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ORIGINAL CONTRIBUTION

Inhibition of Na/K-ATPase promotes myocardial tumor necrosis factor-alpha protein expression and cardiac dysfunction via calcium/mTOR signaling in endotoxemia

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Abstract Tumor necrosis factor-α (TNF-α) is a major pro-inflammatory cytokine that causes cardiac dysfunction during sepsis. Na/K-ATPase regulates intracellular Ca^{2+}, which activates mammalian target of rapamycin (mTOR), a regulator of protein synthesis. The aim of this study was to investigate the role of Na/K-ATPase/mTOR signaling in myocardial TNF-α expression during endotoxemia. Results showed that treatment with LPS decreased Na/K-ATPase activity in the myocardium in vivo and in cultured neonatal cardiomyocytes. Inhibition of Na/K-ATPase by ouabain enhanced LPS-induced myocardial TNF-α protein production, but had no effect on TNF-α mRNA expression. More importantly, ouabain further decreased in vivo cardiac function in endotoxemic mice, which was blocked by etanercept, a TNF-α antagonist. LPS-induced reduction in Na/K-ATPase activity was prevented by inhibition of PI3K, Rac1 and NADPH oxidase using LY294002, a dominant-negative Rac1 adenovirus (Ad-Rac1N17) and apocynin, respectively. To assess the role of Rac1 in Ca^{2+} handling, Ca^{2+} transients in adult cardiomyocytes from cardiomyocyte-specific Rac1 knockout ($Rac1^{CKO}$) and wild-type (WT) mice were determined. LPS increased intracellular Ca^{2+} in WT but not in $Rac1^{CKO}$ cardiomyocytes. Furthermore, LPS rapidly increased mTOR phosphorylation in cardiomyocytes, which was blocked by Rac1N17 and an inhibitor of calmodulin-dependent protein kinases (CaMKs) KN93, but enhanced by ouabain. Rapamycin, an inhibitor of mTOR suppressed TNF-α protein levels without any significant effect on its mRNA expression or global protein synthesis. In conclusion, myocardial Na/K-ATPase activity is inhibited during endotoxemia via PI3K/Rac1/NADPH oxidase activation. Inhibition of Na/K-ATPase activates Ca^{2+}/CaMK/mTOR signaling, which promotes myocardial TNF-α protein production and cardiac dysfunction during endotoxemia.

Keywords Lipopolysaccharide · Rac1 GTPase · Na/K-ATPase · Calcium · mTOR · TNF-alpha

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Introduction

Sepsis is a leading cause of death in hospital intensive care units [7, 10]. Mortality in septic shock or severe sepsis with cardiac dysfunction is 70–90% [37]. Overall mortality due to sepsis is three times higher than myocardial infarction in Canadian hospitals [7]. Myocardial expression of pro-inflammatory cytokines, especially tumor necrosis factor-alpha (TNF-α) contributes to cardiac dysfunction, resulting in patient's death [33, 37, 55]. However, the underlying molecular mechanisms of cardiac TNF-α production during sepsis remain elusive.

Na/K-ATPase catalyzes ATP hydrolysis to actively transport three Na^+ ions out of and two K^+ into the cell, and thus maintains *trans*-membrane gradients of Na^+ and K^+. Changes in intracellular Na^+ regulate intracellular

Ca^{2+} via the Na^+/Ca^{2+} exchanger [34]. In cardiomyocytes, Na/K-ATPase activity is inhibited by phospholemman [22] and glutathionylation induced by NADPH oxidase [13]. In the heart, Na/K-ATPase modulates cardiomyocyte apoptosis [47], contractility [3, 39] and hypertrophy [25] via Ca^{2+}-dependent mechanisms. LPS inhibits Na/K-ATPase activity in the lung [29] and kidney [19]. Inhibition of Na/K-ATPase potentiates LPS-induced cytokine expression including TNF-α in macrophages [40]. We have previously shown that LPS increases intracellular Ca^{2+} in cardiomyocytes [15]; however, changes in cardiac Na/K-ATPase activity during sepsis were not studied. Thus, the role of Na/K-ATPase in myocardial TNF-α expression and cardiac dysfunction during endotoxemia remains unknown.

Mammalian target of rapamycin (mTOR), a Ser/Thr protein kinase, acts as a central regulator of cell growth and metabolism by controlling protein synthesis and other cellular processes [23]. It is sensitive to tuberous sclerosis complex (TSC)1/TSC2 activation and intracellular Ca^{2+} concentration [23, 26, 30, 51]. Studies have shown that mTOR contributes to cardiac hypertrophy [11, 35] and ventricular remodeling after myocardial infarction [6]. LPS activates mTOR [1, 9, 20, 53], however, its role in pro-inflammatory cytokine expression during sepsis remains controversial. For example, mTOR activation by LPS promotes TNF-α and interleukin-12 expression in monocyte-derived dendritic cells [20]. On the contrary, mTOR activation inhibits TNF-α production in monocytes [53], macrophages [1] and HL-1 cells during LPS stimulation [49]. Thus, the effects of mTOR activation are cell-type specific. Importantly, the role of mTOR in myocardial TNF-α protein production during endotoxemia remains unknown.

In the present study, we hypothesized that myocardial Na/K-ATPase activity is inhibited during endotoxemia via PI3K/Rac1/NADPH oxidase pathway. We further hypothesized that inhibition of Na/K-ATPase activates Ca^{2+}-dependent mTOR, leading to enhanced TNF-α protein production in cardiomyocytes during LPS stimulation. To test these hypotheses, cultured cardiomyocytes and in vivo endotoxemia mouse models were employed. Our study demonstrated a novel function of Na/K-ATPase in the regulation of myocardial TNF-α expression and cardiac function during endotoxemia.

Materials and methods

Animals and preparation of neonatal mouse cardiomyocytes

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication #85-23, revised 1996) and experimental protocols were approved by Animal Use Subcommittee at the University of Western Ontario. C57BL/6 wild-type (WT) and Rac1 floxed ($Rac1^{f/f}$) mice [16] were purchased from the Jackson Laboratory (Bar Harbor, Maine). Cre transgenic mice which overexpress Cre recombinase under the control of α-myosin heavy-chain (MHC) promoter were provided by Dr. E. Dale Abel (University of Utah, UT). The generation of cardiomyocyte-specific Rac1 knockout mice ($Rac1^{CKO}$) was achieved by breeding $Rac1^{f/f}$ mice with the Cre transgenic mice [45]. Neonatal cardiomyocytes were isolated and cultured according to methods we have previously described [48]. Cells were treated with LPS (Sigma, Oakville, ON, Canada), apocynin (Sigma), ouabain (Sigma), KN-93 (Sigma), rapamycin (Cell Signaling Technology, Danvers, MA) or infected with adenoviruses.

Adenoviral infection of neonatal cardiomyocytes

Cardiomyocytes were infected with adenoviruses carrying a dominant-negative form of Rac1 (Ad-Rac1N17, Vector Biolabs, Philadelphia, PA), green fluorescence protein (Ad-GFP, a gift from Dr. J. Lipp, Medical University of Vienna, Austria) and LacZ (Ad-LacZ, Vector Biolabs, Philadelphia, PA) at a multiplicity of infection (MOI) of 10 plaque forming units/cell. Adenovirus-mediated gene transfer was applied as previously described [43]. All experiments were started after 48 h of adenoviral infection.

Na/K-ATPase activity assay

Na/K-ATPase activity in neonatal cardiomyocytes and left ventricle (LV) myocardium was determined by a fluorometric method with modifications [2, 27]. Briefly, homogenates were prepared in a buffer containing 250 mM sucrose, 2 mM EDTA, 1.25 mM EGTA, 5 mM NaN_3, and 10 mM Tris (pH 7.4). Homogenates were freeze-thawed four times. 30 μg protein lysates were incubated for 5 min at 37°C in a buffer containing 5 mM $MgCl_2$, 1.25 mM EDTA, 100 mM Tris base (pH 7.4), 1 mM EGTA, and 5 mM NaN_3, with or without 6 mM ouabain. 3-*O*-methylfluorescein phosphate (3-O-MFP, 160 μM) was added to the lysates followed by incubation at 37°C for 1 min. Activated Na/K-ATPase hydrolyzes 3-O-MFP and forms a fluorescent compound 3-O-MF. To activate Na/K-ATPase, 10 mM KCl was added and fluorescence was recorded. The excitation and emission wavelengths were 470 and 515 nm, respectively. The amplitude of the emission was shown to be proportional to the concentration of 3-O-MF, and a standard curve was created using varying concentration of 3-O-MF. K^+-dependent 3-O-MFPase activity was determined by subtracting activity with KCl from the

activity without KCl. Na/K-ATPase activity was determined by subtracting K^+-dependent 3-O-MFPase activity without ouabain from the activity with ouabain and expressed as micromoles of liberated phosphate (Pi) per minute per milligram protein.

Measurement of TNF-α mRNA

Total RNA was extracted from cardiomyocytes and LV myocardium using the Trizol Reagent (Invitrogen, Burlington, ON, Canada), following manufacturer's instructions. TNF-α mRNA levels were determined by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) using the same primers as previously reported [21, 42]. TNF-α mRNA stability was assessed after treatment with actinomycin D, a transcription inhibitor. Neonatal cardiomyocytes were stimulated with LPS (0.1 μg/ml) with or without ouabain (50 μM) for 3 h followed by actinomycin D (5 μg/ml) treatment. After 20, 60 and 120 min of actinomycin D exposure, total RNA was extracted from cardiomyocytes. TNF-α mRNA levels were measured.

Measurement of TNF-α protein

TNF-α protein levels were measured using a mouse TNF-α ELISA kit (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions. The measurements were standardized by cell numbers in cardiomyocyte cultures or mg protein in myocardial tissues.

[^{3}H]-Leucine incorporation

Neonatal cardiomyocytes were seeded into 12-well plates (8×10^5 cells/well). After 48 h, cells were treated with LPS (1 μg/ml) and [^{3}H]-leucine (1 μCi/ml, Amersham, GE Healthcare, Baie d'Urfe, Quebec) with and without rapamycin (10 nM) for 5 h. Cells were then washed three times with ice-cold PBS. Proteins were precipitated with 5% trichloroacetic acid (TCA) for 30 min on ice. Cell precipitates were washed two additional times with TCA and solubilized in 0.2 M NaOH. The radioactivity of [^{3}H]-leucine incorporated into proteins were measured by a liquid scintillation counter (Tri-Carb 2900TR, Perkin-Elmer, Woodbridge, ON).

Adult cardiomyocyte isolation

Cardiomyocytes were isolated from the hearts of adult $Rac1^{f/f}$ and $Rac1^{CKO}$ mice as previously described with modifications [5]. Hearts were mounted on a Langendorff system and perfused with digestion buffer containing 75 μg/mL of liberase blendzyme IV (Roche, Laval, Quebec). Following digestion, cells were resuspended and exposed to a series of sedimentation and resuspension steps in buffer containing increasing concentrations of Ca^{2+} (12.5 μM–1.5 mM). Healthy, rod-shaped myocytes were used for measurements of Ca^{2+} transients.

Intracellular Ca^{2+} transients

Free intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured in isolated ventricular cardiomyocytes using fura-2-AM as previously described [5]. Cells were loaded with 1 μM fura-2-AM and then paced at 0.5, 1, 2 and 4 Hz using a 2.5 ms duration pulse. Fluorescence intensity at 510 nm was measured with alternating 345 and 380 nm excitation using a Deltascan monochrometer system (Photon Technology International, London, ON, Canada). $[Ca^{2+}]_i$ was calculated by the methods of Grynkiewicz [17].

Western blot analysis

Thirty micrograms of protein lysates were subjected to separation on a 10% SDS-PAGE gel, followed by electrotransfer to nitrocellulose membranes. Blots were probed with specific antibodies against total mTOR (1:1,000) and phospho-mTOR (1:1,000, Cell Signaling Technology, Danvers, MA), respectively, as previously reported [42]. Signals were detected by the chemiluminescence detection method and quantified by densitometry. The relative phosphorylation levels of mTOR under various experimental conditions were estimated by taking the ratio of the densitometric signal acquired with anti-phospho-mTOR to the corresponding signal acquired with anti-mTOR.

In vivo LV pressure–volume measurements

Adult male WT mice (8–10 weeks old) were pretreated with etanercept (2 mg/kg, i.p., Amgen, Thousand Oaks, CA, USA), a TNF-α inhibitor containing the extracellular ligand-binding portion of TNF-α receptor 2 and the Fc component of human IgG1 (TNFR2:Fc fusion protein) [18, 42], or saline for 30 min, followed by LPS (0.5 mg/kg, i.p.) or ouabain (2 mg/kg, i.p.) treatment. After 4 h, mice were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (12.5 mg/kg) cocktail. A Millar pressure-conductance catheter (SPR839, 1.4F) was inserted into the LV through the right carotid artery to measure LV pressure, volume and heart rate. The signal was recorded by a PowerLab Chart program and analyzed by a PVAN software (Millar Instruments, Houston, TX), as previously reported [14, 43].

Statistical analysis

Results are presented as mean ± SEM. Differences between two groups were analyzed by unpaired Student

t test. For multigroup comparisons, one or two-way ANOVA followed by Student–Newman–Keuls or Bonferroni post-test was performed. A *P* value less than 0.05 was considered statistically significant.

Results

LPS inhibits myocardial Na/K-ATPase activity

To examine the effect of LPS on Na/K-ATPase activity, neonatal cardiomyocytes isolated from C57BL/6 mice were treated with LPS (1 μg/ml) for 2 h. LPS decreased Na/K-ATPase activity by 44% (Fig. 1a). To study Na/K-ATPase activity in the myocardium in vivo, $Rac1^{f/f}$ mice were treated with LPS (2 mg/kg, i.p.) or saline for 1, 2, 3 or 4 h, after which hearts were isolated and myocardial Na/K-ATPase activity was measured. After 2 h of LPS treatment, myocardial Na/K-ATPase activity was decreased by 73% (Fig. 1b). These data showed that LPS strongly inhibits myocardial Na/K-ATPase activity.

Inhibition of Na/K-ATPase promotes TNF-α protein production during LPS stimulation

TNF-α is a major pro-inflammatory cytokine contributing to cardiac dysfunction during endotoxemia. To elucidate the role of Na/K-ATPase on LPS-induced TNF-α expression, neonatal cardiomyocytes were incubated with ouabain, a selective inhibitor of Na/K-ATPase. A concentration-dependent enhancement of LPS-induced TNF-α protein levels was observed following ouabain treatment (Fig. 2a). Surprisingly, ouabain had no measurable effect on LPS-induced TNF-α mRNA expression (Fig. 2b). Furthermore, ouabain did not alter TNF-α mRNA stability during LPS stimulation (Fig. 2c). To verify these in vitro data, adult mice were treated with vehicle or LPS (0.5 mg/kg, i.p.) with or without ouabain (2 mg/kg). Consistently, ouabain enhanced myocardial TNF-α protein production in response to LPS, without a significant effect on TNF-α mRNA expression (Fig. 2d, e). These data indicate that inhibition of Na/K-ATPase activity enhances LPS-induced TNF-α protein levels without any apparent effects on its mRNA expression or stability in the myocardium.

Inhibition of Na/K-ATPase decreases myocardial function during endotoxemia

To evaluate the effect of ouabain on cardiac dysfunction during endotoxemia, adult mice were treated with LPS (0.5 mg/kg) with or without ouabain (2 mg/kg) for 4 h. LV hemodynamic parameters were determined by using a Millar pressure-conductance catheter (Table 1; Fig. 3). Ouabain increased stroke volume and LV d*P*/d*t* under basal conditions. LPS significantly decreased LV d*P*/d*t* and stroke work, which were further reduced by ouabain. In addition, ouabain significantly decreased cardiac output and maximal power during endotoxemia (Table 1; Fig. 3a–d). To determine if these effects of ouabain were mediated by TNF-α, a TNF-α antagonist etanercept was employed. Mice treated with etanercept and ouabain had significantly better cardiac function compared to ouabain treatment alone during endotoxemia (Fig. 3a–d). These results suggest that ouabain increases cardiac contractile function under basal physiological conditions. In contrast, inhibition of Na/K-ATPase by ouabain further suppresses cardiac function during endotoxemia via enhancement of TNF-α production.

Inhibition of Na/K-ATPase by LPS is mediated by PI3K/Rac1/NADPH oxidase pathway

We have recently shown that the PI3K/Rac1/NADPH oxidase pathway is activated and important for cardiac TNF-α production during endotoxemia [56]. To determine

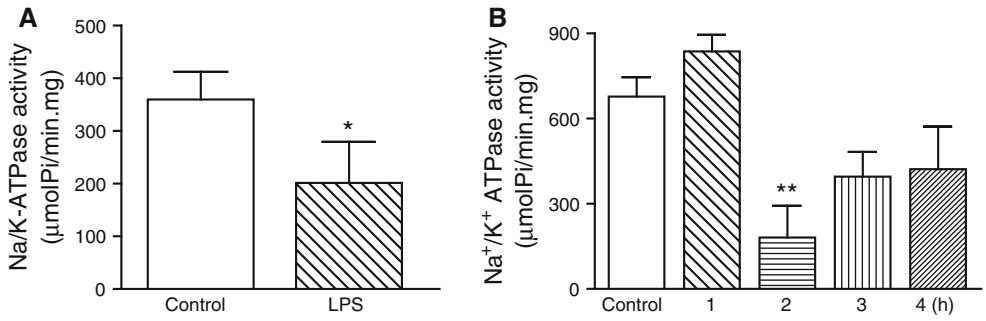

Fig. 1 Inhibition of Na/K-ATPase activity and its effect on TNF-α expression during LPS stimulation. **a** Neonatal cardiomyocytes from WT mice were treated with vehicle or LPS (1 μg/ml) for 2 h. Na/K-ATPase activity in these cells was determined using a fluorometric assay. **b** Adult male $Rac1^{f/f}$ mice were treated with LPS (2 mg/kg, i.p.) for 1, 2, 3 and 4 h. Na/K-ATPase activity in the left ventricular myocardium was determined. $*P < 0.05$, $**P < 0.01$ vs. control

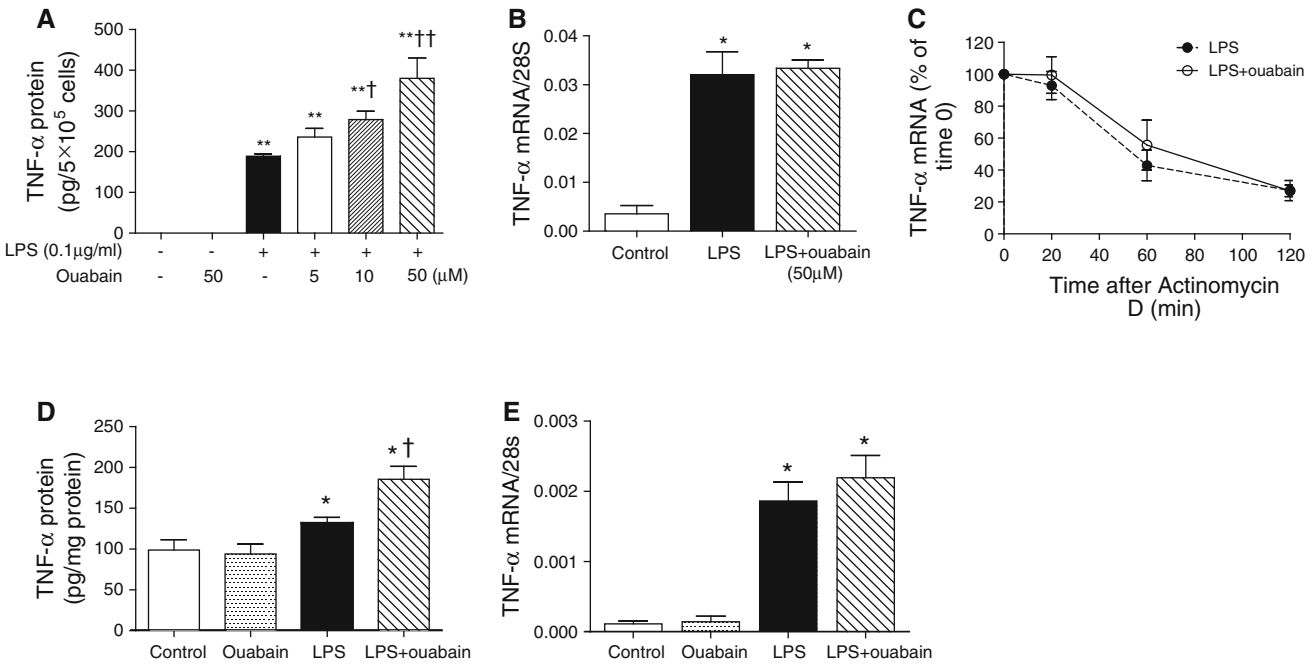

Fig. 2 Ouabain enhances LPS-induced myocardial TNF-α protein expression. Neonatal cardiomyocytes from WT mice were treated with LPS (0.1 μg/ml) with or without ouabain for 3 or 5 h. TNF-α protein levels (**a**) in culture medium and mRNA levels (**b**) were measured by ELISA and real-time RT-PCR, respectively. **c** Stability of TNF-α mRNA was assessed in the presence and absence of actinomycin D, an inhibitor of transcription. TNF-α mRNA levels in cells were measured. Adult male WT mice were treated with LPS (0.5 mg/kg, i.p.) for 2 and 4 h. TNF-α protein (**d**) and mRNA (**e**) levels in heart tissues were measured. Data are means ± SEM from 3–7 independent experiments per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. LPS

Table 1 *In vivo* hemodynamic measurements with or without ouabain and etanercept treatments in mice with endotoxemia

Parameters	Control	Ouabain	LPS	LPS + ouabain	Etanercept	LPS + etanercept + ouabain
n	7	8	8	7	6	7
Heart rate (bpm)	403 ± 22	404 ± 12	468 ± 17	423 ± 20	335 ± 18	442 ± 20
MAP (mmHg)	86.6 ± 5.4	91.9 ± 4.4	70.4 ± 5.8	63.7 ± 3.5	75.7 ± 3.9	71.6 ± 4.9
SLVP (mmHg)	99.4 ± 3.2	112.5 ± 8.2	81.8 ± 4.5	73.6 ± 2.7	94.1 ± 3.7	96.1 ± 4.0
LVEDP (mmHg)	3.9 ± 1.3	4.0 ± 1.3	3.6 ± 1.0	4.6 ± 0.9	3.0 ± 1.1	3.0 ± 0.8
Tau (ms)	10.1 ± 0.7	13.2 ± 1.9	13.8 ± 1.1	18 ± 0.9*†	11.3 ± 0.6	14.5 ± 0.8‡
Maximal power (mW)	10.0 ± 1.6	12.8 ± 2.3	7.0 ± 0.7	2.5 ± 0.5*†	7.2 ± 1.2	5.9 ± 0.6*‡

Adult male WT mice were pretreated with etanercept (2 mg/kg, i.p.), a TNF-α antagonist, or saline for 30 min, followed by LPS (0.5 mg/kg, i.p.) or ouabain (2 mg/kg, i.p.) treatment. After 4 h, cardiac function was determined using a Millar pressure-conductance catheter. Results of LV d*P*/d*t*, stroke work and cardiac output are presented in Fig. 3. Data are mean ± SEM

MAP mean arterial pressure, *SLVP* systolic left ventricular pressure, *LVEDP* left ventricular end-diastolic pressure

* $P < 0.05$ vs. control; † $P < 0.05$ vs. LPS; ‡ $P < 0.05$ vs. LPS + ouabain

if PI3K acts as an up-stream regulator of Na/K-ATPase, the PI3K inhibitor LY294002 was employed. LY294002 had no effect on Na/K-ATPase activity under control conditions but completely restored Na/K-ATPase activity after LPS stimulation in neonatal cardiomyocytes (Fig. 4a). These results were further confirmed in vivo. Adult male WT mice were pretreated with LY294002 (7.5 mg/kg, i.p.) for 30 min followed by LPS stimulation (2 mg/kg, i.p.). As shown in Fig. 4b, LY294002 blocked myocardial Na/K-ATPase activity suppression by LPS, indicating that LPS inhibits cardiac Na/K-ATPase activity via PI3K.

To determine the effect of Rac1 on Na/K-ATPase activity, neonatal cardiomyocytes isolated from WT mice were infected with an adenovirus encoding a dominant-negative form of Rac1 (Ad-Rac1N17), which specifically inhibits Rac1 activity. Overexpression of Rac1N17 restored Na/K-ATPase activity inhibited by LPS (Fig. 4c). Consistently, LPS decreased Na/K-ATPase activity in the

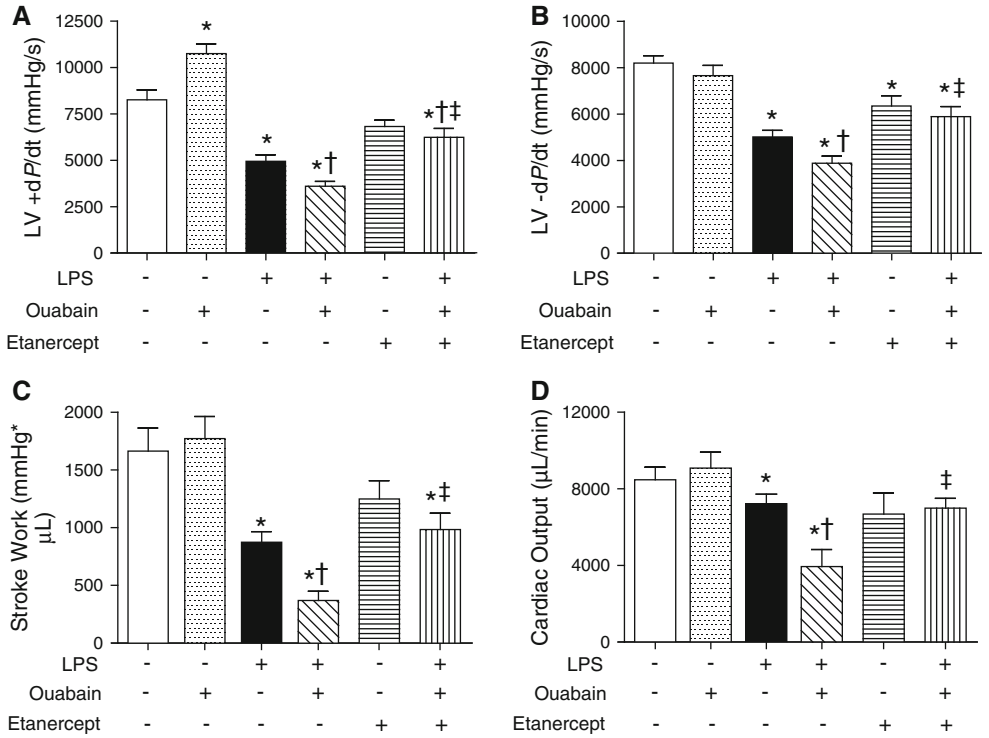

Fig. 3 In vivo cardiac function during endotoxemia. Adult male WT mice were pretreated with etanercept (2 mg/kg, i.p.), a TNF-α antagonist, or saline for 30 min, followed by LPS (0.5 mg/kg, i.p.) or ouabain (2 mg/kg, i.p.) treatment. After 4 h, cardiac function was determined using a Millar pressure-conductance catheter. Changes in LV $+dP/dt$ (**a**), LV $-dP/dt$ (**b**), stroke work (**c**) and cardiac output (**d**) are presented. Data are means ± SEM. $n = 7$–8 per group. $^{*}P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. LPS; $^{\ddagger}P < 0.05$ vs. LPS + ouabain

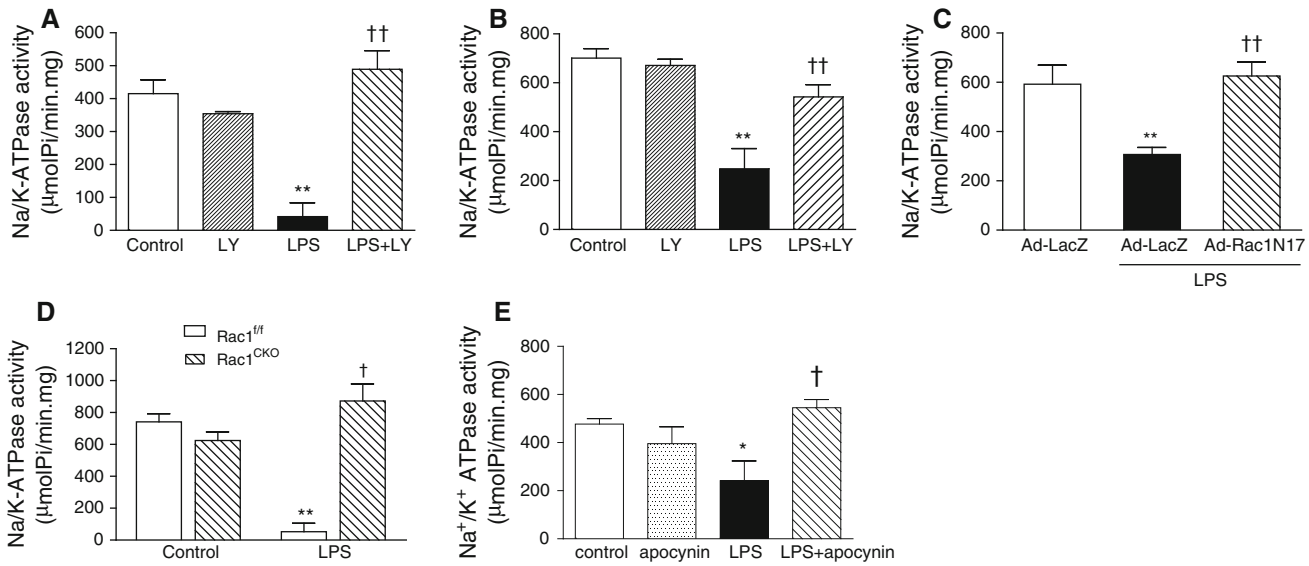

Fig. 4 LPS-induced PI3K, Rac1 and NADPH oxidase activities suppress Na/K-ATPase. **a** WT neonatal cardiomyocytes were treated with LPS (1 μg/ml) with or without LY294002 (10 μM) for 2 h. Na/K-ATPase activity in these cells was measured. **b** Adult male C57BL/6 mice were pretreated with saline (control) or LY294002 (7.5 mg/kg) for 30 min followed by treatment with LPS (2 mg/kg, i.p.) for 2 h. Na/K-ATPase activity in the LV myocardium was determined. **c** WT neonatal cardiomyocytes were infected with Ad-LacZ or Ad-Rac1N17 for 24 h followed by treatment with LPS (1 μg/ml). Na/K-ATPase activity was measured. **d** Na/K-ATPase activity in $Rac1^{f/f}$ and $Rac1^{CKO}$ myocardium were measured after 2 h of LPS treatment (2 mg/kg, i.p.). **e** Cells were incubated with LPS with or without apocynin (400 μM) for 2 h. Na/K-ATPase activity was measured. Data are means ± SEM from 3–5 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control and Ad-LacZ; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. Ad-LacZ + LPS and LPS

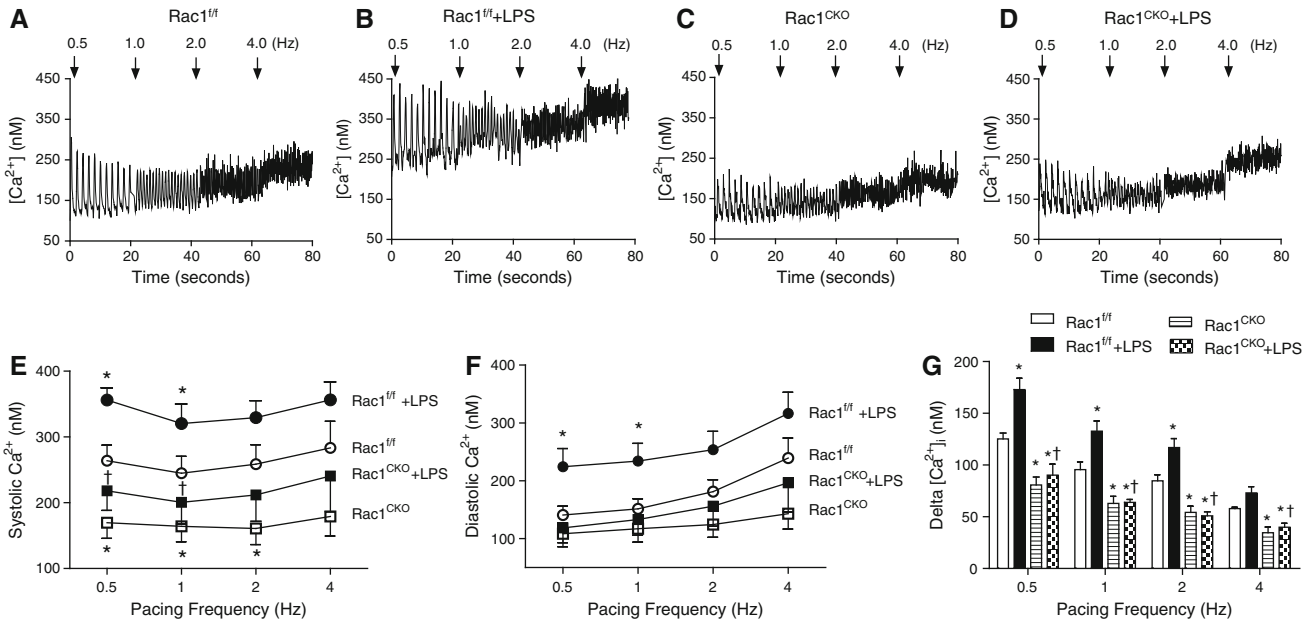

Fig. 5 Rac1 increases LPS-induced intracellular Ca^{2+} transient in cardiomyocytes. Adult cardiomyocytes from $Rac1^{f/f}$ and $Rac1^{CKO}$ hearts treated with vehicle or LPS (5 μg/ml) for 30 min were paced at 0.5, 1, 2 and 4 Hz. Intracellular Ca^{2+} transients were recorded. **a–d** Representative tracings from $Rac1^{f/f}$ and $Rac1^{CKO}$ with and without LPS treatment. Effects of LPS on systolic (**e**), diastolic (**e**) and difference (**g**) between systolic and diastolic intracellular Ca^{2+} concentrations during pacing were determined. Data are mean ± SEM from 4–6 mice per group (8–12 cells). $^{*}P < 0.05$ vs. $Rac1^{f/f}$; $^{\dagger}P < 0.05$ vs. $Rac1^{f/f}$ + LPS

myocardium of $Rac1^{f/f}$ mice but had no effect on $Rac1^{CKO}$ mice (Fig. 4d). To determine if NADPH oxidase regulates Na/K-ATPase activity in response to LPS, neonatal cardiomyocytes were treated with apocynin, a specific inhibitor of NADPH oxidase. Apocynin prevented the diminution of Na/K-ATPase activity in response to LPS (Fig. 4e). Taken together, these data implicate the PI3K/Rac1/NADPH oxidase pathway in the inhibition of myocardial Na/K-ATPase activity during LPS stimulation.

LPS-induced intracellular Ca^{2+} is mediated by Rac1

Inhibition of Na/K-ATPase is known to increase intracellular Ca^{2+} [34]. Since LPS-induced inhibition of Na/K-ATPase is mediated by Rac1 as shown above, the role of Rac1 in intracellular Ca^{2+} during LPS stimulation was studied. To this end, Ca^{2+} transients were recorded using fura-2 in adult cardiomyocytes isolated from $Rac1^{f/f}$ and $Rac1^{CKO}$ mice. These cells were paced over a range of frequencies (0.5–4 Hz) (Fig. 5a–g). $Rac1^{CKO}$ cells showed lower systolic Ca^{2+} compared with $Rac1^{f/f}$ cells, but diastolic Ca^{2+} was similar between these two groups (Fig. 5a–c). In addition, systolic and diastolic Ca^{2+}, and the difference between systolic and diastolic Ca^{2+} concentrations were all significantly increased by LPS stimulation in $Rac1^{f/f}$ cardiomyocytes, but not in $Rac1^{CKO}$ cells (Fig. 5a–c). Collectively, these data suggest that Rac1 is not only required for maintaining cardiac Ca^{2+} homeostasis under basal physiological conditions, but also contributes to increased intracellular Ca^{2+} in response to LPS.

mTOR mediates LPS-induced TNF-α protein expression

To gain insight into the molecular mechanism by which Na/K-ATPase regulates TNF-α protein production, we studied mTOR, a master regulator of protein synthesis, in the regulation of cardiac TNF-α expression during endotoxemia. Our data showed that mTOR phosphorylation was significantly increased in neonatal cardiomyocytes in response to LPS stimulation (Fig. 6a). Inhibition of mTOR by rapamycin resulted in a significant decrease in LPS-induced TNF-α protein levels in a dose-dependent manner without any effect on TNF-α mRNA expression (Fig. 6b, c).

To assess the effects of the above treatments on global protein synthesis, [^{3}H]-leucine incorporation was determined in cultured cardiomyocytes. In response to LPS, [^{3}H]-leucine incorporation showed a trend but not significant increase compared to controls (Fig. 6d). Importantly, treatment with rapamycin did not have a significant effect on [^{3}H]-leucine incorporation during LPS stimulation (Fig. 6d). Taken together, our data showed that inhibition of mTOR by rapamycin selectively decreases LPS-induced TNF-α protein synthesis.

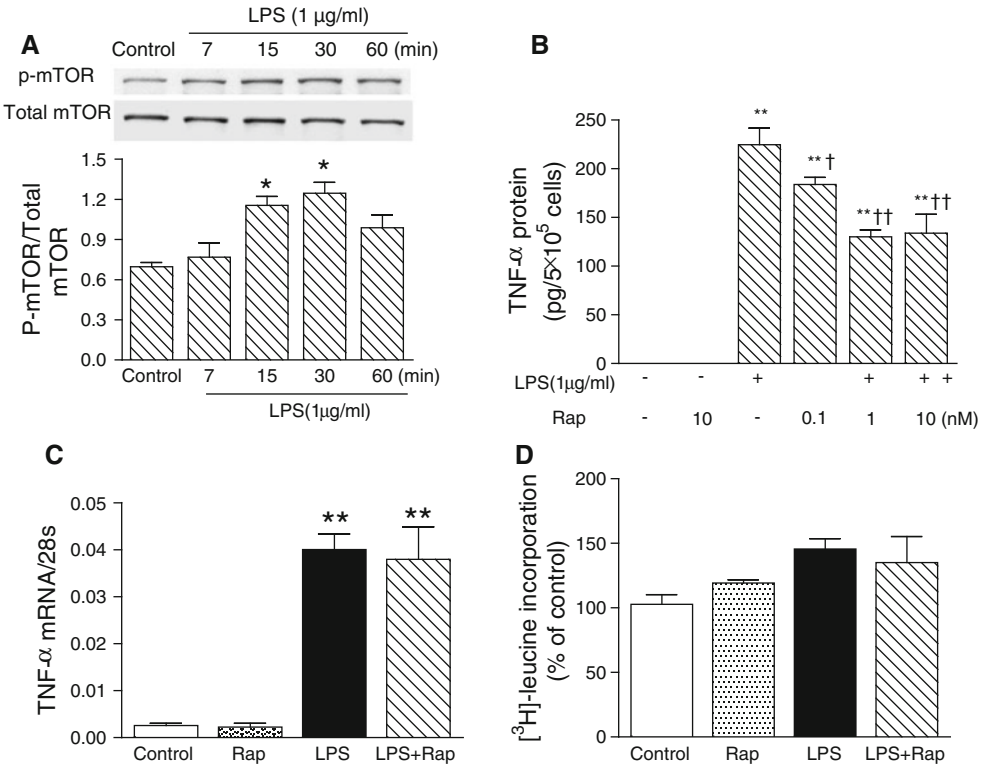

Fig. 6 mTOR activity promotes LPS-induced TNF-α production in cardiomyocytes. **a** WT neonatal cardiomyocytes were cultured in serum-free medium overnight followed by treatment with LPS (1 μg/ml) for 7, 15, 30 and 60 min. Phospho-mTOR was determined by western blotting. Cells were treated with vehicle or LPS (1 μg/ml) with or without rapamycin (Rap). TNF-α protein (**b**) and mRNA (**c**) levels were measured following 5 and 3 h of LPS treatment, respectively. **d** Global protein synthesis in cardiomyocytes was assessed by [^{3}H]-leucine incorporation following 5 h of LPS (1 μg/ml) treatment with or without Rap. Data are mean ± SEM from 3–6 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. LPS

mTOR activation is mediated by Rac1/Na/K-ATPase/CaMK signaling

To determine the role of Rac1 and Na/K-ATPase on mTOR activity during LPS stimulation, neonatal cardiomyocytes were treated with Ad-Rac1N17 and ouabain, respectively. Rac1N17 blocked LPS-induced mTOR phosphorylation (Fig. 7a). On the contrary, ouabain enhanced mTOR phosphorylation induced by LPS (Fig. 7b). Ca^{2+}/calmodulin-dependent protein kinase (CaMK) activation promotes TNF-α expression in monocytes and macrophages [32, 36, 44]. To determine if CaMK mediates mTOR activation, cells were pretreated with KN-93, a CaMK inhibitor. KN-93 blocked LPS-induced mTOR phosphorylation (Fig. 7c). Taken together, these data suggest that Rac1/Na/K-ATPase/CaMK pathway mediates LPS-induced mTOR activation, which promotes TNF-α protein expression.

Discussion

The present study provides the first evidence that LPS negatively regulates cardiac Na/K-ATPase activity via the PI3K/Rac1/NADPH oxidase pathway. Inhibition of Na/K-ATPase activates mTOR apparently via Ca^{2+}, leading to enhanced myocardial TNF-α protein production and impaired cardiac function. Na/K-ATPase/CaMK/mTOR signaling represents a novel molecular mechanism by which LPS stimulates cardiac TNF-α protein production (Fig. 8).

Na/K-ATPase is an important ion transporter and signal transducer located at the cell membrane. It has been demonstrated that Na/K-ATPase activity is decreased during the inflammatory response of the lung, kidney and innate immune system and contributes to pro-inflammatory factor mRNA expression [19, 29, 40]. It is well established that myocardial TNF-α production is a major contributor to cardiovascular dysfunction [8, 12, 28, 31, 50, 54]. Here, we provided both in vivo and in vitro evidence that LPS inhibits Na/K-ATPase activity in the heart. It is interesting to note that Na/K-ATPase inhibition enhances LPS-induced myocardial TNF-α protein production without any apparent effects on TNF-α mRNA expression and stability. Consistently, although ouabain promotes cardiac function under basal physiological conditions, it further suppresses myocardial function during endotoxemia. These results suggest that inhibition of Na/K-ATPase impairs myocardial function via promoting myocardial TNF-α protein translation or inhibiting its degradation during LPS stimulation.

Reactive oxygen species contributes to myocardial TNF-α expression and cardiac dysfunction [24, 41]. Activation of NADPH oxidase inhibits Na/K-ATPase by glutathionylation of its β subunit [13]. We recently demonstrated that LPS activates PI3K/Rac1/NADPH oxidase signaling to promote myocardial TNF-α expression [56]. Further to this observation, the present study showed that

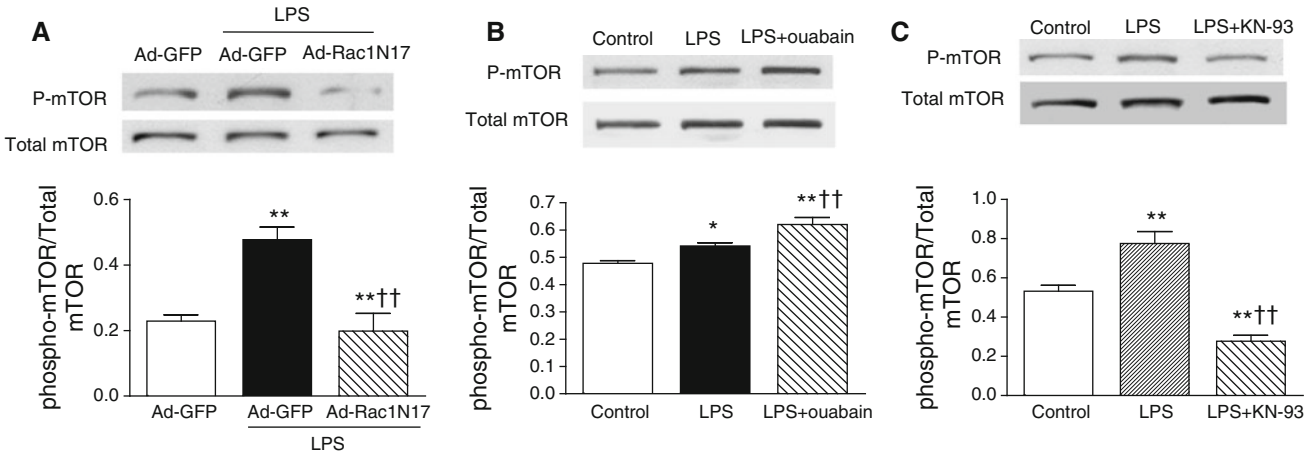

Fig. 7 Role of Rac1, Na/K-ATPase and CaMK in LPS-induced mTOR activation in cardiomyocytes. **a** WT neonatal cardiomyocytes cultured in serum-free medium were infected with Ad-Rac1N17 followed by LPS treatment (1 μg/ml) for 15 min. **b** and **c** Cultured cardiomyocytes were treated with LPS (1 μg/ml) with ouabain (**b** 50 μM) or KN-93 (**c** 10 μM) for 15 min. Phospho-mTOR was detected by western blotting. Data are mean ± SEM from 3–4 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. LPS

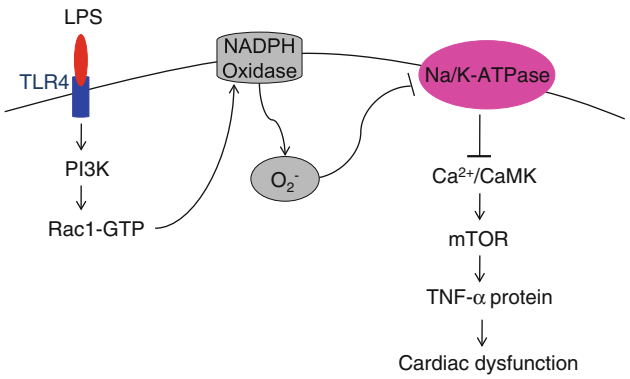

Fig. 8 Involvement of Na/K-ATPase/mTOR signaling pathway leading to TNF-α protein production and cardiac dysfunction during LPS stimulation. LPS suppresses Na/K-ATPase activity via PI3K/Rac1/NADPH oxidase pathway in the myocardium. Inhibition of Na/K-ATPase activates mTOR via a Ca^{2+}/CaMK-dependent mechanism. Activated mTOR promotes TNF-α protein production and results in cardiac dysfunction. TLR4, toll-like receptor 4

reduction of myocardial Na/K-ATPase activity during LPS stimulation was prevented by inhibition of PI3K, Rac1 or NADPH oxidase activities. The present study therefore demonstrated that inhibition of Na/K-ATPase activity enhances LPS-induced TNF-α expression via the PI3K/Rac1/NADPH oxidase pathway.

It is well established that inhibition of Na/K-ATPase activity increases intracellular Ca^{2+} via the Na^{+}/Ca^{2+} exchange [34]. We have previously shown that intracellular Ca^{2+} levels are increased by LPS in cardiomyocytes and contribute to TNF-α expression [15]. To study the role of Rac1, a negative regulator of Na/K-ATPase in Ca^{2+} homeostasis, we measured Ca^{2+} transients in cardiomyocytes and found that Rac1-deficient cardiomyocytes exhibited significantly lower intracellular Ca^{2+} levels compared with $Rac1^{f/f}$ cells during basal conditions and in response to LPS, suggesting that activation of Rac1 increases intracellular Ca^{2+} via inhibition of Na/K-ATPase.

Activation of mTOR stimulates global protein synthesis by directly phosphorylating eukaryotic initiation factor 4E-binding protein and in the longer term increases levels of ribosomal proteins [23, 46]. LPS activates mTOR in phagocytes [1, 20, 53], bone marrow cells [9] and HL-1 cells (a cardiomyocyte cell line) [49]; however, the role of mTOR in pro-inflammatory cytokine expression is cell-type specific. For example, rapamycin, an mTOR inhibitor decreased the production of pro-inflammatory cytokines such as IL-12 and TNF-α in dendritic cells [20]. In contrast, rapamycin enhanced LPS-induced TNF-α mRNA and protein levels in monocytes [53] and macrophages [1]. Similarly, overexpression of mTOR in HL-1 cells decreased TNF-α protein levels stimulated by LPS [49]. In the present study, we showed that mTOR was rapidly and transiently activated between 15 and 30 min after LPS stimulation in neonatal cardiomyocytes cultured in serum-free medium. Our data showed that Na/K-ATPase activity was decreased in the cells cultured in medium containing 10% FBS after 2 h of LPS stimulation. The mismatch between the time points of Na/K-ATPase and mTOR activation is possibly due to different culture conditions with and without serum. Rapamycin decreased TNF-α protein levels after LPS stimulation but had no apparent effect on TNF-α mRNA expression or global protein synthesis. Our data suggest that mTOR is important in LPS-induced TNF-α protein synthesis in cardiomyocytes.

Ca^{2+} is an important regulator of mTOR activation in various types of cells [26, 30, 51]. In primary mouse

neurons, glutamatergic activation of mTOR is Ca^{2+}/CaMK-dependent [30]. In islet beta cells during glibenclamide stimulation, Ca^{2+} activates mTOR via PKA/PKB-dependent and -independent pathways [51]. In PC-12 cells, hypoxia-stimulated influx of Ca^{2+} leads to an activation of mTOR via PKC-α [26]. We have previously shown that intracellular Ca^{2+} mediates LPS-induced TNF-α protein production in cardiomyocytes [15, 55]. The present study showed that LPS-induced Rac1 activation inhibits Na/K-ATPase and subsequently results in increased intracellular Ca^{2+} in cardiomyocytes. CaMK is a downstream target of Ca^{2+} and contributes to LPS-induced TNF-α production [32, 36, 44]. To study the role of CaMK, a downstream effector of Ca^{2+}, in mTOR activation, a selective inhibitor of CaMK was employed. Our data showed that inhibition of CaMK decreased mTOR activity in response to LPS. Furthermore, LPS-induced mTOR activity was blocked by inhibition of Rac1 but enhanced by the inhibition of Na/K-ATPase. These results suggest that Rac1/Na/K-ATPase pathway may activate mTOR via a Ca^{2+}/CaMK-dependent mechanism during LPS stimulation.

Interestingly, mTOR promotes the translation of cellular mRNAs that have a 5′ terminal oligopyrimidine (TOP) tract adjacent to the cap site [46]. The 5′TOP tracts, which are found in all mRNAs that encode ribosomal proteins, enhance translation upon mTOR activation [38]. Examination of the 5′ untranslated region of TNF-α mRNA reveals that both the mouse (GenBank: U68414) and human (GenBank: NM_000594) forms contain a 5′ CTCCCTC sequence, which conforms to the established consensus for regulatory 5′TOP motifs [46]. The presence of this putative 5′TOP motif further supports our hypothesis that TNF-α is controlled at the translational level by mTOR in cardiomyocytes during LPS stimulation. On the contrary, pre-treatment with LPS triggers late preconditioning, which decreases TNF-α production and cardiac injury following myocardial ischemia and reperfusion [4, 52].

In conclusion, our study demonstrated that LPS inhibits Na/K-ATPase activity via PI3K/Rac1/NADPH oxidase pathway in the myocardium. Inhibition of Na/K-ATPase activity promotes myocardial TNF-α protein production and cardiac dysfunction by Ca^{2+}/CaMK-dependent mTOR activation in cardiomyocytes. This Na/K-ATPase/Ca^{2+}/mTOR pathway provides novel insight into the signal transduction mechanisms that regulate myocardial TNF-α expression and cardiac function, and may have implications in the treatment of sepsis (Fig. 8).

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Conflict of interest None declared.

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