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Cardiovascular Research 75 (2007) 408–416

Cardiovascular Research

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Role of neuronal nitric oxide synthase in lipopolysaccharide-induced tumor necrosis factor-alpha expression in neonatal mouse cardiomyocytes

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Received 1 December 2006; received in revised form 14 March 2007; accepted 25 March 2007
Available online 30 March 2007
Time for primary review 18 days

Abstract

Objective: Neuronal nitric oxide synthase (nNOS) has been shown to regulate intracellular calcium in cardiomyocytes. Calcium in turn modulates extracellular signal-related kinase (ERK) signaling, which is important in tumor necrosis factor-alpha (TNF-α) expression during lipopolysaccharide (LPS) stimulation. However, the role of nNOS in LPS-induced TNF-α expression is not known. We hypothesized that nNOS suppresses LPS-induced TNF-α expression by inhibiting the calcium/ERK signaling pathway.
Methods and results: Cultured neonatal mouse cardiomyocytes were challenged with LPS for 4 h. While there was no change in the basal Ca^{2+} concentration, LPS increased peak Ca^{2+} levels. LPS stimulation increased TNF-α mRNA and protein levels in wild-type cells however, the responses were enhanced in $nNOS^{-/-}$ cardiomyocytes. Treatment with an antisense oligonucleotide against nNOS also significantly enhanced TNF-α expression during LPS stimulation. Furthermore, LPS-induced ERK phosphorylation was significantly increased in the $nNOS^{-/-}$ compared to wild-type cardiomyocytes. The enhanced TNF-α expression in $nNOS^{-/-}$ cardiomyocytes was abrogated by an L-type calcium channel blocker verapamil or ERK1 siRNA. Finally, myocardial ERK phosphorylation and TNF-α expression were increased while cardiac function was decreased in endotoxemia in $nNOS^{-/-}$ compared to wild-type mice.
Conclusions: nNOS inhibits LPS-induced TNF-α expression in cardiomyocytes and improves cardiac function in endotoxemia. The inhibitory role of nNOS is mediated by a reduction in L-type calcium channel-dependent ERK signaling in cardiomyocytes.

Keywords: Nitric oxide; Nitric oxide synthase; Calcium; TNF-α; Signal transduction; Sepsis; Cardiomyocyte

1. Introduction

Lipopolysaccharide (LPS) is a major structural component of Gram-negative bacteria and a key mediator of the bodies' response to infection [1]. LPS induces expression of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), which are involved in the pathogenesis of sepsis [2]. During sepsis, increased TNF-α production causes myocardial depression through changes in calcium homeostasis and desensitization of cardiac myofilaments to calcium [3]. Studies from our laboratory have indicated that cardiomyocytes are a major source of TNF-α in the myocardium during sepsis and that inhibition of TNF-α production improves myocardial function and survival in septic mice [4]. Despite the well-established benefits of TNF-α inhibition during endotoxemia, the mediators of increased TNF-α expression are not well understood.

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doi:10.1016/j.cardiores.2007.03.020

Mitogen activated protein kinases (MAPKs) are serine/threonine kinases that are involved in the control of cellular activities such as cell division, gene expression and cell survival [5]. Extracellular signal-regulated kinases (ERKs) are a subset of MAPKs that are activated in the heart during oxidative stress and sepsis [6,7]. In sepsis, the phosphorylation of ERK fosters the expression of TNF-α in several cell types including cardiomyocytes [8,9]. Studies have shown that ERK activation involves a signaling cascade of small GTP binding proteins, and a series of protein kinase-dependent phosphorylations [10]. However, it has been noted that ERK can also be activated by intracellular calcium and through the calcium sensitive signaling protein, calmodulin [8,11,12]. These studies suggest that intracellular calcium levels can modulate TNF-α expression. Indeed, calcium channel blockade is associated with decreased TNF-α levels in a rat septic shock model [13]. Recently, a correlation between calcium levels, ERK phosphorylation and TNF-α expression was established in mononuclear cells following *Mycobacterium bovis* infection [14]. However, the upstream regulators of calcium-dependent TNF-α expression have not been investigated and the role of calcium in regulating ERK phosphorylation and TNF-α expression in cardiomyocytes is unclear.

Neuronal nitric oxide synthase (nNOS) is a constitutively expressed enzyme found predominantly in neuronal cells that produces low levels of nitric oxide during neurotransmission. In cardiomyocytes, nNOS has been localized to the sarcoplasmic reticulum (SR), a critical location for calcium cycling [15]. A recent study by Sears et al. [16] found that nNOS inhibition (genetically, or pharmacologically) resulted in increased cardiac contractility and increased intracellular calcium, indicating that nNOS is an important regulator of intracellular calcium levels in the heart. We recently demonstrated that eNOS enhances TNF-α production in cardiomyocytes after LPS treatment suggesting a role for NOS isoforms in the pathogenesis of sepsis [17]. However, the role of nNOS in LPS-induced TNF-α expression has not been clarified. Considering the role of nNOS in intracellular calcium regulation, it is possible that nNOS may modulate TNF-α expression in sepsis.

The present study sought to investigate the role of nNOS in the expression of TNF-α during sepsis. We hypothesized that nNOS suppresses LPS-induced TNF-α expression in cardiomyocytes. We further hypothesized that the suppression of LPS-induced TNF-α expression is mediated by an inhibition of calcium-dependent ERK activation.

2. Methods

2.1. Animals

Breeding pairs of C57BL/6 and nNOS$^{-/-}$ mice were purchased from the Jackson Laboratory and a breeding program was implemented at our animal care facilities. All animals were provided water and food *ad libitum* and housed in a temperature and humidity controlled facility with 12-hour 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

Cardiomyocyte cultures were prepared as described previously with modifications [18]. Neonatal mouse hearts were removed and then cooled, washed and minced in sodium bicarbonate, Ca^{2+}, and Mg^{2+}-free Hanks balanced salt solution (D-Hanks, Sigma, St. Louis, MO). Cardiomyocytes were dispersed by 10 min of incubation with 22.5 μg/mL liberase 4 (Roche, Laval, Quebec) in D-Hanks at 37 °C with gentle agitation. Cells were collected by centrifugation at 200 *g* for 5 min and re-suspended in 10% FCS-containing M199 medium (Sigma, St. Louis, MO). Removal of non-cardiomyocytes was achieved through 90 min of pre-plating on 35 mm Falcon plates (Benton Dickson, NJ) after which the cardiomyocytes were collected by rinsing, and centrifugation at 200 *g* for 5 min. Cells were incubated in a humidified atmosphere at 37 °C with 5% CO_2 on Falcon plates pre-coated with 1% gelatin. Subconfluence was achieved after 48 h of culture after which treatments with various drugs were performed.

2.3. nNOS blockade using an antisense oligodeoxynucleotide (nNOS ASODN)

In order to account for any compensatory mechanisms active in the nNOS$^{-/-}$ cardiomyocytes, an antisense oligodeoxynucleotide (ASODN) designed against nNOS in the region that codes for the "cardiac" isoform, nNOSμ with the following sequence, 5′-AAC GTG TGC TCT TCC ATG G-3′ was employed. Either a 1 μM or 5 μM solution of the nNOS ASODN was applied to the cardiomyocytes in serum-free M199 24 h prior to 4 h of LPS or control treatment. A mismatched-ODN sequence, 5′-AAGCTGTCCTGTTGCATCC-3′ was used to serve as the control. The effectiveness in specifically attenuating nNOS protein expression by nNOS ASODN was confirmed by Western blot analysis.

2.4. ERK1 inhibition using siRNA

In order to inhibit ERK expression, a small interfering RNA (siRNA) against ERK1 was obtained (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were transfected with 100 nM of ERK1 siRNA, scrambled siRNA (Santa Cruz), or equivalent volume vehicle (transfection reagent without siRNA) as described in our previous studies [9,19].

2.5. Measurement of TNF-α protein levels

TNF-α protein levels were measured using an ELISA kit (ALPCO Diagnostics) for mouse TNF-α as described in our

previous reports [4,17]. In the case of *in vitro* experiments, TNF-α release into 100 μL media was measured. In the case of *in vivo* experiments, TNF-α protein levels were measured from the left ventricular (LV) myocardium. LV tissues were homogenized in phosphate-buffered saline and 200 μg total protein from each sample were used for TNF-α ELISA analysis.

2.6. Measurement of TNF-α and nNOS mRNA levels

Total RNA was extracted from the cardiomyocytes using the Trizol Reagent (Invitrogen) following the manufacturer's instructions. Semi-quantitative RT-PCR was performed as described previously [17]. The sense and antisense primers for TNF-α gene were 5′-CCG ATG GGT TGT ACC TTG TC-3′ and 5′-GGG CTG GGT AGA GAA TGG AT-3′, respectively. The sense and antisense primers for nNOS were 5′ GAG CAG AGC GGC CTT ATC CA 3′ and 5′ TCT CCG CTG TCC TGG AGG TT 3′ respectively. The nNOS primers were designed for Exon 1 which is the region of knockout for this line. To ensure a fixed amount of initial mRNA, parallel glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was performed: the sense primer was 5′ AAA GGG CAT CCT GGG CTA CA 3′, and the antisense primer was 5′ CAG TGT TGG GGG CTG AGT TG 3′. PCR products were separated on 0.8–1.2% agarose. Densitometric analysis was done using Gel Pro Analyzer 3.1 (Media Cybernetics). Data are expressed as the ratio of TNF-α/GAPDH.

2.7. Western blot analysis

The expression of nNOS, eNOS, and iNOS, as well as the phosphorylation status of ERK1/2 was assessed by Western blotting. Aliquots of 40–60 μg were subjected to SDS-PAGE using 10% gels and transferred to nitrocellulose membranes. Blots were probed with antibodies to eNOS, iNOS, phosphorylated ERK1/2, total ERK1/2 (Cell Signaling, 1/1000), nNOS (BD Transduction, 1/750), and actin (loading control, Sigma, 1/5000) and incubated with horseradish peroxidase-conjugated secondary antibodies (BioRad, 1/2000). Detection was performed using the Pierce Supersignal detection method according to the manufacturer's instructions. Data are expressed as the ratio of nNOS, eNOS, iNOS/actin, or phosphorylated /total ERK1/2 proteins.

2.8. Measurement of intracellular calcium

Ca^{2+} transients were measured in isolated neonatal myocytes using fura-2 as described by Sathish et al. [20]. Briefly, cells were seeded on glass coverslips and loaded with 1 μM fura-2 AM (Molecular Probes) for 30 min at 35 °C in M199 containing 10% FCS. Coverslips were removed from the culture-plates and placed in a glass perfusion chamber (0.75 mL vol.) containing modified HEPES buffer (130 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM HEPES, pH 7.4) on the microscope and continuously perfused with bathing solution at 2–3 mL/min at room temperature. Intracellular calcium ($[Ca^{2+}]_i$) responses to LPS (10 μg/mL) were measured in spontaneously beating cardiomyocytes using a Deltascan monochrometer-based system (Photon Technology International, London, ON) connected to a Nikon inverted microscope (Nikon, Melville, NY) operated using Felix software (Photon Technology International).

Fluorescence intensity was measured as described previously [21] with alternating 345 and 380 nm light for excitation and 510 nm emission. The methods of Grynkiewicz et al. [22] were used for calibration of $[Ca^{2+}]_i$ with $[Ca^{2+}]_i = [K_d(R - R_{min})/(R_{max} - R)]Sf_2/Sb_2$, where R_{min} and R_{max} are the ratios of fluorescence intensity at 345 nm to fluorescence intensity at 380 nm with Ca^{2+}-free and saturated conditions, respectively, and Sf_2/Sb_2 is the ratio of fluorescence of Ca^{2+}-free to Ca^{2+}-bound indicator measured at 380 nm. A dissociation constant (K_d) of 225 nM was used for the binding of Ca^{2+} to fura-2, viscosity factor of 0.6 and data were corrected for background fluorescence.

The number of contraction–relaxation cycles quantified varied slightly between each independent experiment but a minimum of five contraction–relaxation cycles were quantified per reading and readings were taken in at least duplicate for each cell culture.

2.9. Endotoxemia and isolated mouse heart preparation

Adult mice (male, aged 5–6 months) were treated with LPS (4 mg/kg, IP) 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 [9]. Briefly, hearts were perfused with Krebs-Henseleit buffer at 2 mL/min constant flow. The perfusion buffer was maintained at 37°C and bubbled continuously with a mixture of 95% O2 and 5% CO2. A 6-0 silk suture was placed through the apex of the left ventricle and threaded through a lightweight rigid coupling rod, which was connected to a force-displacement transducer (FT03, Grass Instrument Co.). Contractile force and heart rate were measured by PowerLab Chart program (ADInstruments, Mountain View, CA). The heart work was calculated by multiplying the force (g) by the heart rate (bpm) and normalized to heart weight. At the end of cardiac function measurements, LV myocardial tissue was collected for determination of ERK1/2 phosphorylation and TNF-α protein expression.

2.10. Data analysis

All results are expressed as the mean±SEM. One-way analysis of variance (ANOVA) followed by the Student Newman–Keul's post hoc test was used as appropriate to detect differences between treatment groups within a genotype. Two-way ANOVA followed by the Bonferroni post hoc test was used to detect differences between genotypes. Statistical significance was assigned when a P value was less than 0.05.

3. Results

3.1. LPS-induced TNF-α expression is enhanced in nNOS$^{-/-}$ cardiomyocytes

Previous studies have shown that LPS administration induces TNF-α production in cultured cardiomyocytes [23] and myocardium [24]. To determine if LPS-induced TNF-α expression is regulated by nNOS, cultured neonatal cardiomyocytes from wild-type (WT) and nNOS$^{-/-}$ mice were treated with LPS (10 μg/mL) for 4 h. TNF-α mRNA and protein levels were examined by RT-PCR and ELISA, respectively. Importantly, the muscle isoform of nNOSμ mRNA was absent in nNOS$^{-/-}$ mice (Fig. 1A). However, very low levels nNOS protein were detected in the hearts of nNOS mice (Fig. 1A). This is consistent with the previous studies showing that this line of nNOS$^{-/-}$ mice has low levels of nNOS expression due to non-muscle splice variants (nNOSβ and nNOSγ) [25]. LPS treatment significantly increased TNF-α mRNA (Fig. 1B) and protein levels (Fig. 1C) in cultured cardiomyocytes. Furthermore, nNOS$^{-/-}$ cardiomyocytes produced significantly more TNF-α in response to LPS challenge than their WT counterparts (Fig. 1).

To determine the effects of LPS on nNOS protein expression, WT cardiomyocytes were treated with LPS (10 μg/mL) for 2, 4, and 8 h. Cells treated with M199 alone served as controls. LPS did not significantly alter nNOS protein levels after 2, 4, or 8 h as measured by Western blot analysis (data not shown).

3.2. nNOS inhibition by ASODN increases LPS-induced TNF-α expression

To eliminate the effects of any compensatory mechanisms that may exist in nNOS$^{-/-}$ mice, WT cardiomyocytes were treated with nNOS ASODN for 24 h prior to LPS exposure (4 h, 10 μg/mL). A mismatched oligodeoxynucleotide (MM-ODN) was employed to serve as a control. To verify the specificity of knockdown of nNOS by ASODN, nNOS, eNOS, and iNOS protein levels were determined by Western blots. While nNOS ASODN (5 μM) markedly inhibited nNOS expression, it had no effect on eNOS protein expression (Fig. 2A). As expected, iNOS protein expression was undetectable under our experimental conditions (Fig. 2A). Application of nNOS ASODN (5 μM) significantly increased TNF-α mRNA expression (Fig. 2B) and TNF-α

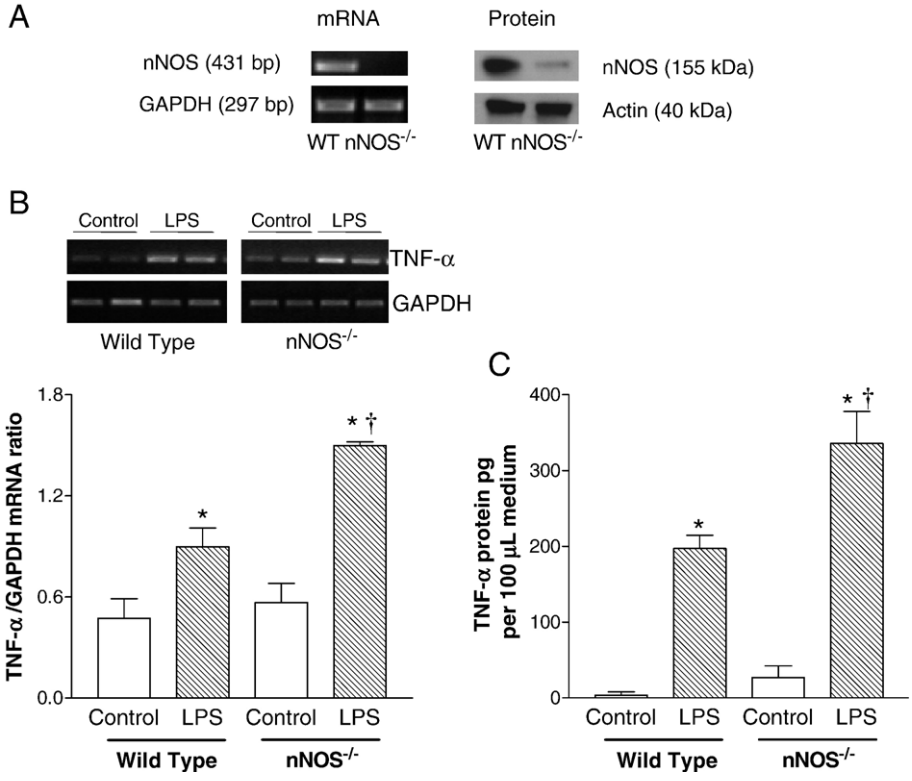

Fig. 1. Effects of LPS on TNF-α mRNA expression and protein levels in neonatal cardiomyocytes from wild-type (WT) and nNOS deficient (nNOS$^{-/-}$) mice. A, PCR and Western blots demonstrating knockout/knockdown of nNOS in nNOS$^{-/-}$ hearts. Gels are nNOS mRNA/protein shown with corresponding loading controls (GAPDH and actin, respectively). B, Cardiomyocytes were treated with LPS (10 μg/mL) for 4 h and TNF-α mRNA expression was measured by RT-PCR. Data is expressed as the ratio of TNF-α:GAPDH, $n=3–5$ independent experiments per group. Upper panel is a representative gel. C, Cardiomyocytes were treated with LPS for 4 h and TNF-α protein levels were measured by ELISA. Data is expressed as the amount of TNF-α protein in 100 μL of media, $n=9–10$ independent experiments per group. *$P<0.05$ vs. corresponding controls, †$P<0.05$ vs. WT with LPS.

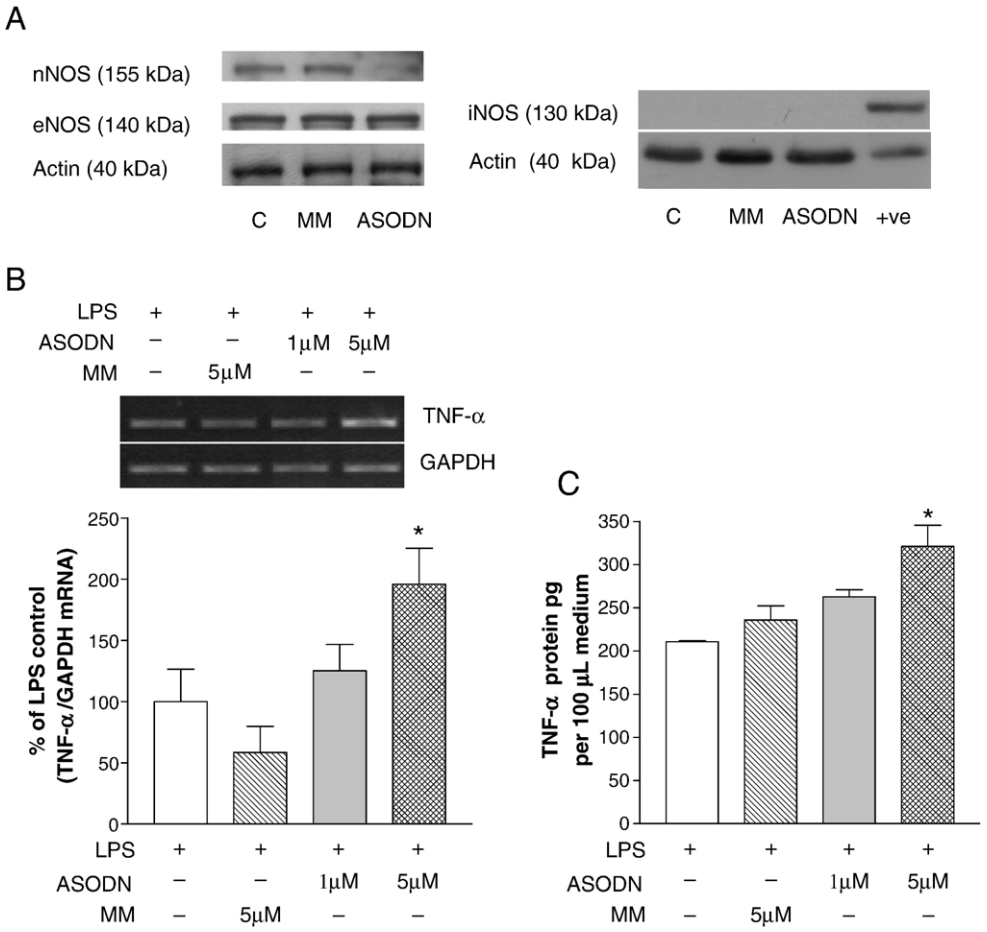

Fig. 2. Effects of nNOS antisense oligodeoxynucleotide (ASODN) on LPS-induced TNF-α. A, Western blots demonstrating specific knockdown of nNOS expression by ASODN. Representative blots are nNOS, eNOS and iNOS protein expression with actin as a loading control. Since iNOS protein was not detectable in cardiomyocytes, a positive control (+ve) was included. B, Cardiomyocytes were treated with LPS (10 μg/mL) for 4 h after a 24 hour pre-treatment with the indicated ODN. Cells were collected for RT-PCR analysis. Data is expressed as the TNF-α:GAPDH ratio as a percentage of LPS control. C, Cardiomyocytes were treated with LPS for 4 h after a 24 hour pre-treatment with the indicated ODN and TNF-α protein levels were measured by ELISA. Data is expressed as the amount of TNF-α/100 μL medium. $n=4$ independent experiments per group, *$P<0.05$ vs. LPS control. MM, mismatched.

protein levels (Fig. 2C) induced by LPS as compared to the MM-ODN treated cardiomyocytes ($P<0.05$). The application of nNOS ASODN alone did not cause any significant changes in TNF-α mRNA or protein levels. Additionally, the application of MM-ODN (5 μM) did not have any significant effects on LPS-induced TNF-α expression (Fig. 2B and C).

3.3. LPS-induced ERK1/2 phosphorylation is enhanced in $nNOS^{-/-}$ cardiomyocytes

ERK phosphorylation is an important regulator of LPS-induced TNF-α expression in cardiomyocytes [9]. To determine the role of ERK phosphorylation in the enhanced TNF-α expression during LPS stimulation in $nNOS^{-/-}$ cardiomyocytes, ERK1/2 phosphorylation was examined. Basal ERK1/2 phosphorylation was not different between WT and $nNOS^{-/-}$ cardiomyocytes (Fig. 3). LPS treatment resulted in significant increases in ERK1/2 phosphorylation in both WT and $nNOS^{-/-}$ cells. However, ERK1/2 phosphorylation increased by a further 75% in $nNOS^{-/-}$ compared to WT cardiomyocytes, suggesting an enhancement of ERK1/2 phosphorylation in the $nNOS^{-/-}$ cells (Fig. 3).

3.4. ERK inhibition diminishes LPS-induced TNF-α expression

In order to establish a causal relationship between ERK phosphorylation and downstream TNF-α expression, 100 nM ERK1 siRNA, or scrambled control siRNA was applied to cardiomyocytes 24 h prior to LPS or control treatment. Our recent studies have demonstrated that this siRNA inhibits ERK1 mRNA expression [19]. In the present study, treatment with ERK1 siRNA markedly decreased ERK1 protein expression with little effect on ERK2 while scrambled siRNA had no effect (Fig. 4A). Furthermore, ERK1 siRNA significantly decreased LPS-induced TNF-α protein levels as compared to scrambled or vehicle treated controls in both WT and $nNOS^{-/-}$ cardiomyocytes (Fig. 4B).

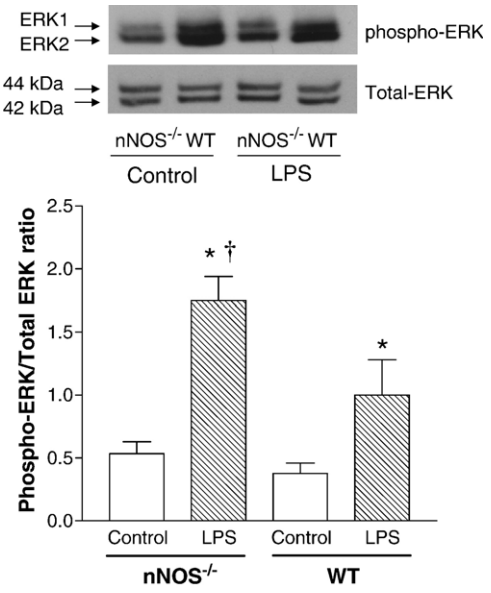

Fig. 3. Effects of LPS and nNOS on ERK phosphorylation in neonatal mouse cardiomyocytes. Cardiomyocytes from wild-type (WT) and nNOS deficient ($nNOS^{-/-}$) mice were treated with LPS (10 μg/mL) for 4 h and ERK1/2 phosphorylation was examined by Western blot analysis. Top panel shows a representative blot. Lower panel shows quantitative data expressed as the ratio of phosphorylated ERK1/2 to total ERK1/2. $n=7$ independent experiments per group. *$P<0.05$ vs. corresponding controls, †$P<0.05$ vs. WT with LPS.

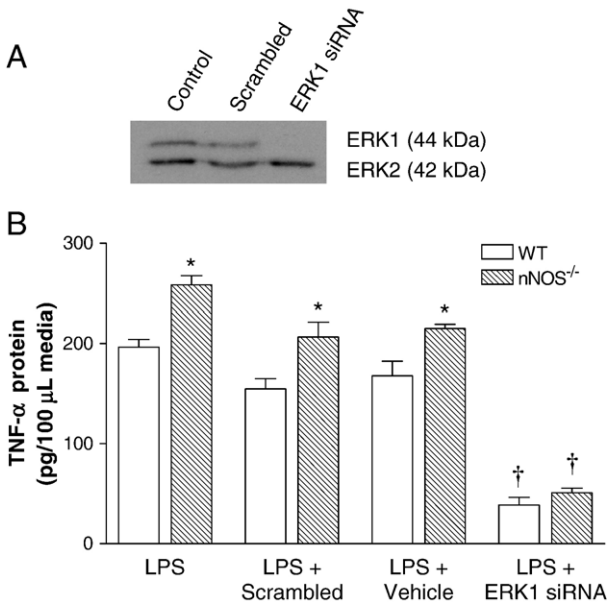

Fig. 4. Effects of ERK1 siRNA on LPS-induced TNF-α protein expression. A, Western blot demonstrating specific knockdown of ERK1 by ERK1 siRNA. Cardiomyocytes were treated with media (control), scrambled siRNA, or ERK1 siRNA (100 nM) for 24 h. B, Cardiomyocytes were treated with media, scrambled siRNA, vehicle (transfection reagent), or ERK1 siRNA for 24 h prior to LPS administration (10 μg/mL for 4 h). TNF-α protein levels were measured by ELISA and are expressed as the amount of TNF-α protein/100 μL culture medium. $n=3$–7 independent experiments per group. *$P<0.05$ vs. WT counterpart, †$P<0.05$ vs. all other corresponding LPS treated groups.

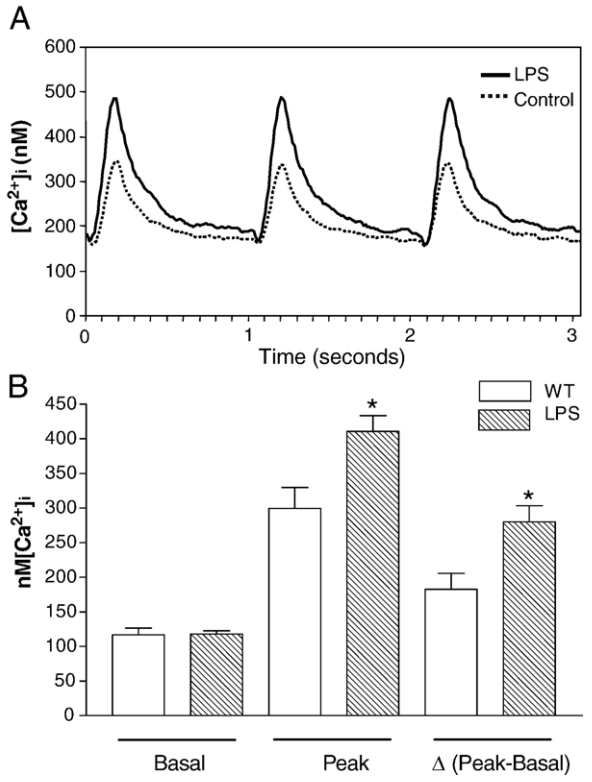

Fig. 5. Effects of LPS on intracellular calcium concentrations in wild-type neonatal cardiomyocytes. Cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by microspectro-fluorometry in spontaneously beating cardiomyocytes loaded with fura-2 (1 μM). Cardiomyocytes were treated with LPS (10 μg/mL) for 30 min. A, Representative traces for control and LPS treated neonatal myocytes have been aligned and superimposed to illustrate increased peak $[Ca^{2+}]_i$ in LPS treated neonatal myocytes. B, Data expressed as the average basal, peak and Δ (peak-basal) calcium concentrations for 5 contraction–relaxation cycles. *$P<0.05$ vs. control, $n=4$ independent experiments.

3.5. LPS increases calcium transients in cardiomyocytes

To determine the role of calcium in mediating LPS-induced TNF-α expression, cardiomyocytes were cultured and loaded with the calcium sensitive dye fura-2 (1 μM). Cardiomyocytes were then exposed to LPS treatment (10 μg/mL) or control HEPES buffer for 30 min. After LPS treatment, diastolic $[Ca^{2+}]_i$ did not change, however, the peak amplitude of $[Ca^{2+}]_i$ transients was significantly increased with respect to control (Fig. 5).

3.6. Inhibition of L-type calcium channels attenuates LPS-induced TNF-α expression

To determine if elevated calcium levels following LPS administration are responsible for ERK1/2 phosphorylation and TNF-α expression, WT and $nNOS^{-/-}$ cardiomyocytes were pre-treated with a L-type calcium channel inhibitor verapamil (100 μM) for 4 h before LPS (10 μg/mL) challenge. In the absence of verapamil pre-treatment, $nNOS^{-/-}$ cardiomyocytes exhibited increased TNF-α levels following

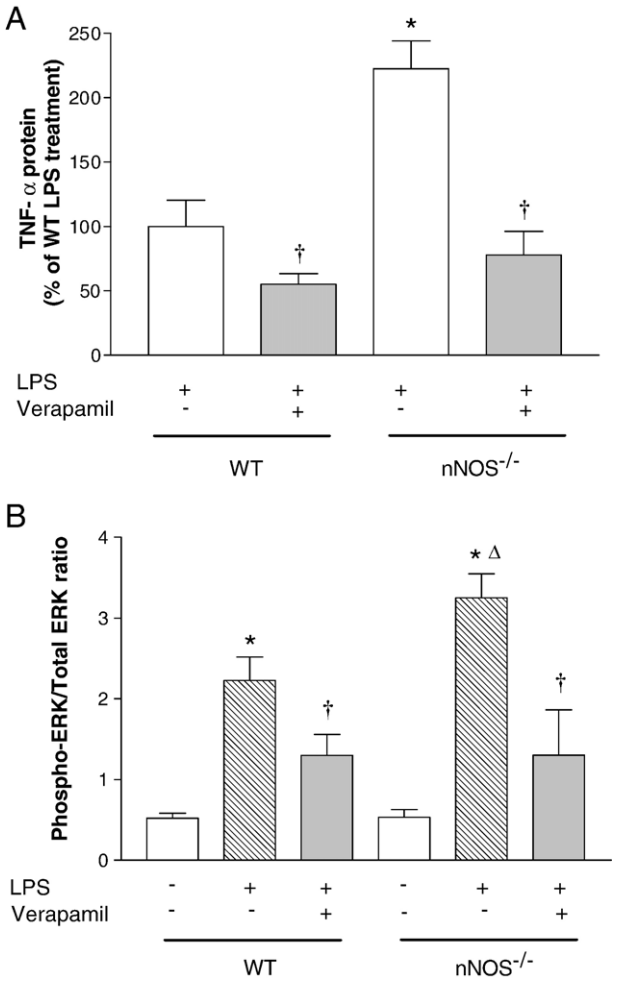

Fig. 6. Effects of calcium channel inhibition on LPS-induced TNF-α expression and ERK1/2 phosphorylation. Cultured wild-type (WT) and nNOS$^{-/-}$ cardiomyocytes were treated concurrently with verapamil (100 μM) and LPS (10 μg/mL) for 4 h. A, TNF-α protein levels in 100 μL culture medium were measured by ELISA. Data is expressed as % of wild-type cells with LPS treatment. *P<0.05 vs. WT LPS, †P<0.05 vs. corresponding LPS alone. B, ERK1/2 phosphorylation in WT and nNOS$^{-/-}$ cardiomyocytes determined by Western blotting. Data is expressed as the ratio of phosphorylated ERK (P-ERK) to total ERK levels. *P<0.05 vs. corresponding untreated controls, †P<0.05 vs. corresponding LPS alone, ΔP<0.05 vs. WT+LPS. n=5–8 independent experiments per group.

LPS administration (Fig. 6A). However, pre-treatment with verapamil significantly decreased TNF-α protein expression induced by LPS in both WT and nNOS$^{-/-}$ cardiomyocytes (Fig. 6A). Furthermore, pre-treatment with verapamil also significantly attenuated LPS-induced ERK1/2 phosphorylation in both WT and nNOS$^{-/-}$ cells (Fig. 6B).

3.7. Myocardial TNF-α expression and cardiac function in endotoxemia

To explore the role of nNOS in myocardial depression during endotoxemia, WT and nNOS$^{-/-}$ mice were treated with LPS (4 mg/kg, IP). Four hours after LPS treatment, nNOS$^{-/-}$ mice were more lethargic than WT mice, indicating much severe symptoms of endotoxemia in nNOS$^{-/-}$ mice. Cardiac function was measured in isolated hearts to avoid reflex influences and changes in loading conditions of the heart during endotoxemia. Although there was no significant difference in heart rate between the two groups, the heart work was significantly decreased in nNOS$^{-/-}$ compared with WT mice (P<0.05, Fig. 7A). Furthermore, myocardial TNF-α protein levels were significantly increased in nNOS$^{-/-}$ mice under both control and endotoxemic conditions compared to corresponding WT mice (P<0.05, Fig. 7B). Myocardial ERK phosphorylation was increased in endotoxemia in nNOS$^{-/-}$ compared to WT mice (P<0.05, Fig. 7C).

4. Discussion

The present study examined the role of nNOS in LPS-induced TNF-α expression in cardiomyocytes. The major findings were as follows. First, LPS-induced TNF-α expression was enhanced in cardiomyocytes lacking the nNOS gene. This effect was mediated by ERK signaling as ERK1/2 phosphorylation was further increased in LPS-treated nNOS$^{-/-}$ cardiomyocytes and the enhanced TNF-α expression during LPS stimulation was inhibited by an ERK1 siRNA. Second, LPS treatment significantly increased intracellular calcium transients in cardiomyocytes. Third, LPS-induced ERK1/2 phosphorylation and TNF-α expression were decreased by the inhibition of L-type calcium channels with verapamil. Finally, cardiac function was decreased in endotoxemia in nNOS$^{-/-}$ compared to WT mice. These results suggest that nNOS inhibits LPS-induced TNF-α expression in cardiomyocytes and improves cardiac function in endotoxemia. The effects of nNOS are mediated at least in part by inhibition of calcium-dependent ERK1/2 signaling.

Nitric oxide synthase (NOS) has three isoforms: nNOS, inducible (iNOS) and endothelial NOS (eNOS). Cardiomyocytes constitutively express both nNOS and eNOS while iNOS can be induced in cardiomyocytes with a minimum of 6 h of LPS treatment [17]. To avoid any potential effects of iNOS expression on our study results, experiments were carried out at 4 h after LPS treatment when iNOS was not detectable [17]. NO modulates cardiac TNF-α production. In this regard, we recently demonstrated that NO derived from eNOS promotes LPS-induced TNF-α expression in cardiomyocytes [17]. However, the role of nNOS in LPS-induced TNF-α expression is not known. In the present study, basal TNF-α mRNA and protein levels were similar between nNOS$^{-/-}$ and WT cardiomyocytes. After LPS challenge, TNF-α expression was enhanced in the nNOS$^{-/-}$ cardiomyocytes and in cardiomyocytes treated with nNOS ASODN. Our study demonstrated for the first time that nNOS inhibits LPS-induced TNF-α expression in cardiomyocytes. These effects of nNOS are in direct opposition to the effects of eNOS found in our previous studies [17], suggesting a Ying and Yang relationship between nNOS and eNOS in the regulation of TNF-α expression during LPS stimulation. The unique actions of nNOS in cardiomyocytes

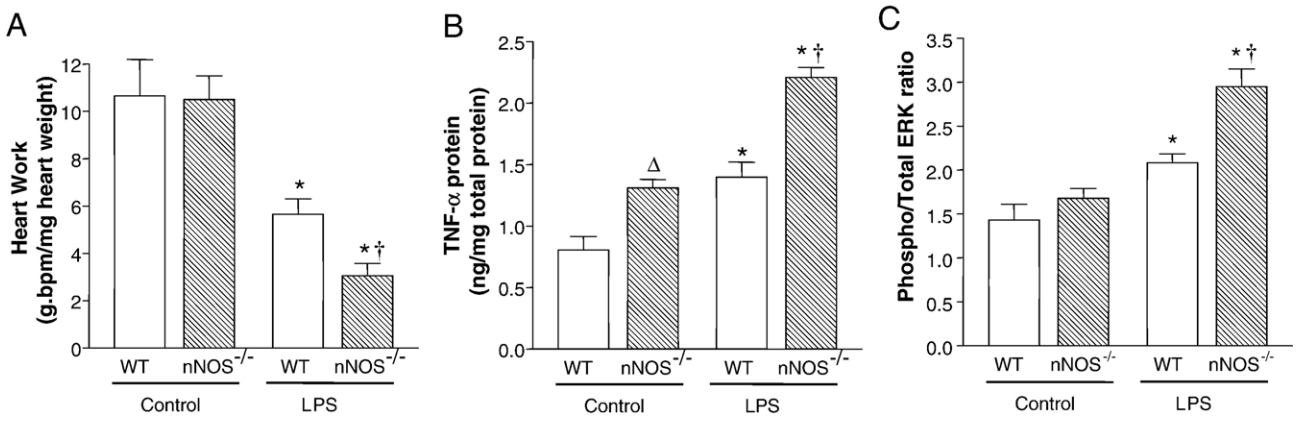

Fig. 7. Myocardial TNF-α expression and cardiac function in endotoxemia in wild-type (WT) and nNOS$^{-/-}$ mice. Adult mice were treated with lipopolysaccharide (LPS, 4 mg/kg, IP) for 4 h. A, Cardiac function was measured as heart work (g·bpm/mg heart weight) in isolated hearts. B, Myocardial TNF-α protein levels (ng/mg total protein) were determined by ELISA. C, ERK phosphorylation was determined by Western blot analysis. *$P<0.05$ vs. corresponding controls, †$P<0.05$ vs. WT+LPS treatment, Δ$P<0.05$ vs. WT controls. $n=5$–6 per group.

prompted us to further investigate its signal transduction mechanisms on the LPS-induced TNF-α expression in cardiomyocytes.

nNOS is located in the sarcoplasmic reticulum of cardiomyocytes and plays an important role in cardiomyocyte Ca^{2+} homeostasis [15,16]. Studies have shown that deficiency in nNOS results in intracellular Ca^{2+} overload in cardiomyocytes mainly through activation of L-type Ca^{2+} channels [16]. As increases of Ca^{2+} entry from L-type Ca^{2+} channels activate ERK [8,26], we hypothesized that LPS-induced ERK phosphorylation was increased in nNOS$^{-/-}$ cardiomyocytes, leading to increased TNF-α expression. In the present study, we demonstrated that treatment with LPS significantly increased the size of the Ca^{2+} transient in the WT cardiomyocytes. LPS stimulation also increased ERK1/2 phosphorylation in cardiomyocytes. Furthermore, we demonstrated that LPS-induced ERK phosphorylation was enhanced in nNOS$^{-/-}$ cardiomyocytes. These data suggest that nNOS has an inhibitory role in LPS-induced ERK1/2 activation in cardiomyocytes. To further demonstrate the contribution of L-type Ca^{2+} channels in the enhanced ERK1/2 phosphorylation, cardiomyocytes were treated with an L-type Ca^{2+} channel blocker verapamil. Consistent with our hypothesis, treatment with verapamil significantly decreased LPS-induced ERK1/2 phosphorylation in both nNOS$^{-/-}$ and WT cardiomyocytes.

Recent studies from our lab and others have shown that ERK1/2 phosphorylation is an important mediator of LPS-induced TNF-α expression [8,9,27]. To demonstrate the role of ERK activation in the enhanced TNF-α expression in NOS$^{-/-}$ cardiomyocytes during LPS stimulation, ERK1 siRNA was employed in the present study to selectively inhibit ERK1 expression in cardiomyocytes. Treatment with an ERK1 siRNA significantly inhibited LPS-induced TNF-α expression in nNOS$^{-/-}$ cardiomyocytes. These data support the notion that enhanced ERK phosphorylation contributes to the enhanced TNF-α expression during LPS stimulation in nNOS$^{-/-}$ cardiomyocytes.

Myocardial TNF-α expression contributes to the impaired cardiac function in sepsis. To further study the physiological significance of enhanced LPS-induced ERK phosphorylation and TNF-α expression in nNOS$^{-/-}$ cardiomyocytes, an *in vivo* model of sepsis was employed in the present study. Myocardial ERK1/2 phosphorylation and TNF-α protein levels were increased while cardiac function was decreased in endotoxemia in nNOS$^{-/-}$ compared to WT mice. The results suggest that nNOS inhibits myocardial TNF-α production and improves cardiac function in endotoxemia. Unexpectedly, myocardial TNF-α protein levels were significantly increased in control nNOS$^{-/-}$ hearts by the Langendorff procedure, which involves a brief period of ischemia (less than 5 min) during heart isolation followed by reperfusion for about 30 min. However, the increase in myocardial TNF-α was not associated with increases in ERK activation or changes in cardiac function. The physiological significance and underlying mechanisms of ischemia/reperfusion-induced TNF-α expression in nNOS$^{-/-}$ hearts require further investigation.

Previous studies on nNOS expression following LPS administration have shown inconsistent results. For example, nNOS mRNA expression increased in the paraventricular nucleus [28] and vena cava [29] of mice after 2 h of intraperitoneal LPS injection. However, in adult rat cardiac myocytes, nNOS mRNA decreased after 6 h of LPS treatment [30]. Thus, the effect of LPS on nNOS expression may be tissue- and time-dependent. In the present study, LPS treatment for up to 8 h did not have any effects on nNOS protein expression in the cultured neonatal mouse cardiomyocytes. Our data suggest that the inhibitory effects on LPS-induced ERK1/2 phosphorylation and TNF-α expression appear to be derived from the nNOS constitutively expressed in cardiomyocytes.

In summary, the present study demonstrated a novel inhibitory role for nNOS in the regulation of LPS-induced TNF-α expression in cardiomyocytes. This inhibition is

achieved by a reduction in L-type calcium channel-dependent ERK signaling. Furthermore, nNOS$^{-/-}$ mice exhibited significantly enhanced myocardial TNF-α expression and diminished cardiac function during endotoxemia. We conclude that nNOS plays an important role in the regulation of TNF-α expression and cardiac function in sepsis.

Acknowledgements

This study was supported by operating grants from the Canadian Institutes of Health Research (#MGP-14653, Q. Feng; MOP-10019, S. Sims) and the Heart & Stroke Foundation of Ontario (HSFO, T-5306, Q. Feng). Dr. Feng is a HSFO Career Investigator and a recipient of a Premier's Research Excellence Award (PREA) from the Province of Ontario. N. Geoghegan-Morphet was supported by an NSERC Graduate Scholarship. D. Burger was supported by a Master's Studentship Award from HSFO. V. Sathish was supported by a postdoctoral fellowship from the HSFO Program Grant in Heart Failure.

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