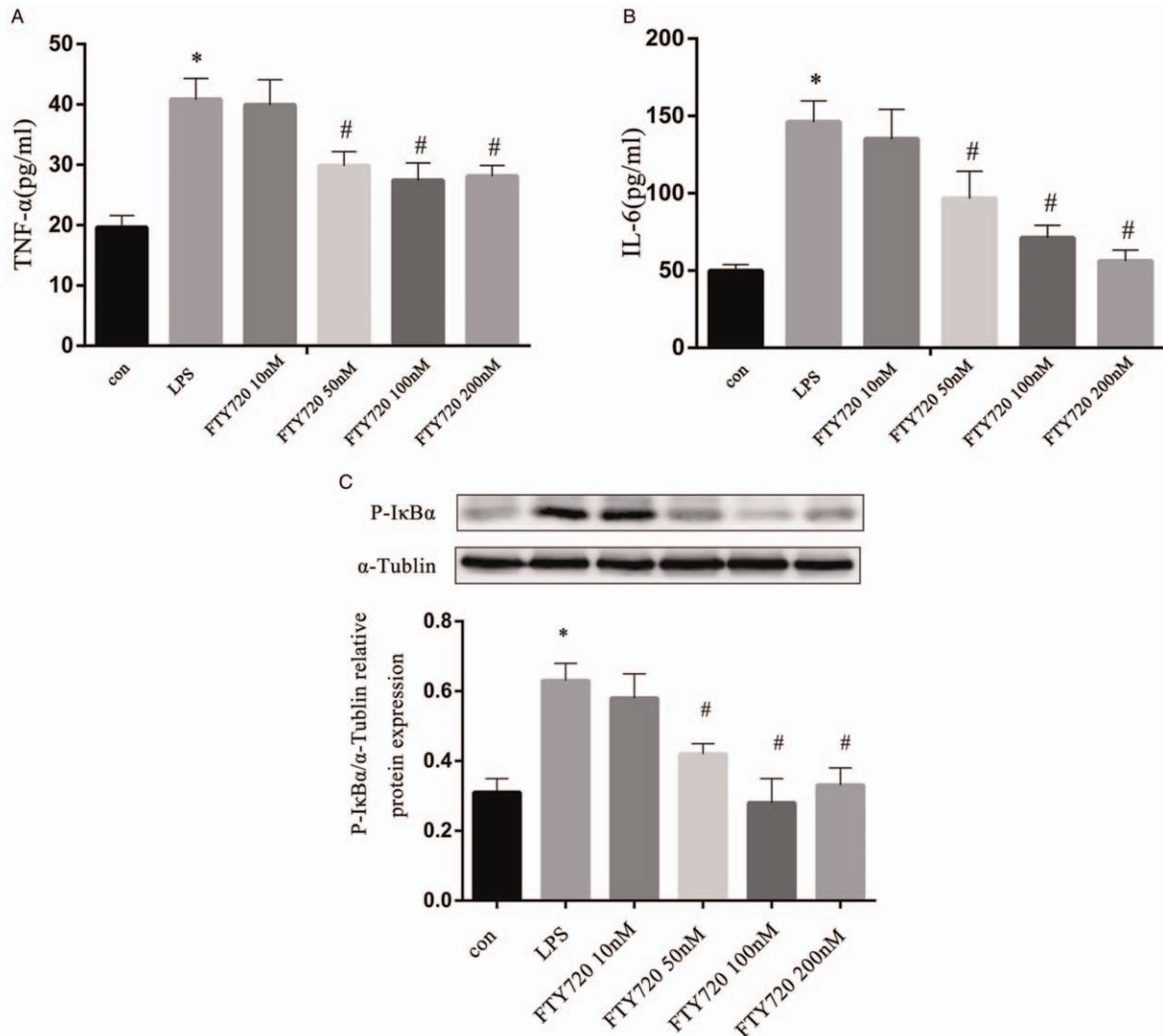


FIG. 3. FTY720 reduces the secretion of TNF- α and IL-6 via the NF- κ B pathway. The secretion of inflammatory cytokines such as TNF- α and IL-6 was inhibited by FTY720. Different concentrations of FTY720 (50 nM–200 nM) could decrease the expression of P-I κ B α . The data are expressed as the means \pm SD of three independent experiments. The method of pairwise comparison in one-way ANOVA was used. * $P < 0.05$ compared with the con group. # $P < 0.05$ compared with the LPS group. LPS indicates lipopolysaccharide.

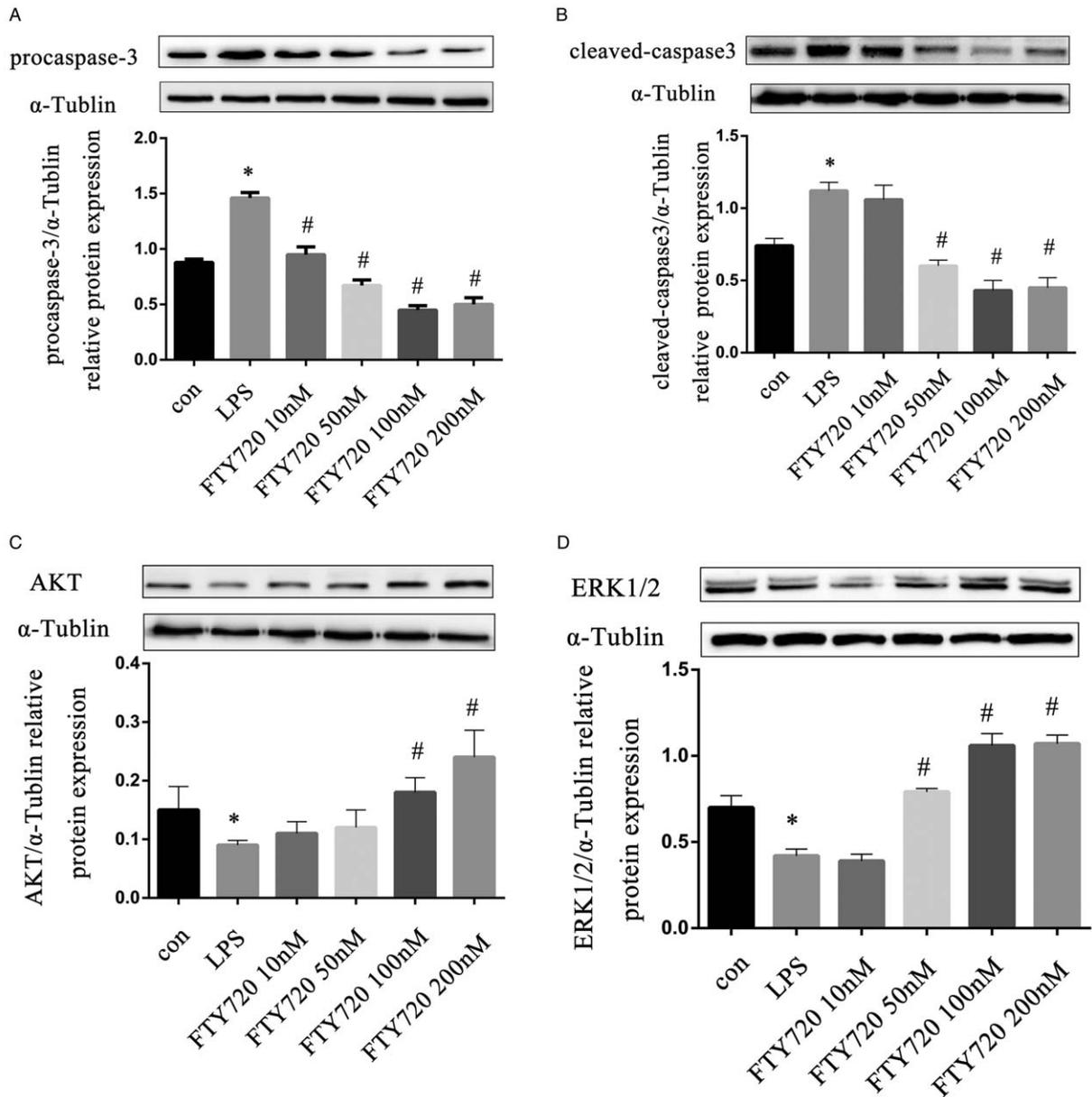


FIG. 4. **FTY720 inactivates caspase-3, while it oppositely activates the AKT and ERK1/2 pathways of LPS-induced cardiomyocytes.** The effects of FTY720 on the expression of caspase-3, AKT, and ERK 1/2. A and B, Caspase-3 activation by LPS was inhibited by FTY720. C and D, FTY720 induced a dose-dependent increase in the expression of AKT and ERK 1/2. Equal gel loading was confirmed using an antibody against α-tubulin. The data are expressed as the means ± SD of 3 independent experiments. The method of pairwise comparison in one-way ANOVA was used. *P* < 0.05 compared with the con group. #*P* < 0.05 compared with the LPS group. LPS indicates lipopolysaccharide.

mechanisms consist of a wide array of derangements that include exaggerated systemic inflammation, impaired micro-circulation, and tissue hypoperfusion leading to the development of multi-organ dysfunction and death (12). Inflammatory cytokines play important roles in this physiopathological process (1). LPS is a major component of gram-negative bacteria and mainly causes endotoxemia of the patients. Numerous studies have shown that the administration of LPS could bring about an inflammatory reaction that involved Toll-like receptors (13). Myocardial dysfunction was a common complication of sepsis due to pro-inflammatory cytokines. The patients with sepsis and multi-organ dysfunction had a higher proportion of

cardiac arrest, and their prognosis is worse (14). To date, most studies have taken the systemic inflammatory response as research objects. However, some studies have shown that the levels of plasma cytokines were not consistent with clinical outcome (15). How does LPS influence the autocrine of cardiomyocytes? Cardiomyocytes also express many phenotypic identification receptors such as CD14 and TLR4. The experiments presented here also demonstrated that LPS can induce cardiomyocytes to secrete inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-10 approximately in a dose- and time-dependent manner (Table 1 and Fig. 1). The expression of P-IκBα is also upregulated (Fig. 2E). Therefore,

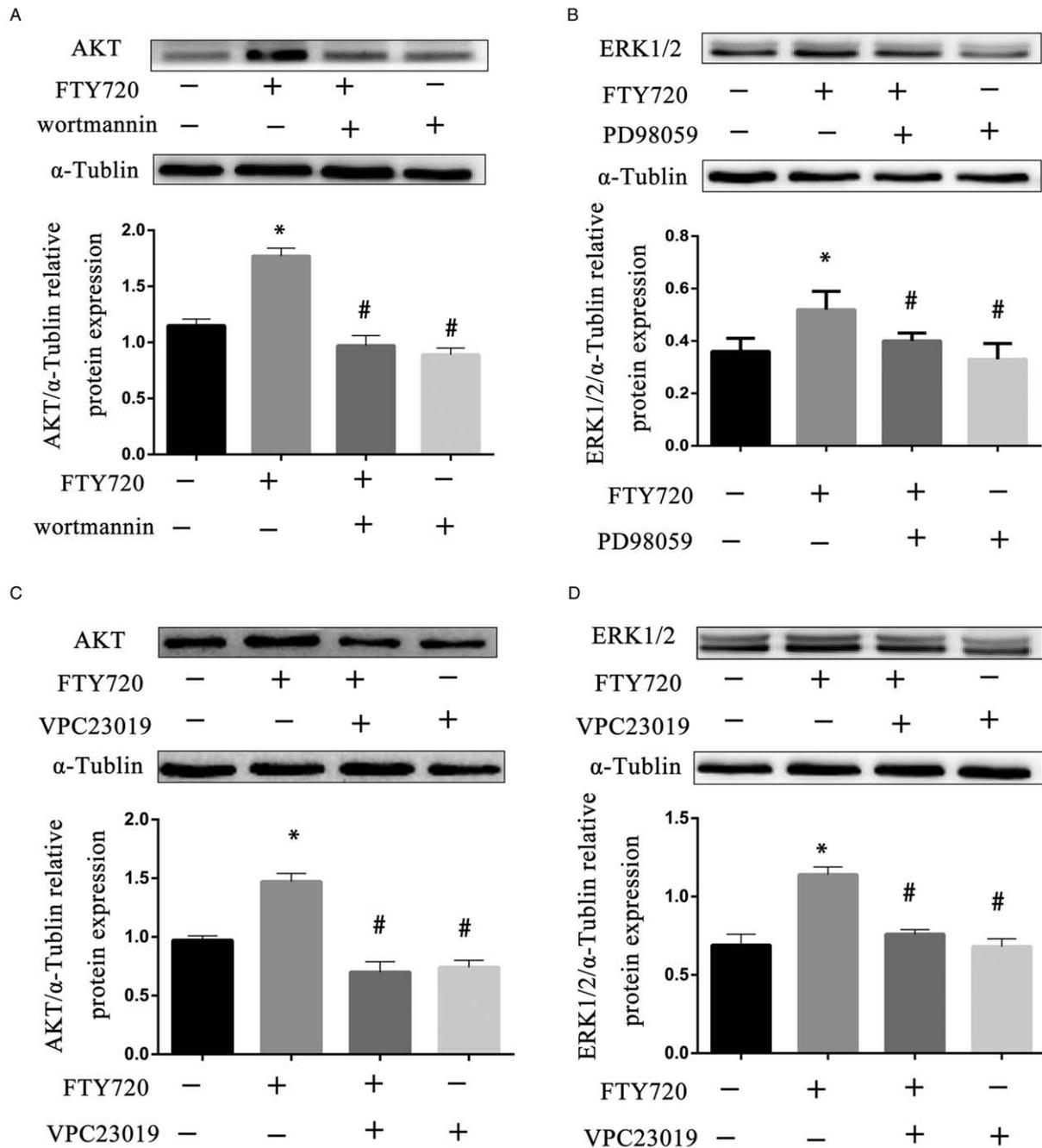


FIG. 5. **FTY720 activates AKT and ERK 1/2 through S1P1 and S1P3 receptors in H9C2 cardiomyocytes.** Cardiomyocytes were treated or not with the S1P1 and S1P3 receptor antagonist VPC (A, C), the ERK inhibitor PD (B), or the AKT inhibitor Wortmannin (D). The expression of ERK1/2 and AKT was significantly reduced by PD, VPC, and Wortmannin. The data were analyzed by one-way ANOVA.

we drew the conclusion that LPS stimulation can result in not only the activation of systemic inflammation but also the local inflammation of cardiomyocytes. The NF- κ B pathway is activated. The number of cardiomyocytes relates to cardiac function. Caspase-3 is an important and well-known protein in regulating cell apoptosis. Pro-inflammatory cytokines such as TNF- α could activate caspase-3 and induce cell apoptosis. Wang et al. (11) discovered that the upregulated expression of proteins such as TNF- α and IL-1 β accompanied with the activation of caspase-3, which occurred in cardiomyocytes

subjected to H/R injury. In this experiment, LPS activates the apoptotic pathway of caspase-3, meanwhile inactivates the prosurvival pathways of AKT and ERK1/2 (Fig. 2, A–D).

S1P is a bioactive lipid mediator that is widely expressed in the circulatory system (4). FTY720 is a S1P receptor agonist, and its structure is similar to sphingosine. Several studies have shown that S1P/FTY720 participated in the inflammatory response. Based on the result of reduced S1P levels in serum or plasma of sepsis patients, Winkler et al. (16) illustrated that S1P could restore endothelial integrity, dampen the

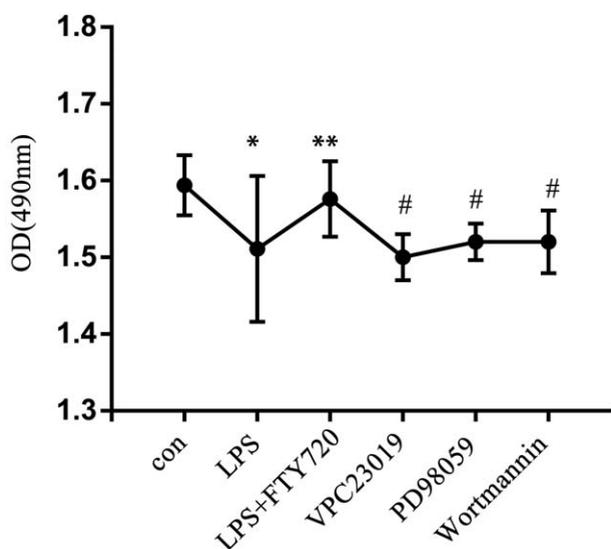


FIG. 6. **Cell proliferation.** Cell proliferation was measured using MTT. The inhibition of cell proliferation by LPS was reversed by FTY720. The OD values were decreased by VPC, PD, and Wortmannin. The method of pairwise comparison in one-way ANOVA was used. * $P < 0.05$ compared with the con group. ** $P < 0.05$ compared with the LPS group. # $P < 0.05$ compared with the LPS+FTY720 group. LPS indicates lipopolysaccharide.

inflammatory host response, and improve organ function in sepsis. The short-term use of S1P1 agonists may be therapeutic in influenza through suppressing the cytokine storm and possibly in other acute respiratory diseases (17). Mice that were engineered to lack plasma S1P showed vascular leakage, which was reversed by an S1P1 agonist (18). S1P alleviated vascular leakage and inflammatory cell infiltration in LPS-induced lung injury in mice, which was consistent with the result by Camerer et al. (5, 10). Concerning the association between S1P/FTY720 and cardiovascular system, many studies have shown a cardioprotection effect of S1P. S1P could protect cardiomyocytes against hypoxia/reoxygenation injury through resisting apoptosis (11). S1P treatment was beneficial and decreased the infarct size after myocardial infarction through the PI3K/AKT pathway (19). In view of these reports, we explored the role of FTY720 on endotoxigenic cardiomyocytes and the potential mechanism (Fig. 6).

In our study, the H9C2 cells were treated with different concentrations of FTY720. The ELISA method was used to determine the change of cytokine levels. The data showed that FTY720 could reduce the levels of TNF- α , IL-6 and the protein expression of P-I κ B α as shown in Figure 3, which suggested the suppressive effect on the inflammation of FTY720 and the NF- κ B signal pathway involved. The expression of caspase-3 was inhibited, which implied an inhibitory effect of apoptosis. FTY720 inactivated apoptotic signaling pathway of caspase-3 and activated prosurvival signaling of ERK1/2 and AKT (Fig. 4), which were widely accepted of FTY720 to promote the growth and survival of cells. The prosurvival effect was supported by the result of the MTT assay.

A more extensive application of specific S1P receptor agonists makes it possible to further investigate the S1P receptors.

The receptors of S1P1 and S1P3 are widely expressed on the myocardium cell membrane. S1P1 was the receptor that enhanced the survival of cardiomyocytes during hypoxia (20). Consistent with this finding, the use of S1P1 specific agonist SEW2871 showed the importance of S1P1 in cardioprotection. Theilmeyer et al. (6) demonstrated that S1P protected the heart against I/R injury through regulating PI3K/AKT pathway via the S1P3. However, another researcher demonstrated that S1P2 also played an important role on cardiomyocytes during I/R injury (21). In this experiment, our results showed that FTY720 activated ERK1/2 and AKT through S1P1 and S1P3 receptors, which was proved by a selective S1P1 and S1P3 receptor antagonist VPC23019 (Fig. 5). FTY720 does not interact with S1P2. Therefore, the role of S1P2 remains uncertain.

Overall, our studies have demonstrated a protective role of S1P in endotoxigenic cardiomyocytes cultured *in vitro*. The local inflammation of cardiomyocytes is an important part of the systemic inflammatory response. S1P decreases the levels of TNF- α and IL-6 by an autocrine mechanism in H9C2. This result partly answers the question as to why the levels of plasma cytokines were not consistent with the clinical outcome. This study may offer new strategies to alleviate the cardiovascular dysfunction in sepsis.

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