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Disruption of phospholipase Cγ1 signalling attenuates cardiac tumor necrosis factor-α expression and improves myocardial function during endotoxemia

Tianqing Peng[1,2,3]*, E Shen[1,2], Jue Fan[1], Yan Zhang[1], J. Malcolm O. Arnold[2,4], and Qingping Feng[1,2,4]*

[1]*Center for Critical Illness Research, Lawson Health Research Institute, VRL 6th Floor, 800 Commissioners Road, London, Ontario, Canada N6A 4G5;* [2]*Department of Medicine;* [3]*Department of Pathology, and* [4]*Department of Physiology and Pharmacology, University of Western Ontario, London, Ontario, Canada N6A 4G5*

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Aims Lipopolysaccharide (LPS) induces tumor necrosis factor-α (TNF-α) expression in cardiomyocytes, which contributes to myocardial dysfunction during sepsis. The purpose of this study was to investigate the role of phosphatidylinositol (PI) phospholipase Cγ1 (PLCγ1) in cardiac TNF-α expression, and myocardial dysfunction during endotoxemia.

Methods and results In cultured mouse neonatal cardiomyocytes, LPS increased PLCγ1 phosphorylation. Knockdown of PLCγ1 with specific siRNA or inhibition of PLCγ1 with U73122 attenuated TNF-α expression induced by LPS. This action of PLCγ1 was mediated through inositol-1,4,5-trisphosphate (IP3)/IP3 receptor (IP3R) pathways since blocking either IP3 or IP3R decreased LPS-induced TNF-α expression. In contrast, neither diacylglycerol agonist nor antagonist had any evident effect on LPS-induced TNF-α expression in cardiomyocytes. To investigate the role of PLCγ1 in endotoxemia *in vivo*, wild-type and heterozygous PLCγ1 knockout ($PLC\gamma1^{+/-}$) mice were pre-treated with either U73122, or its inactive analog U73343, or vehicle for 15 min, followed by LPS for 4 h. Inhibition of PLCγ1 by U73122 or by heterozygous deletion of the PLCγ1 gene decreased cardiac TNF-α expression. More importantly, LPS-induced myocardial dysfunction was also attenuated in $PLC\gamma1^{+/-}$ mice or by U73122 treatment.

Conclusion PLCγ1 signalling induces cardiac TNF-α expression and myocardial dysfunction during LPS stimulation. The action of PLCγ1 on TNF-α expression is mediated through IP3/IP3R pathways. The present results suggest that PLCγ1 may be a potential therapeutic target for myocardial dysfunction in sepsis.

1. Introduction

Sepsis and subsequent multiple organ failure remain the major cause of morbidity and mortality in intensive care units.[1] The heart is one of the most common organs adversely affected by sepsis and myocardial dysfunction puts septic patients at high risk of developing multi-organ failure, which is associated with a high mortality.[2,3] Endotoxins or lipopolysaccharides (LPS) of gram-negative bacteria are important pathogens responsible for myocardial dysfunction during sepsis.[4,5] LPS via toll-like receptor-4 induces production of pro-inflammatory cytokines,[6] among which tumor necrosis factor-alpha (TNF-α) has been suggested to be one of the major factors responsible for myocardial depression during sepsis.[4,7] In response to LPS, cardiomyocytes produce TNF-α, which may be an important local source of TNF-α production in heart. High levels of TNF-α production mediate contractile dysfunction in intact animals, isolated hearts and cardiomyocytes during sepsis.[8–10] Thus, understanding regulation of cardiac TNF-α expression may point to novel therapeutic strategies to treat or prevent myocardial dysfunction in sepsis. We have recently demonstrated that activation of NAD(P)H oxidaze-mediated mitogen-activated protein kinase (MAPK) is required for LPS-induced TNF-α expression in cardiomyocytes and blocking either NAD(P)H oxidaze or MAPK pathway improves myocardial function during endotoxemia.[7,11,12] However, molecular mechanisms of LPS-induced TNF-α expression in cardiomyocytes are still not fully understood.

Phospholipase C (PLC) is central to the core machinery for the phosphoinositide signal transduction pathway.[13,14] Phosphatidylinositol (PI)-phospholipase C (PI-PLC) comprises

* Corresponding authors. Tel: +1 519 685 8300 Ext. 55441; fax: +1 519 685 8341. *E-mail address*: tpeng2@uwo.ca; qfeng@uwo.ca

a family of four classes including PLCβ, γ, δ, and ϵ, encoded by different genes.[13] Each class includes several isozymes: four isozymes of PLCβ, two isozymes of PLCγ, and four isozymes of PLCδ. Activation of PI-PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to produce two important intracellular second messengers, inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). These intracellular lipid signalling molecules modulate various cellular responses. It has been shown that inhibition of PLC by their general inhibitors decreases TNF-α expression in macrophages and keratinocytes during LPS stimulation,[15,16] suggesting a role of PLC. However, a causal role of PI-PLC in cardiomyocyte TNF-α expression and myocardial dysfunction remains to be determined in sepsis. In addition, the contribution of distinct isoform of PLC to cardiac dysfunction in sepsis has not been demonstrated since cardiomyocytes express at least three isoforms: PLCβ, PLCγ, and PLCδ.[17]

PLCγ is a major substrate for tyrosine kinases.[13,14] Studies have shown that inhibition of tyrosine kinases blocks LPS-induced TNF-α expression.[18] These data suggest that PLCγ may play a role in LPS-induced TNF-α expression in cardiomyocytes. Since PLCγ1 is the predominant isoform expressed in cardiomyocytes,[19] the present study is focused on the contribution of PLCγ1 isoform in sepsis. We demonstrated that PLCγ1 signalling regulates LPS-stimulated TNF-α expression in cardiomyocytes and that PLCγ1 plays an important role in myocardial depression during endotoxemia.

2. Methods

2.1 Animals

Wild-type C57BL/6 mice were purchased from the Jackson Laboratory. Heterozygous PLCγ1 knockout ($PLC\gamma1^{+/-}$) mice were generously provided by Dr Demin Wang (The Blood Research Institute, The Blood Center of Southeastern Wisconsin, Milwaukee, WI, USA). Genotyping of $PLC\gamma1^{+/-}$ mice was conducted by using polymerase chain reaction (PCR) as described previously.[20] All animals were provided water and food *ad libitum* and housed in a temperature and humidity controlled facility with 12 h light and dark cycles. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). Experimental protocols were approved by the Animal Use Subcommittee at the University of Western Ontario.

2.2 Neonatal mouse cardiomyocyte culture

Isolation and cultures of neonatal mouse cardiomyocytes were conduced as described previously.[7,11,12] After 48 h of culture, treatments with various drugs were performed.

2.3 Reagents

LPS (*Salmonella typhosa*), U73122, U73343, heparin, dantrolene, 1-hexadecyl-2-acetyl glycerol (HAG) and 1-oleoyl-2-acetyl-sn-glycerol (OAG) were purchased from Sigma or Calbiochem.

2.4 Phospholipase Cγ1 knockdown using small interfering RNA

In order to knock down PLCγ1 expression, a small interfering RNA (siRNA) against PLCγ1 was obtained (Santa Cruz Biotechnology, Santa Cruz, CA) and a scramble siRNA was employed as control. Transfection was performed using TransMessenger Transfection Reagent (Qiagen) according to manufacturer's protocol as described in our previous studies.[11,21]

2.5 Plasmids and transfection

Transfections were performed using Lipofectamine™ Transfection Reagent (Invitrogen) following the manufacturer's instruction. Cells were transfected with GFP-IP3R-(224–605) or pEGFP-C1 (null vector). GFP-IP3R-(224–605) was from Dr Tamas Balla (National Institutes of Health, MD, USA).

2.6 Measurement of tumor necrosis factor-α protein levels

TNF-α release into the media was measured using an ELISA kit (ALPCO Diagnostics) for mouse TNF-α as described in our previous reports.[7,11,12]

2.7 Analysis of tumor necrosis factor-α mRNA by real-time reverse-transcriptase polymerase chain reaction

Total RNA was extracted from cardiomyocytes using the Trizol Reagent (Gibco-BRL) following the manufacturer's instruction. Real-time reverse-transcriptase PCR (RT–PCR) for TNF-α and GAPDH was performed using the same primers as previously described.[11,21,22]

2.8 Western blot analysis

Expression of PLCγ1 and GAPDH protein was determined by western blot analysis using specific antibodies against PLCγ1 and GAPDH (cell signalling, 1/1000), respectively.

2.9 Measurement of phospholipase Cγ1 phosphorylation

Assessment of the phosphorylation of PLCγ1 (Tyr783) was accomplished by western blotting analysis using antibodies against phospho-PLCγ1 (cell signalling, 1/1000), respectively, as described previously.[11,21,22]

2.10 Endotoxemia and Isolated Mouse Heart Preparation

Adult mice (male, aged 2–3 months) were treated with LPS (4 mg/kg, i.p.) to induce endotoxemia. After 4 h of LPS treatment, mouse hearts were isolated and perfused in a Langendorff system to measure cardiac function as we previously described.[11]

2.11 Statistical analysis

All data were given as mean ± SD or SEM from at least three independent experiments. Measurements for all *in vitro* experiments were made in duplicate or triplicate. Differences between two groups were compared by unpaired Student's *t*-test. For multi-group comparisons, ANOVA followed by Student–Newman–Keuls test was performed. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Contribution of phospholipase Cγ1 to tumor necrosis factor-α expression in lipopolysaccharide-stimulated cardiomyocytes

Our recent study and others have shown that LPS induces PLCγ1 activation in cardiomyocytes.[22,23] In the present study, we also showed that LPS treatment elevated the levels of PLCγ1 phosphorylation (*Figure 1A*). These data support that LPS increases PLCγ1 activation in cardiomyocytes.

To investigate the role of PLCγ1 in TNF-α expression in LPS-stimulated cardiomyocytes, we used siRNA to specifically down-regulate PLCγ1 expression. Cardiomyocytes were transfected with either PLCγ1 siRNA or scramble siRNA as control. Twenty-four hours after transfection, cardiomyocytes were incubated with LPS for 4 h and the efficacy of siRNA on PLCγ1 protein and TNF-α mRNA were analysed. Transfection of PLCγ1 siRNA decreased PLCγ1 protein in cardiomyocytes, compared with a scramble siRNA (*Figure 1B*), suggesting that the PLCγ1 siRNA achieved a successful knockdown. In response to LPS, TNF-α mRNA was decreased by 61% in PLCγ1 siRNA-transfected compared with scramble siRNA-transfected cardiomyocytes (*Figure 1C*). These data suggest that PLCγ1 signalling induces TNF-α expression in LPS-stimulated cardiomyocytes.

The contribution of PLCγ1 to TNF-α expression in response to LPS was also examined by using a selective pharmacological inhibitor, U73122. An inactive analogue, U73343 was used as a negative control. Cardiomyocytes were pre-treated with either U73122 or U73343 (10 μM) for 20 min, followed by LPS (10 μg/mL) for 4 h. In agreement with a recent study,[24] U73122 treatment decreased PLCγ1 phosphorylation, suggesting an inhibition of PLCγ1 activation (*Figure 2A*). In line with the effect of PLCγ1 siRNA, TNF-α mRNA and protein were significantly decreased in U73122-treated compared with U73343-treated cardiomyocytes (*Figure 2B* and *C*). This result further supports the fact that inhibition of PLCγ1 down-regulates LPS-induced TNF-α expression in cardiomyocytes.

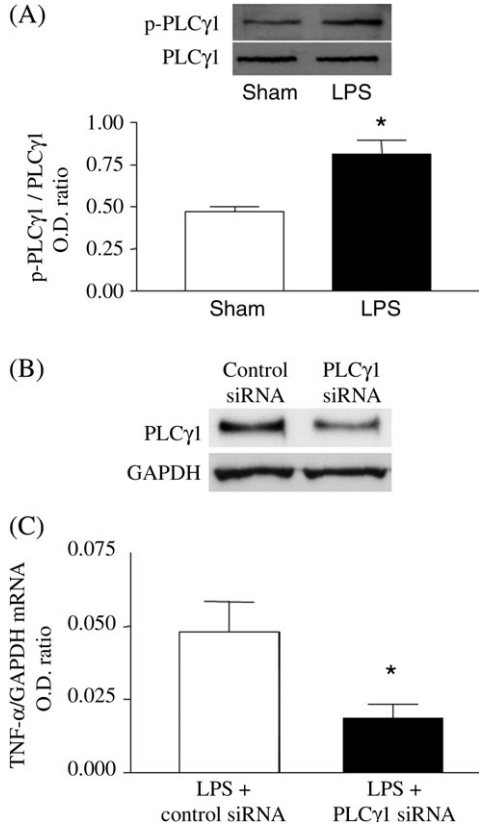

Figure 1 Role of phospholipase Cγ1 in cardiomyocyte tumor necrosis factor-α expressison. (*A*) Cardiomyocytes were treated with either lipopolysaccharide (10 μg/mL) or vehicle. Phospholipase Cγ1 phosphorylation was determined by western blot analysis at 30 min after lipopolysaccharide treatment. A representative blot (upper panel) and quantitative data (lower panel) show a significant increase in phospholipase Cγ1 phosphorylation in cardiomyocytes after lipopolysaccharide treatment. (*B*), (*C*) After transfection with phospholipase Cγ1 small interfering RNA or a scramble small interfering RNA, cardiomyocytes were incubated with either vehicle or lipopolysaccharide (10 μg/mL) for 4 h. The levels of phospholipase Cγ1 protein and tumor necrosis factor-α mRNA were determined, respectively. (*B*) is a representative western blot for PLCγ1 protein expression from three different experiments. C shows down-regulation of tumor necrosis factor-α mRNA expression after PLCγ1 knockdown in LPS-stimulated cardiomyocytes. Data are mean ± SD from three independent experiments. *$P < 0.05$ vs. Sham or lipopolysaccharide + control small interfering RNA.

3.2 Role of IP3/IP3R in cardiomyocyte tumor necrosis factor-α expression during lipopolysaccharide stimulation

Activation of PLCγ1 produces IP3 and DAG, both of which trigger the down-stream signalling pathways. To investigate the down-stream pathways which mediate the role of PLCγ1 in LPS-induced TNF-α expression, we first used HAG, a DAG antagonist to block and OAG, a DAG agonist to mimic DAG. As shown in *Figure 3A*, neither pre-treatment with HAG (∼100 μM) nor OAG (∼50 μM) changed basal or LPS-stimulated TNF-α expression in cardiomyocytes. This result suggests that DAG is not involved in PLCγ1-mediated TNF-α expression during LPS stimulation. We then examined

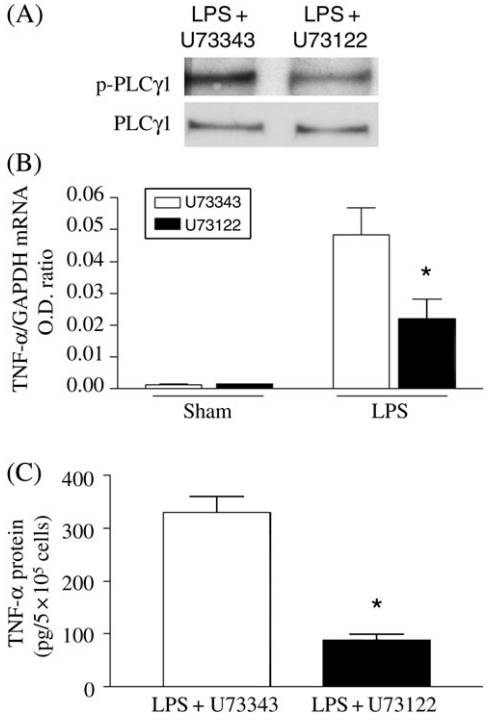

Figure 2 Effects of U73122 on phospholipase Cγ1 phosphorylation and tumor necrosis factor-α expression in lipopolysaccharide-stimulated cardiomyocytes. Cardiomyocytes were pre-incubated with either U73122 or U73343 (10 μM) for 20 min, followed by lipopolysaccharide (10 μg/mL) for 4 h. Phospholipase Cγ1 phosphorylation (Tyr783) and tumor necrosis factor-α expression were measured, respectively. (*A*) A representative blot from three independent experiments shows a decrease in lipopolysaccharide-induced PLCγ1 phosphorylation after U73122 treatment. (*B*), (*C*) The levels of tumor necrosis factor-α mRNA (*B*) and protein (*C*) are attenuated in U73122-treated cardiomyocytes during lipopolysaccharide stimulation. Data are means ± SD of 3–4 independent experiments (*$P < 0.05$ vs. lipopolysaccharide + U73343).

the role of the IP3-dependent pathway in LPS-induced TNF-α expression. Recent studies have shown that cellular IP3 can be modulated by over-expression of the exogenous ligand-binding domain of its receptor.[25–27] An intensely investigated IP3-binding module is the IP3-binding domain of the IP3R. The N-terminal cytoplasmic region of the human type-I IP3R (residues 1–604) binds IP3 with comparable affinity to the full-length IP3R.[28] Removal of residues 1–223 (suppressor region) further increases the affinity for IP3.[29] Thus, the ligand-binding domain of the human type-I IP3R (residues 224–605) behaves as effective mobile cytosolic IP3 buffer and over-expression of this domain attenuates the induced intracellular Ca^{2+} signal in different cells.[25–27] To modulate IP3 signalling, we over-expressed the ligand-binding domain of the human type-I IP3R (residues 224–605) [IP3R-(224–605)] in cardiomyocytes by using a recombinant plasmid GFP-IP3R-(224–605), which contains genes encoding IP3R-(224–605) fused with enhanced green fluorescence protein (EGFP).[27] Cardiomyocytes were transfected with either GFP-IP3R-(224–605) or pEGFP-C1 as control. Twenty-four hours after transfection, expression of GFP-IP3R-(224–605) was verified by RT–PCR for detection of both IP3R-(224–605) and EGFP mRNA in cardiomyocytes (*Figure 3B*). In response to LPS, TNF-α protein was

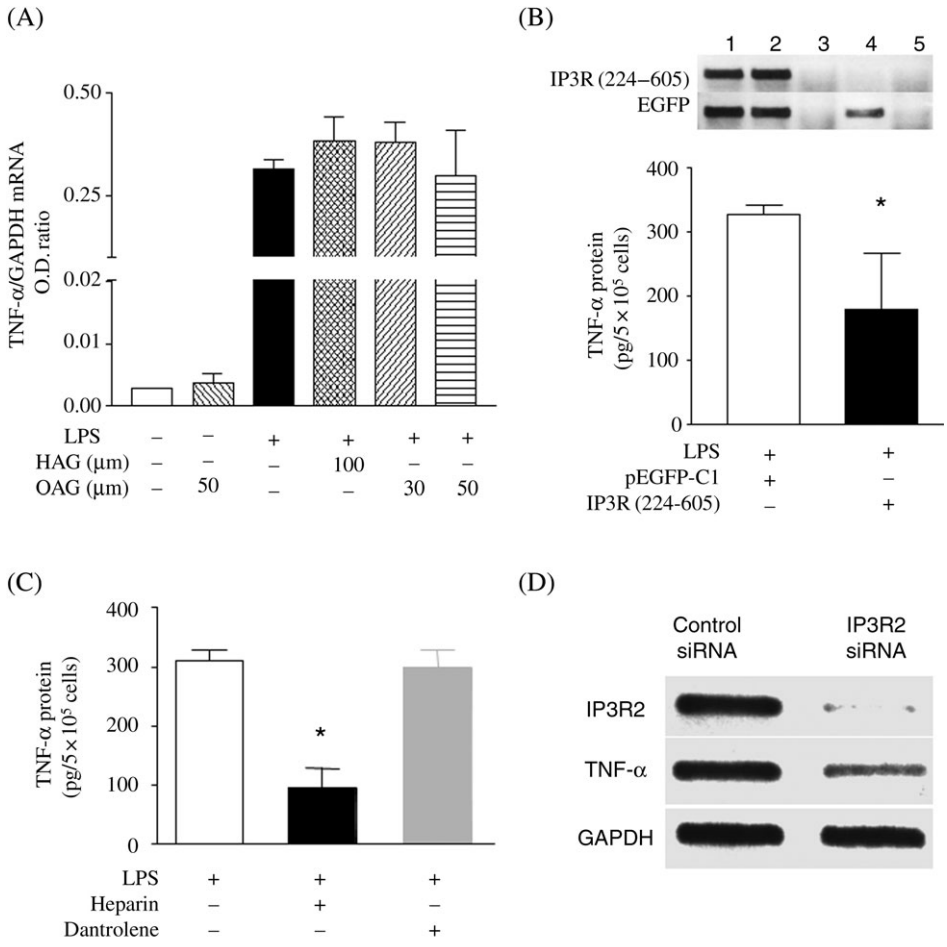

Figure 3 Role of diacylglycerol and inositol-1,4,5-trisphosphate pathways in lipopolysaccharide-stimulated tumor necrosis factor-α expression. (*A*) Cardiomyocytes were pre-treated with either 1-hexadecyl-2-acetyl glycerol (100 μM) or 1-oleoyl-2-acetyl-sn-glycerol (30 and 50 μM) for 20 min, followed by lipopolysaccharide (10 μg/mL) for 4 h. tumor necrosis factor-α mRNA was measured by real-time reverse-transcriptase polymerase chain reaction analysis. Neither 1-hexadecyl-2-acetyl glycerol nor 1-oleoyl-2-acetyl-sn-glycerol treatment changed tumor necrosis factor-α mRNA expression in lipopolysaccharide-stimulated cardiomyocytes. (*B*) (Upper panel) cardiomyocytes were transfected with either GFP-IP3R-(224–605) or pEGFP-C1. Reverse-transcriptase polymerase chain reaction was conducted to detect the expression of EGFP and IP3R-(224–605). A representative reverse-transcriptase polymerase chain reaction amplification for both EGFP and IP3R-(224–605) mRNA is shown from three independent experiments (Lane 1: GFP-IP3R-(224–605) positive control; Lane 2: GFP-IP3R-(224–605) transfected cells; Lane 3: GFP-IP3R-(224–605) transfected cells without RT; Lane 4: pEGFP-C1 transfected cells; Lane 5: pEGFP-C1 transfected cells without RT). (*B*) (Lower panel), 24 h after transfection with either GFP-IP3R-(224–605) or pEGFP-C1, cardiomyocytes were incubated with lipopolysaccharide (10 μg/mL) for 4 h and tumor necrosis factor-α protein was measured. Over-expression of GFP-IP3R-(224–605) significantly decreased lipopolysaccharide-induced tumor necrosis factor-α protein expression in cardiomyocytes. (*C*) Cardiomyocytes were incubated with lipopolysaccharide (10 μg/mL) in the presence of either heparin (50 μM) or dantrolene (20 μM) or vehicle for 4 h. tumor necrosis factor-α protein was measured. Heparin but not dantrolene significantly decreased tumor necrosis factor-α protein expression during lipopolysaccharide stimulation. (*D*) After transfection with IP3R2 small interfering RNA or a scramble small interfering RNA, cardiomyocytes were incubated with lipopolysaccharide (10 μg/mL) for 4 h. The levels of IP3R2 and tumor necrosis factor-α mRNA were determined by reverse-transcriptase polymerase chain reaction. A representative reverse-transcriptase polymerase chain reaction amplification for IP3R2, tumor necrosis factor-α and GAPDH mRNA from 3 independent experiments shows that IP3R2 small interfering RNA attenuated IP3R2 and tumor necrosis factor-α mRNA expression induced by lipopolysaccharide, whereas GAPDH mRNA expression was not altered. Data are means ± SD of three to four independent experiments (*$P < 0.05$ vs. lipopolysaccharide).

significantly attenuated in GFP-IP3R-(224–605)-transfected, compared with pEGFP-C1-transfected cardiomyocytes (*Figure 3B*). The data supports the involvement of IP3 signalling in LPS-induced TNF-α expression. To further demonstrate the contribution of IP3 signalling, we examined the role of IP3Rs in TNF-α expression during LPS stimulation since the IP3 signalling is largely dependent on the binding to its receptors, IP3Rs.[30,31] Cardiomyocytes were incubated with LPS in the presence or absence of an IP3R antagonist, heparin or a ryanodine receptor antagonist, dantrolene for 4 h. Blocking IP3R, but not ryanodine receptor, significantly decreased TNF-α protein expression during LPS stimulation (*Figure 3C*). This result suggests an important role of IP3R in LPS-induced TNF-α expression. To substantiate the results obtained by heparin, we employed siRNA to specifically knock down IP3R in cardiomyocytes. IP3R has three isoforms. Since IP3R2 is the predominant isoform expressed both in ventricular and in atrial myocytes.[32,33] We chose to study IP3R2 isoform in the present study. Transfection of IP3R2 siRNA decreased IP3R2 expression in cardiomyocytes, compared with a scramble siRNA (*Figure 3D*), suggesting that the IP3R2 siRNA achieved a successful knockdown. In response to LPS, TNF-α mRNA was attenuated in IP3R2 siRNA-transfected compared with scramble siRNA-transfected cardiomyocytes (*Figure 3D*). Taken together, our data suggest that IP3/IP3R signalling contributes to cardiomyocyte TNF-α expression during LPS stimulation.

3.3 Role of phospholipase Cγ1 in myocardial tumor necrosis factor-α expression during endotoxemia

To demonstrate the role of PLCγ1 in LPS-induced TNF-α expression in endotoxemia *in vivo*, PLCγ1$^{+/-}$ mice were employed since PLCγ1 null (PLCγ1$^{-/-}$) mice die *in utero*[34] and are not available for the present study. It has been shown that PLCγ1 protein was reduced by ∼50% and the PLCγ1-related signalling was effectively blocked in PLCγ1$^{+/-}$ compared with wild-type mice.[34] To confirm the heterozygous knockout of PLCγ1 protein in PLCγ1$^{+/-}$ hearts, we analysed PLCγ1 protein levels by western blot. As expected, the levels of PLCγ1 protein were reduced by about half in PLCγ1$^{+/-}$ compared with wild-type littermate hearts (data not shown). We then treated PLCγ1$^{+/-}$ and their wild-type littermates with either vehicle or LPS (4 mg/kg, i.p.). Four hours later, we harvested the hearts to examine TNF-α mRNA expression. Basal levels of TNF-α mRNA expression were not significantly altered in PLCγ1$^{+/-}$ compared with wild-type hearts. In response to LPS, TNF-α mRNA levels decreased by 36% in PLCγ1$^{+/-}$ hearts (*Figure 4A*). To further demonstrate the role of PLCγ1 in TNF-α expression, wild-type mice were treated with either U73122 or its inactive analogue U73343 (9 mg/kg, i.p.) for 15 min, followed by LPS (4 mg/kg, i.p.) for 4 h. U73122 significantly attenuated TNF-α mRNA expression by 70% (*Figure 4B*), compared with U73343. Taken together, these results suggest that PLCγ1 signalling induces TNF-α expression in the adult myocardium *in vivo* during LPS stimulation.

3.4 Role of phospholipase Cγ1 in myocardial dysfunction of endotoxemia

To explore the role of PLCγ1 in myocardial depression induced by endotoxemia, PLCγ1$^{+/-}$ mice and their wild-type littermates were given vehicle or LPS (4 mg/kg, i.p.). Four hours later, cardiac function was assessed in isolated hearts to avoid systemic reflex influences. Although there was no change in heart rate, heart work and rate of contraction were significantly reduced in endotoxemic mice compared with sham animals (*Figure 5*), indicating myocardial depression. Deletion of one allele of PLCγ1 restored heart work and rate of contraction and relaxation without affecting heart rate in endotoxemic mice (*Figure 5*). These results demonstrate that PLCγ1 contributes to myocardial dysfunction in endotoxemic mice.

The role of PLCγ1 in myocardial depression induced by endotoxemia was also examined by using a pharmacological inhibitor, U73122. Wild-type mice were pre-treated with U73122 or its inactive analogue U73343 (9 mg/kg, i.p.) for 15 min, followed by vehicle or LPS (4 mg/kg, i.p.) for 4 h. Cardiac function was assessed as above. U73122 had no effect on sham animals, but significantly increased heart work and rate of contraction and relaxation without affecting heart rate in endotoxemic mice, compared with U73343 (*Figure 6*). These data further support the contribution of PLCγ1 to myocardial dysfunction in endotoxemia.

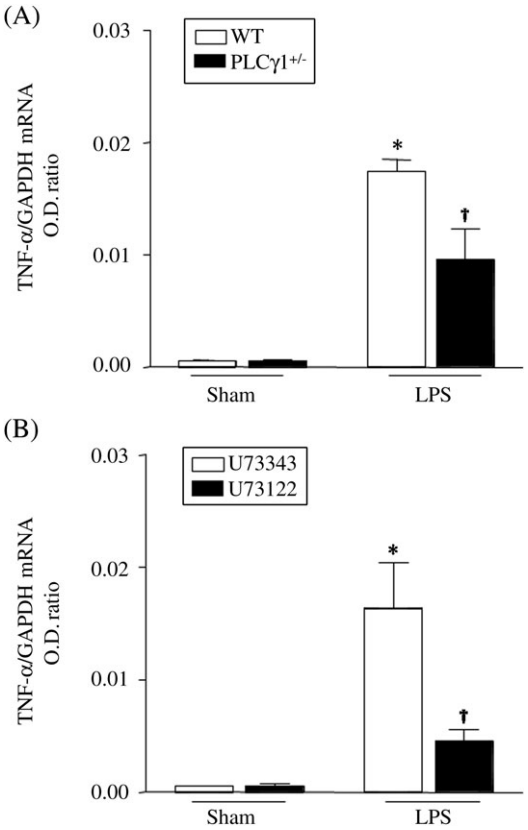

Figure 4 Role of phospholipase Cγ1 in myocardial tumor necrosis factor-α expression during endotoxemia phospholipase Cγ1$^{+/-}$ and littermate wild-type mice were treated with either vehicle or lipopolysaccharide (4 mg/kg, i.p) for 4 h. Wild-type mice were pre-treated with either U73122 or U73343 (9 mg/kg, i.p.) for 15 min, followed by vehicle or lipopolysaccharide (4 mg/kg, i.p.) for 4 h. Myocardial tumor necrosis factor-α mRNA was determined lipopolysaccharide-induced tumor necrosis factor-α mRNA levels were significantly attenuated in PLCγ1$^{+/-}$ (*A*) or U73122-treated (*B*) compared with wild-type or U73343-treated hearts, respectively. Data are means ± SD of six to nine hearts (*$P < 0.05$ vs. Sham. †$P < 0.05$ vs. wild-type or U73343).

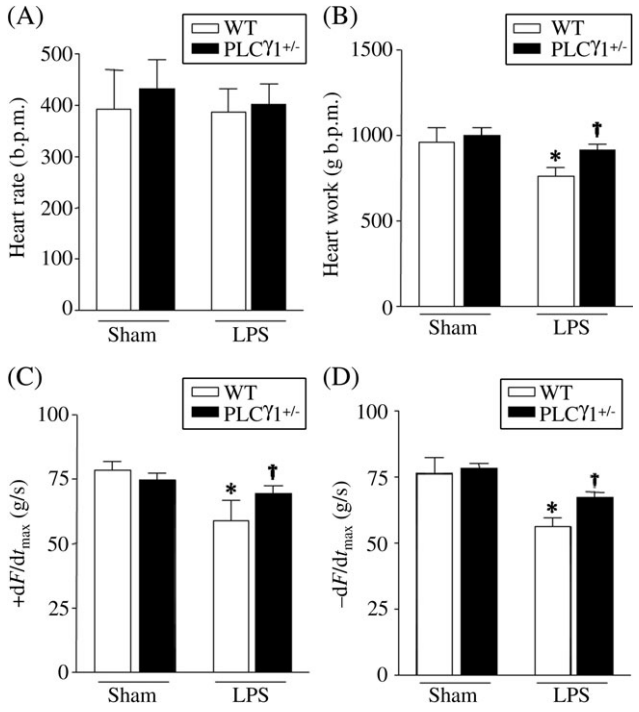

Figure 5 Myocardial dysfunction in phospholipase $C\gamma1^{+/-}$ and littermate wild-type mice with endotoxemia. Phospholipase $C\gamma1^{+/-}$ and littermate wild-type mice were treated with lipopolysaccharide (4 mg/kg, i.p.). Four hours later, contractile function of the heart was determined. Changes in heart rate (*A*), heart work (*B*), rate of contraction ($+dF/dt_{max}$, *C*) and relaxation ($-dF/dt_{min}$, *D*) are presented. Heart work and rate of contraction and relaxation were significantly improved in phospholipase $C\gamma1^{+/-}$ compared with littermate wild-type mice. Data are mean ± SEM, $n = 7$–9 per group, $^{*}P < 0.05$ vs. Sham in wild-type; $^{\dagger}P < 0.05$ vs. lipopolysaccharide in phospholipase $C\gamma1^{+/-}$.

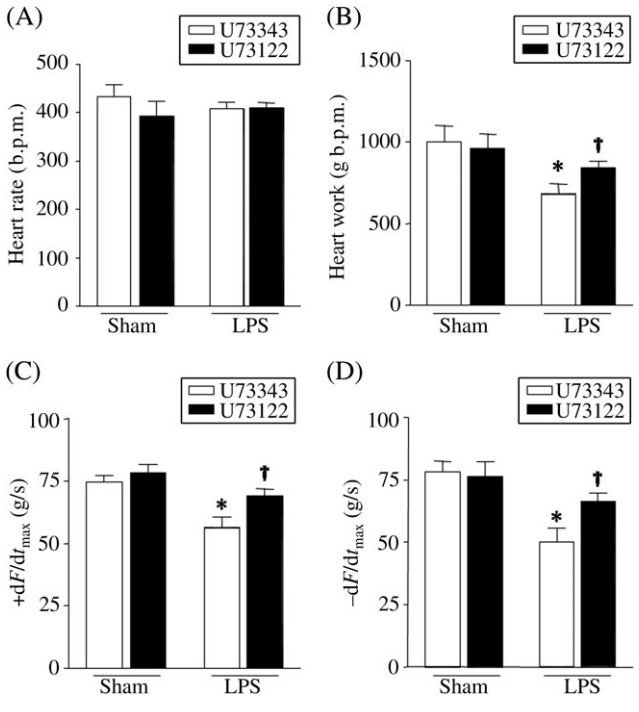

Figure 6 Effect of U73122 on myocardial function in endotoxemia. Wild-type mice were pre-treated with either U73122 or U73343 (9 mg/kg, i.p.) for 15 min, followed by vehicle or lipopolysaccharide (4 mg/kg, i.p.) for 4 h. Changes in heart rate (*A*), heart work (*B*), rate of contraction ($+dF/dt_{max}$, *C*) and relaxation ($-dF/dt_{min}$, *D*) are presented. Heart work and rate of contraction and relaxation were significantly improved in U73122 + lipopolysaccharide compared with U73343 + lipopolysaccharide. Data are mean ± SEM, $n = 7$–9 per group, $^{*}P < 0.05$ vs. Sham; $^{\dagger}P < 0.05$ vs. lipopolysaccharide + U73122.

4. Discussion

The present study used cardiomyocytes and an *in vivo* model of endotoxemia to investigate the role of PLCγ1 in LPS-induced TNF-α expression and myocardial dysfunction. We demonstrated for the first time that PLCγ1 signalling induces cardiac TNF-α expression during LPS stimulation and that PLCγ1 activation contributes to myocardial dysfunction induced by endotoxemia. The action of PLCγ1 on TNF-α expression is mediated through IP3/IP3R signalling pathways. Thus, PLCγ1 represents a novel signalling pathway leading to cardiac TNF-α expression.

Cardiomyocytes express PLCβ, PLCγ, and PLCδ.[17] Previous studies have suggested that PLC is implicated in ischaemia/reperfusion-induced cardiac dysfunction, cardiac hypertrophy, and diabetic cardiomyopathy.[35–37] However, it remains unclear whether PLC activation contributes to myocardial dysfunction in sepsis. The present study investigated the role of PLCγ1 in LPS-induced myocardial dysfunction. Our data showed that LPS-induced myocardial dysfunction was restored in $PLC\gamma1^{+/-}$ or PLCγ1 inhibitor-treated mice. These results demonstrated that PLCγ1 activation contributes to myocardial depression in endotoxemia. Mechanisms by which PLCγ1 mediates myocardial dysfunction are not fully understood. Studies have suggested that proinflammatory factors, in particular, TNF-α, interleukins and inducible nitric oxide synthase have emerged as initial contributors to myocardial dysfunction in sepsis.[38] Therefore, data from the present study suggest that up-regulation of TNF-α production may be one of the potential mechanisms by which PLCγ1 mediates myocardial dysfunction in endotoxemia since blocking TNF-α preserves myocardial function.[7] Other mechanisms may also contribute to PLCγ1-mediated myocardial dysfunction in our model of sepsis, which are beyond the scope of the present study but merit future studies.

The role of TNF-α in myocardial depression during endotoxemia has been addressed. However, mechanisms by which LPS induces TNF-α expression in cardiomyocytes are not fully understood. It is well established that the effects of LPS are mediated via toll-like receptors.[6] However, LPS signalling downstream of toll-like receptors remains largely unknown in cardiomyocytes. Our recent studies and others have demonstrated that NAD(P)H oxidaze and MAPK, in particular ERK1/2 and p38, are important in mediating TNF-α expression in cardiomyocytes.[7,11,12,39] The present study aimed to investigate the role of PLCγ1 in LPS induced TNF-α expression in cardiomyocytes. Consistent with our recent study and others,[22,23] the present result also showed that LPS increased PLCγ1 phosphorylation in cultured cardiomyocytes. Using cultured neonatal mouse cardiomyocytes and an *in vivo* model of endotoxemia, we have provided definitive evidence that support a pivotal role of PLCγ1 signalling in TNF-α expression in the heart during endotoxemia. It is currently unclear if there are any cross-talks between PLCγ1 and NAD(P)H oxidaze in LPS-stimulated cardiomyocytes. Our preliminary study showed that inhibition of PI-PLC or deficiency of PLCγ1

had no evident effect on NAD(P)H oxidaze activation in LPS-stimulated hearts as assessed by cytochrome c reduction assay (data not shown). These results suggested that PLCγ1 does not act upstream of the NAD(P)H oxidaze. Whether PLCγ1 is downstream or parallel signalling of NAD(P)H oxidaze requires further investigation.

Although PLCγ activation produces both IP3 and DAG, our data suggest that the role of PLCγ in LPS-induced TNF-α expression is mediated via IP3-dependent pathway. This conclusion is based on the following data. Inhibition of IP3 significantly decreased LPS-induced TNF-α expression in cardiomyocytes during LPS stimulation. Furthermore, treatment with IP3R antagonist or knockdown of IP3R2 attenuated TNF-α expression in LPS-stimulated cardiomyocytes. In contrast, incubation with either DAG antagonist (∼100 μM) or agonist (∼50 μM) did not change basal or LPS-stimulated TNF-α expression. The downstream signalling of IP3/IP3R in regulation of cardiomyocyte TNF-α expression remains to be determined. IP3 binds to its receptors (IP3R) and elevates $[Ca^{2+}]_i$,[40–42] our recent study and others showed that LPS increases the amplitude of $[Ca^{2+}]_i$ transients and inhibition of L-type calcium channel attenuates TNF-α expression.[43,44] These studies suggest that IP3R-mediated Ca^{2+} release may be an important downstream signalling in regulation of TNF-α expression. It has been demonstrated that elevation of $[Ca^{2+}]_i$ modulates Raf/MEK/ERK1/2 MAPK pathway.[45] We have shown that ERK1/2 is required for TNF-α expression in LPS-stimulated cardiomyocytes[11] and PLCγ1 signalling induces ERK1/2 MAPK activation during LPS stimulation.[22] Thus, it is most likely that ERK1/2 MAPK acts downstream of PLCγ1 signalling in regulation of TNF-α expression in LPS-stimulated cardiomyocytes. Taken together, LPS activates PLCγ1, which induces IP3 production and elevation of $[Ca^{2+}]_i$. Elevation of $[Ca^{2+}]_i$ promotes ERK1/2 activation, leading to TNF-α expression. However, the present study showed that inhibition of PLCγ1 signalling decreases but not abrogates LPS-induced TNF-α expression in cardiomyocytes and fails to completely restore myocardial function in endotoxemic mice. These data suggest the presence of additional signalling mechanism in parallel leading to TNF-α expression in LPS-stimulated cardiomyocytes. In this regard, our recent study has demonstrated that PLCγ1/ERK1/2 pathway is activated in parallel with p38 MAPK in response to LPS in cardiomyocytes.[22] Thus, LPS activates toll-like receptors,[6] which results in a parallel activation of PLCγ1/ERK1/2 and p38, leading to TNF-α expression in cardiomyocytes.

In summary, the present study provided promising evidence that PLCγ1 signalling induces LPS-induced TNF-α expression in the heart. The downstream signalling pathway involves activation of IP3/IP3R and ERK1/2 MAPK. Furthermore, PLCγ1 activation contributes to myocardial dysfunction during endotoxemia. The cause of myocardial dysfunction in sepsis is probably multi-factorial, it is therefore important to identify individual contributing factor and the underlying mechanisms to generate worthwhile therapeutic targets. Since none of the available strategies targeting outside-in signalling in cardiomyocytes show adequate effectiveness in sepsis, the potential of targeting intracellular signalling pathways may open up new therapeutic avenues for sepsis. Thus, the finding that PLCγ1 contributes to myocardial dysfunction may have potential clinical implications in sepsis.

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