

Endothelial Nitric-oxide Synthase Enhances Lipopolysaccharide-stimulated Tumor Necrosis Factor- α Expression via cAMP-mediated p38 MAPK Pathway in Cardiomyocytes*

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The purpose of this study was to investigate the role of endothelial nitric-oxide synthase (eNOS), cAMP, and p38 MAPK in tumor necrosis factor- α (TNF- α) expression induced by lipopolysaccharide (LPS). LPS dose- and time-dependently induced phosphorylation of p38 MAPK and TNF- α expression in neonatal mouse cardiomyocytes. TNF- α expression was preceded by p38 MAPK phosphorylation, and selective inhibition of p38 MAPK abrogated LPS-induced TNF- α expression. Deficiency in eNOS decreased basal and LPS-stimulated TNF- α expression in cardiomyocytes. NOS inhibitor L-NAME attenuated LPS-induced p38 MAPK phosphorylation and TNF- α production in wild-type cardiomyocytes, whereas NO donor 2,2'-(hydroxynitrosohydrazono)bis-ethanamine (DETA-NO) (2 μ M) or overexpression of eNOS by adenoviral gene transfer restored the response of eNOS^{-/-} cardiomyocytes to LPS. These effects of NO were mediated through cAMP-dependent pathway based on the following facts. First, deficiency in eNOS decreased basal levels of intracellular cAMP, and DETA-NO elevated intracellular cAMP levels in eNOS^{-/-} cardiomyocytes. Second, a cAMP analogue 8-Br-cAMP mimicked the effect of NO in eNOS^{-/-} cardiomyocytes. Third, either inhibition of cAMP or cAMP-dependent protein kinase attenuated LPS-stimulated p38 MAPK phosphorylation and TNF- α production in wild-type cardiomyocytes. In conclusion, eNOS enhances LPS-stimulated TNF- α expression in cardiomyocytes. Activation of p38 MAPK is essential in LPS-stimulated TNF- α expression. Moreover, the effects of NO on LPS-stimulated TNF- α expression are mediated through cAMP/cAMP-dependent protein kinase-dependent p38 MAPK pathway in neonatal cardiomyocytes.

function in intact animals, isolated hearts, and cardiomyocytes (3–5). TNF- α -induced myocardial dysfunction has two distinct phases. The early phase, which occurs within minutes after TNF- α exposure, is related to sphingosine production leading to disturbances of intercellular Ca²⁺ homeostasis (6). The late phase, which occurs hours after TNF- α exposure, is mediated by iNOS expression, apoptosis, and inhibition of both pyruvate dehydrogenase activity and mitochondrial function (7, 8). In contrast to the detrimental paradigm, treatment of TNF- α improves survival of TNF- α ^{-/-} mice infected with encephalomyocarditis virus in a dose-dependent manner by increasing viral clearance (9). Deficiency in TNF- α receptor function increased infarct size after myocardial ischemia (10). These studies suggest that TNF- α has a beneficial role in viral infections and myocardial ischemia.

Lipopolysaccharide (LPS) of Gram-negative bacteria has been recognized as a causative agent in myocardial depression during sepsis. Cardiomyocytes produce TNF- α in response to LPS exposure (4, 11). However, the signaling pathways of LPS-induced TNF- α expression in cardiomyocytes have not been fully defined. Previous studies have been focused on Toll-like receptors (TLR) (12–14) and nuclear factor- κ B (NF- κ B) in cardiomyocytes (14, 15). Little is known about the signaling downstream of TLR and upstream of NF- κ B. A crucial role of p38 MAPK has been demonstrated in TNF- α expression induced by LPS in neutrophils (16). However, whether p38 MAPK is involved in LPS-stimulated TNF- α expression in cardiomyocytes remains to be determined.

Nitric-oxide synthase has three isoforms that serve different physiological functions as a result of their pattern of expression and the amount of nitric oxide (NO) produced upon activation (17, 18). Neuronal nitric-oxide synthase is mainly expressed in neuronal cells and regulates neurotransmission (19). Inducible nitric-oxide synthase (iNOS) is expressed during inflammation or cytokine stimulation and produces high levels of NO that may be toxic to undesired microbes or tumor cells but may also harm healthy tissues (8, 17, 20). Endothelial nitric-oxide synthase (eNOS) is constitutively expressed in a variety of cells including endothelial cells and cardiomyocytes. Activation of eNOS produces low levels of NO, which has been implicated as a signaling molecule of many cellular processes, including

Tumor necrosis factor- α (TNF- α),¹ a pro-inflammatory cytokine with multiple biological actions (1, 2), impairs contractile

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¹ The abbreviations used are: TNF- α , tumor necrosis factor- α ; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NO, nitric oxide; NOS, nitric-oxide synthase; eNOS, endothelial nitric-oxide

synthase; iNOS, inducible nitric-oxide synthase; AC, adenylate cyclase; GC, guanylate cyclase; PKA, cAMP-dependent protein kinase; L-NAME, N^ω-nitro-L-arginine methyl ester; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3- α]quinoxalin-1-one; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; NF- κ B, nuclear factor- κ B; DETA-NO, 2,2'-(hydroxynitrosohydrazono)bis-ethanamine; TLR, Toll-like receptors; SNAP, S-nitroso-N-acetylpenicillamine.

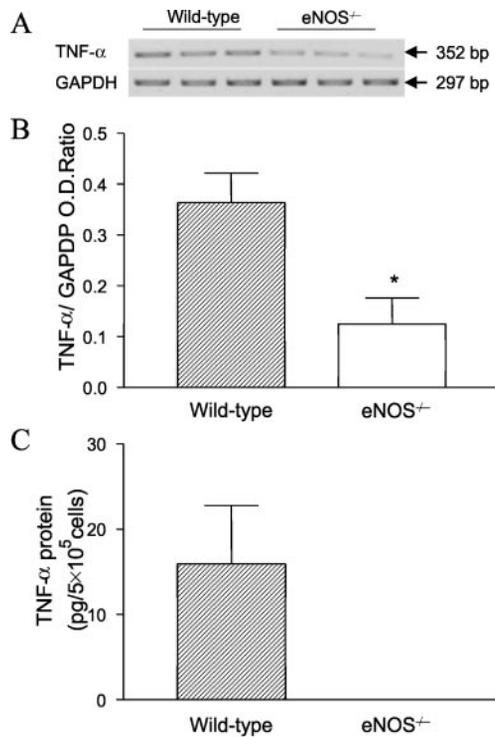


FIG. 1. Basal TNF- α expression in neonatal cardiomyocytes. Neonatal cardiomyocytes were prepared from wild-type and eNOS^{-/-} mice as indicated under "Experimental Procedures." After 48 h of cell culture, TNF- α protein was measured in culture medium by ELISA, and TNF- α mRNA was analyzed in cardiomyocytes by RT-PCR. *A*, representative gel of TNF- α and GAPDH amplification. *B*, TNF- α /GAPDH optical density ratio. TNF- α mRNA expression was significantly decreased in eNOS^{-/-} mouse cardiomyocytes, compared with the wild-type cardiomyocytes. *C*, very low levels of TNF- α protein were measured in the culture medium of wild-type cardiomyocytes but were not detectable in eNOS^{-/-} mouse cardiomyocytes. Measurements were made in triplicate. Data are means \pm S.D. from 3 to 6 independent experiments. *, $p < 0.05$ versus wild-type.

endothelium-dependent relaxation of blood vessels, angiogenesis, inhibition of platelet aggregation, and modulation of myocardial function (17, 21, 22). These actions of NO have been ascribed to either the production of cGMP via soluble guanylate cyclase (GC) and activation of cGMP-dependent protein kinase or the production of cAMP via adenylate cyclase (AC) and activation of cAMP-dependent protein kinase (PKA) in cardiomyocytes (23, 24). Exogenous NO donors have been demonstrated to induce TNF- α production in differentiated U937 cells and in the myocardium (24, 25). However, it remains unknown if endogenously produced NO from eNOS modulates TNF- α expression during LPS stimulation in cardiomyocytes.

The purpose of this study was to investigate the role of eNOS in regulation of TNF- α expression upon LPS stimulation and to determine the contribution of p38 MAPK, cGMP, as well as cAMP to LPS-induced TNF- α expression in neonatal mouse cardiomyocytes.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Neonatal Mouse Cardiomyocytes—C57BL/6 wild-type, iNOS^{-/-} (stock number 2609), and eNOS^{-/-} (stock number 2684) mice were purchased from The Jackson Laboratory. A breeding program was carried out to produce neonates. All animals were used in accordance with the guidelines of the Animal Care Committee at the University of Western Ontario, Canada. The neonatal cardiomyocytes were prepared according to methods we described previously (8). Briefly, ventricular myocardial tissues from wild-type, iNOS^{-/-}, or eNOS^{-/-} mice born within 24 h were minced in a nominally Ca²⁺- and Mg²⁺-free Hanks' balanced solution. Cardiomyocytes were dispersed by 0.625 mg/ml collagenase (type II) at 37 °C for 40 min. The isolated cells were pre-plated for 90 min to remove non-cardiomyocytes. The cardio-

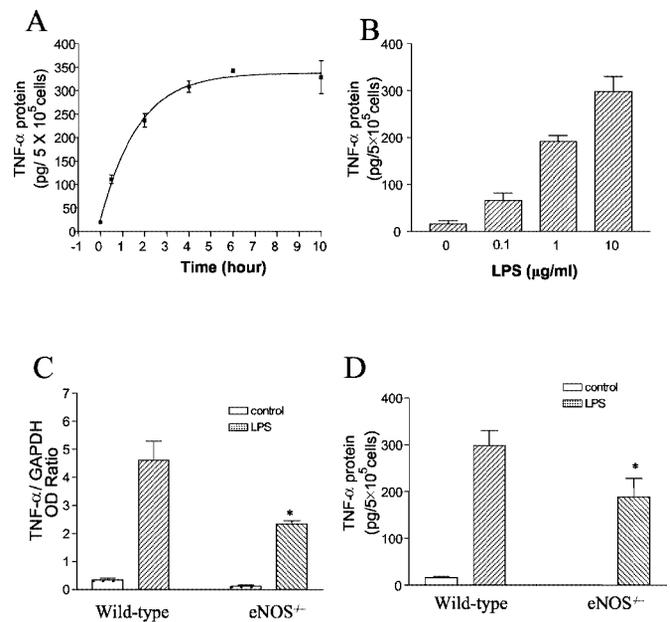


FIG. 2. LPS induction of TNF- α expression in wild-type and eNOS^{-/-} neonatal cardiomyocytes. After 48 h of culture, cardiomyocytes were treated by LPS. *A*, time course of LPS-induced TNF- α protein production. TNF- α protein was increased within 30 min and reached the maximal levels after 4 h of LPS treatment (10 μ g/ml). *B*, LPS (0.1–10 μ g/ml) dose-dependently induced TNF- α protein release in wild-type cardiomyocytes. After LPS treatment (10 μ g/ml), TNF- α mRNA (*C*) and protein release (*D*) were significantly decreased in eNOS^{-/-} neonatal cardiomyocytes, compared with wild-type cardiomyocytes. LPS were treated for 4 h in *B–D*. Measurements were made in triplicate. Data are mean \pm S.D. from 3 to 6 independent experiments. *, $p < 0.05$ versus wild-type.

myocytes were plated in M199 medium containing 10% fetal calf serum in 35-mm Petri dishes pre-coated with 1% gelatin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. After 48 h of cell culture, cardiomyocytes were treated with different drugs either alone or in combination.

Adenoviral Infection of Neonatal Mouse Cardiomyocytes—Cardiomyocytes from eNOS^{-/-} mice were infected with adenoviral vectors containing bovine eNOS gene (Adv-eNOS, a gift from Dr. Z. Katusic) or containing green fluorescence protein (Adv-GFP, a gift from Dr. J. Lipp) as a negative control at a multiplicity of infection of 10 plaque-forming units/cell. Adenovirus-mediated gene transfer was implemented by adding a minimal volume of the medium 199 with 2% fetal calf serum containing gene-carrying adenoviruses. After culture for 2 h, the full volume of culture medium containing 10% fetal calf serum was supplied. All experiments were performed after 24 h of adenoviral infection.

Drugs—Salmonella typhosa LPS, N^ω-nitro-L-arginine methyl ester (L-NAME), 1H-(1,2,4)oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), 2,2'-(hydroxynitrosodiazono)bis-ethanamine (DETA-NO), S-nitroso-N-acetylpenicillamine (SNAP), cycloheximide, and 8-Br-cGMP were purchased from Sigma. SB203580, SB202190, 8-Br-cAMP, SQ22536, and H89 were purchased from Calbiochem.

Measurement of TNF- α Protein and Intracellular cAMP and cGMP—TNF- α levels in the culture media were determined using a mouse TNF- α ELISA Kit (ALPCO Diagnostics) according to the manufacturer's instructions. The values were standardized with the number of cardiomyocytes. Intracellular cAMP or cGMP levels were measured by a cAMP or cGMP enzyme immunoassay (EIA) system (Amersham Biosciences) following the manufacturer's instructions. The values were presented as the levels of cAMP or cGMP to total proteins.

Analysis of TNF- α mRNA by Semi-quantitative Reverse Transcriptase (RT)-PCR—Total RNA was extracted from the cardiomyocytes using the Trizol Reagent (Invitrogen) following the manufacturer's instructions. Semi-quantitative RT-PCR was performed as we described previously (8, 26) with modifications. The DNA oligonucleotide primers for TNF- α were selected from the published sequence of TNF- α gene (GenBankTM accession number M13049). 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. Negative controls using H₂O and samples without reverse transcription were

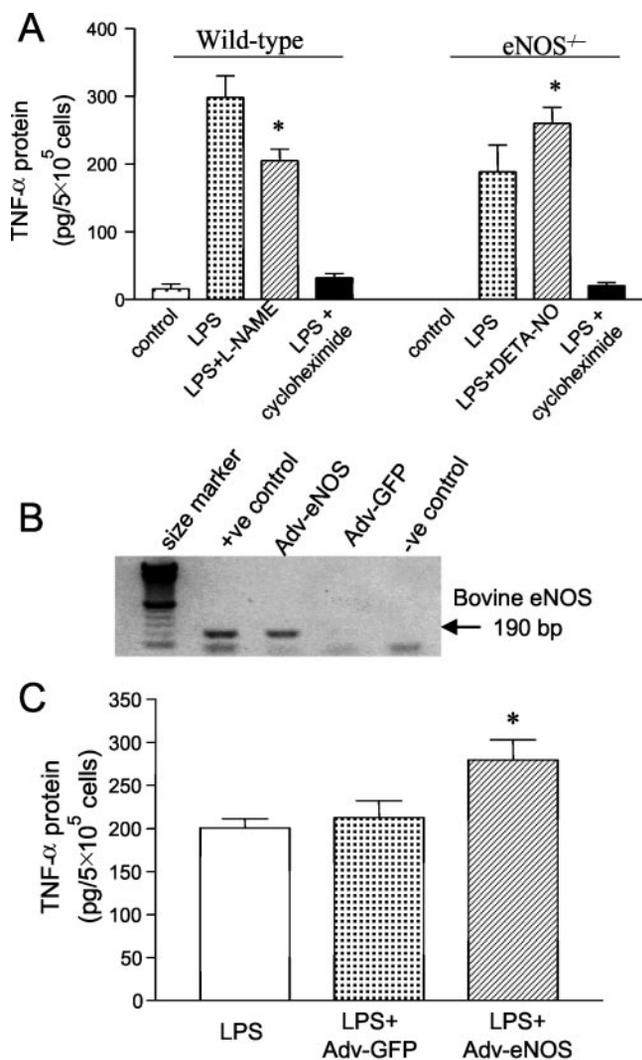


FIG. 3. Effects of NO on LPS-induced TNF- α production in cardiomyocytes. Neonatal cardiomyocytes were isolated from wild-type and eNOS^{-/-} mice and cultured for 48 h. **A**, wild-type cardiomyocytes were treated with LPS (10 μ g/ml) alone or in the presence of nitric-oxide synthase inhibitor L-NAME (500 μ M). L-NAME decreased LPS-induced TNF- α production in wild-type cardiomyocytes by 35% (*, $p < 0.05$ versus wild-type LPS). eNOS^{-/-} cardiomyocytes were treated with LPS (10 μ g/ml) alone or in the presence of NO donor DETA-NO (2 μ M) for 4 h. DETA-NO increased LPS-induced TNF- α in eNOS^{-/-} cardiomyocytes by 30% (*, $p < 0.05$ versus eNOS^{-/-} LPS). Treatment of protein synthesis inhibitor cycloheximide (20 μ M) abolished LPS-induced TNF production in both wild-type and eNOS^{-/-} cardiomyocytes. **B**, confirmation of eNOS mRNA expression by RT-PCR after bovine eNOS gene was re-introduced in eNOS^{-/-} cardiomyocytes. DNA from Adv-eNOS was used as a positive control for amplification of bovine eNOS expression and total RNA from Adv-eNOS-infected eNOS^{-/-} cardiomyocytes without RT as a negative control. **C**, eNOS^{-/-} cardiomyocytes were infected with Adv-eNOS or Adv-GFP for 24 h before treatment with LPS. Adv-eNOS infection increased LPS-induced TNF- α protein production in eNOS^{-/-} cardiomyocytes by 26% (*, $p < 0.05$ versus LPS+Adv-GFP). Measurements were made in triplicate. Data are means \pm S.D. from 3 to 7 independent experiments.

included in every run. Each sample was amplified three times consecutively in a thermal cycler (Hybaid). To ensure a fixed amount of initial mRNA, parallel glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was performed using the following primers based on GAPDH gene sequence (GenBankTM accession number M17701): sense 5' AAA GGG CAT CCT GGG CTA CA 3', and antisense 5' CAG TGT TGG GGG CTG AGT TG 3'. Semi-quantitative RT-PCR detection was based on the optimal conditions for each set of primers derived from calibration curves (data not shown). cDNAs were exponentially amplified for 28 cycles and 27 cycles for the TNF- α and GAPDH genes, respectively. PCR products were separated on 1.2% agarose. The pho-

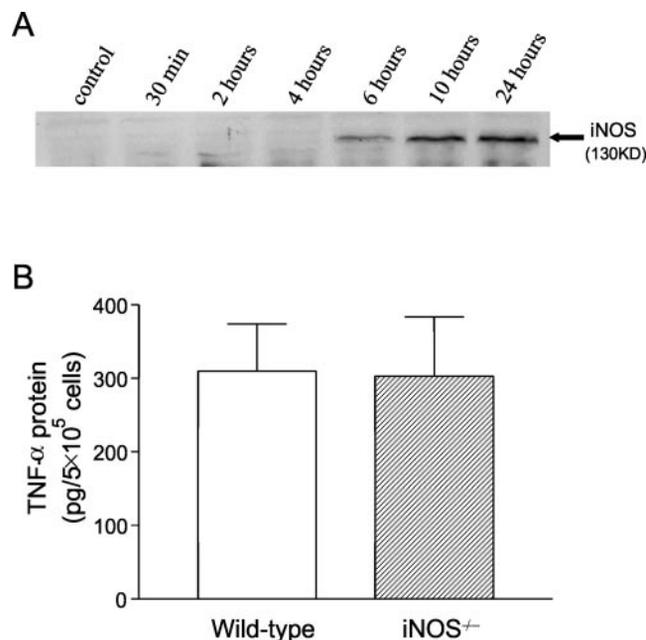


FIG. 4. Involvement of iNOS in LPS-induced TNF- α protein production in cardiomyocytes. **A**, the time course of iNOS protein expression in cultured neonatal cardiomyocytes in response to LPS. After addition of LPS (10 μ g/ml) in culture medium, the cells were harvested at different times for detection of iNOS protein by Western blotting. Expression of iNOS protein (130 kDa) was detectable only 6 h after LPS treatment. The figure is representative of 3 independent Western blot experiments. **B**, wild-type and iNOS^{-/-} cardiomyocytes were treated with LPS for 4 h. TNF- α production in the culture medium from both cardiomyocytes was measured. Compared with wild-type cardiomyocytes, deficiency in iNOS had no effect on LPS-induced TNF- α protein production ($p =$ not significant). Measurements were made in triplicate. Data are means \pm S.D. from 4 independent experiments.

tographed DNA bands were scanned and analyzed by a densitometer. TNF- α to GAPDH ratio for each sample was calculated.

Analysis of p38 MAPK Phosphorylation—Assessment of the phosphorylation status of p38 MAPK in cardiomyocytes was accomplished by Western blotting (26, 27). Aliquots containing 30 μ g of protein were subjected to SDS-PAGE using 12% gels, followed by electrotransfer to nitrocellulose membranes. Blots were probed with antibodies against p38 MAPK/phospho-p38 MAPK (New England Biolabs, 1:1000), followed by incubation with horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Detection was performed using the enhanced chemiluminescence detection method. Signals were determined by a densitometer. Levels of phosphorylated p38 MAPK to total p38 MAPK were presented.

Nitrite Measurement—The formation of nitrite in culture medium was used as an indicator of NO release by neonatal cardiomyocytes. The nitrite concentration in the supernatant was measured by Griess reaction as described previously (8). The concentration was expressed as nmol/mg cell protein.

Detection of iNOS Protein by Western Blot Analysis—Total 30 μ g of protein in each sample was subjected to SDS-PAGE using 10% gels, followed by electrotransfer to ECL membranes. Expression of iNOS protein was determined by probing the blots using specific antibodies against iNOS (Transduction Laboratory, 1:2000), followed by an ECL detection method (27). Inducible NOS was detected as a 130-kDa band.

Statistical Analysis—All data were given as mean \pm S.D. Differences between the two groups were compared by an unpaired Student's t test. For multigroup comparisons, analysis of variance followed by Student-Newman-Keuls test was performed. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Decreased basal TNF- α Expression in eNOS^{-/-} Cardiomyocytes—To determine basal levels of TNF- α expression in cardiomyocytes from wild-type and eNOS^{-/-} mice, levels of TNF- α mRNA and protein were examined. TNF- α mRNA was detectable in wild-type cardiomyocytes (Fig. 1, A and B). However,

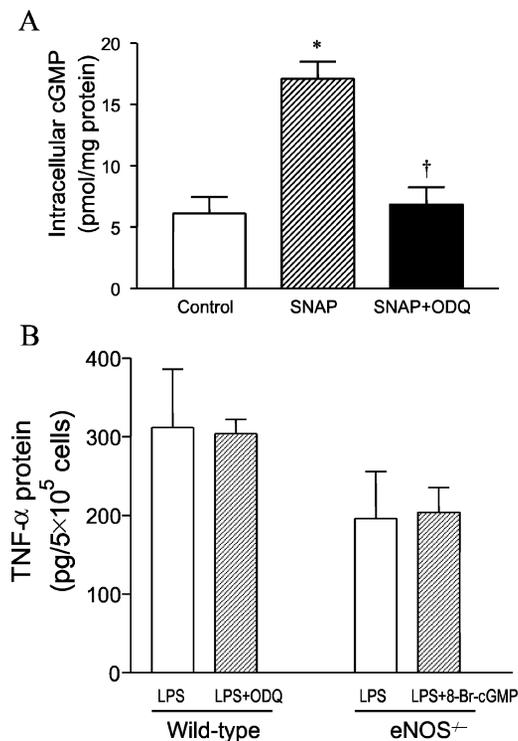


FIG. 5. Effects of cGMP on LPS-stimulated TNF- α expression. Wild-type and eNOS^{-/-} cardiomyocytes were cultured for 48 h after isolation. *A*, wild-type mouse cardiomyocytes were treated with a selective guanylate cyclase inhibitor ODQ (100 μ M) alone or in combination with the NO donor SNAP (10 μ M) for 2 h. Intracellular cGMP level was measured by ELISA. SNAP increased cGMP levels by \approx 2.5-fold, compared with the basal level in cardiomyocytes (*, $p < 0.01$ versus control, $n = 3$). ODQ completely blocked SNAP-stimulated increase in cGMP production (†, $p < 0.01$ versus SNAP, $n = 3$). *B*, wild-type mouse cardiomyocytes were pretreated with ODQ (100 μ M) for 30 min followed by incubation of LPS (10 μ g/ml) for 4 h. Pretreatment with ODQ did not have any effect on TNF- α production induced by LPS in wild-type cardiomyocytes ($p =$ not significant). Treatment of 8-Br-cGMP (10 μ M) for 4 h did not have any effect on TNF- α production induced by LPS in eNOS^{-/-} cardiomyocytes ($p =$ not significant). Measurements were made in triplicate. Data are means \pm S.D. from 3–5 independent experiments.

TNF- α mRNA expression was significantly decreased by 65.6% ($p < 0.05$) in eNOS^{-/-} cardiomyocytes, compared with wild-type cardiomyocytes (Fig. 1*B*). Similarly, very low levels of TNF- α protein were detected in the culture medium of wild-type cardiomyocytes, whereas no detectable TNF- α protein was found in the culture medium of eNOS^{-/-} cardiomyocytes (Fig. 1*C*). The results demonstrate that deficiency in eNOS decreases basal TNF- α expression at the transcriptional level in neonatal mouse cardiomyocytes.

Attenuation of LPS-stimulated TNF- α Expression in eNOS^{-/-} Cardiomyocytes—Previous studies have shown that LPS administration induces production of TNF- α in cultured cardiomyocytes (11) and in the myocardium (4). To investigate whether LPS-stimulated TNF- α expression is modulated by eNOS, TNF- α mRNA and protein expression were measured in wild-type and eNOS^{-/-} cardiomyocytes after LPS treatment. Fig. 2*A* illustrates the time course of TNF- α protein production induced by LPS (10 μ g/ml) in cardiomyocytes. Up-regulation of TNF- α protein expression occurred in less than 30 min and peaked around 4–6 h after LPS challenge. Based on this time course response to LPS, 4 h were chosen for all subsequent experiments. LPS dose-dependently induced TNF- α protein release in wild-type cardiomyocytes (Fig. 2*B*). The levels of TNF- α mRNA (Fig. 2*C*) and protein (Fig. 2*D*) in response to LPS were significantly decreased by 49.2 and 33.9% ($p < 0.05$) in eNOS^{-/-} cardiomyocytes, com-

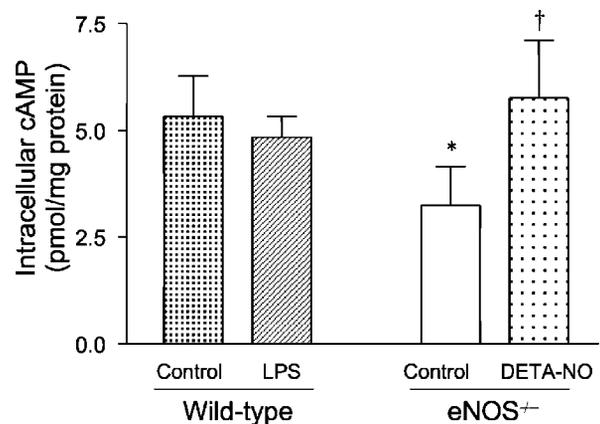


FIG. 6. Effects of DETA-NO on intracellular cAMP production in cardiomyocytes. Intracellular cAMP levels were decreased in eNOS^{-/-} compared with wild-type cardiomyocytes (*, $p < 0.05$ versus wild-type control). Addition of NO donor DETA-NO (2 μ M) to eNOS^{-/-} cardiomyocytes for 30 min elevated the intracellular cAMP levels similar to wild-type cardiomyocytes (†, $p < 0.05$ versus eNOS^{-/-} control). LPS (10 μ g/ml) had no effect on intracellular cAMP production in wild-type cardiomyocytes after treatment for 30 min. Measurements were made in triplicate. Data are means \pm S.D. from 4 independent experiments.

pared with wild-type cardiomyocytes. Consistent with basal TNF- α expression, LPS-stimulated TNF- α expression is also decreased in eNOS^{-/-} cardiomyocytes.

The Role of NO in LPS-stimulated TNF- α Expression—To assess if enhancement of LPS-stimulated TNF- α expression in the wild-type cardiomyocytes was due to NO production from eNOS, three lines of experiments were conducted. First, the NOS inhibitor L-NAME was employed in wild-type cardiomyocytes to eliminate endogenously generated NO. Wild-type cardiomyocytes were treated with LPS (10 μ g/ml) and L-NAME (500 μ M) for 4 h. L-NAME treatment decreased LPS-induced TNF- α production to the levels similar to eNOS^{-/-} cardiomyocytes in response to LPS ($p < 0.05$, Fig. 3*A*). Second, basal nitrite level in eNOS^{-/-} cardiomyocytes was lower than that of wild-type cardiomyocytes (8.3 \pm 0.5 versus 14.4 \pm 1.3 nmol/mg cell protein, $n = 5$ per group, $p < 0.05$). Addition of low concentration of DETA-NO (2 μ M) raised nitrite levels (16.8 \pm 2.5 nmol/mg cell protein) in eNOS^{-/-} cardiomyocytes to those of the wild-type cardiomyocytes. DETA-NO increased basal TNF- α protein from undetectable levels in eNOS^{-/-} cardiomyocytes to 3.8 \pm 2.7 pg/5 \times 10⁵ cells and increased LPS-induced TNF- α protein production by 32.6% ($p < 0.05$, Fig. 3*A*). LPS-induced TNF- α protein production in both eNOS^{-/-} and wild-type cardiomyocytes was completely inhibited by protein synthesis inhibitor cycloheximide (Fig. 3*A*). Third, the effect of eNOS on LPS-induced TNF- α expression was further confirmed by using adenoviral mediated eNOS gene transfer. Expression of re-introduced eNOS gene in eNOS^{-/-} cardiomyocytes was detected by RT-PCR using bovine eNOS-specific primers (Fig. 3*B*). Adv-eNOS infection increased TNF- α production by about 26% ($p < 0.05$, Fig. 3*C*) in response to LPS in eNOS^{-/-} cardiomyocytes. Thus, the presence of NO was able to restore TNF- α production of eNOS^{-/-} cardiomyocytes in response to LPS.

Because LPS has been demonstrated to induce iNOS expression in cardiomyocytes (7), we then investigated whether iNOS-derived NO was involved in the regulation of LPS-stimulated TNF- α expression in our experiments. Fig. 4*A* showed that iNOS protein expression was only detectable at 6 h but not at 4 h after LPS treatment in cardiomyocytes. To exclude further the contribution of iNOS in LPS-induced TNF- α production in our study, iNOS^{-/-} cardiomyocytes were employed. After 4 h of LPS treatment, TNF- α production was not different between

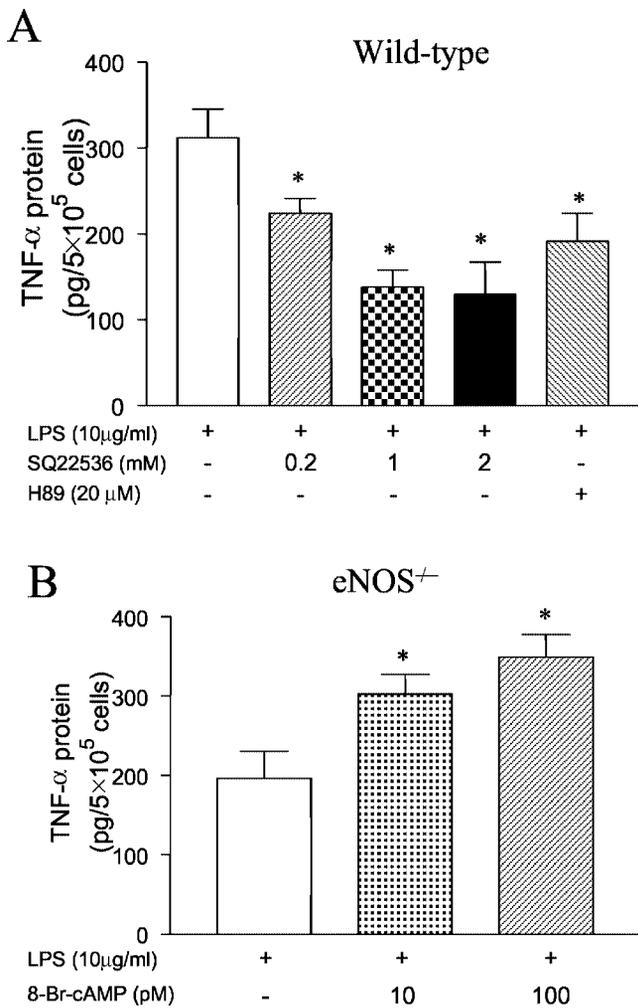


FIG. 7. Effects of cAMP and PKA on LPS-stimulated TNF- α expression. Wild-type and eNOS^{-/-} cardiomyocytes were cultured for 48 h after isolation. *A*, wild-type mouse cardiomyocytes were pretreated with a selective adenylate cyclase inhibitor SQ22536 (0.2–2 mM) or a specific PKA inhibitor H89 (20 μ M) for 30 min followed by incubation of LPS (10 μ g/ml) for 4 h. Pretreatment with SQ22536 or H89 attenuated TNF- α production induced by LPS (*, $p < 0.05$ versus LPS alone). *B*, eNOS^{-/-} cardiomyocytes were treated with LPS alone or 8-Br-cAMP (10 to 100 pM) for 4 h. Treatment of 8-Br-cAMP (10–100 pM) enhanced LPS-induced TNF- α production (*, $p < 0.05$ versus LPS alone). Measurements were made in triplicate. Data are mean \pm S.D. from 3 to 5 independent experiments.

iNOS^{-/-} and wild-type cardiomyocytes (Fig. 4*B*), suggesting that iNOS was not involved in TNF- α production in the present study. Our results strongly support the notion that NO production from eNOS enhances LPS-stimulated TNF- α expression.

Role of cGMP in LPS-stimulated TNF- α Expression—It has been widely recognized that NO exerts cellular effects via both cGMP-dependent and -independent mechanisms (23, 24). In the present study, we tested the cGMP-dependent pathway by using a selective GC inhibitor ODQ and a membrane-permeable cGMP analogue 8-Br-cGMP. ODQ completely blocked NO donor SNAP-induced cGMP production (Fig. 5*A*). However, it did not have any effect on LPS-induced TNF- α protein production in wild-type cardiomyocytes (Fig. 5*B*). Furthermore, no effect of 8-Br-cGMP was observed on LPS-induced TNF- α expression in eNOS^{-/-} cardiomyocytes (Fig. 5*B*). The results suggest that eNOS-derived NO regulates LPS-stimulated TNF- α expression through a mechanism independent of cGMP.

Role of cAMP in LPS-stimulated TNF- α Expression—Studies have demonstrated that NO also activates cAMP-dependent pathway (23). We therefore hypothesized that deficiency in

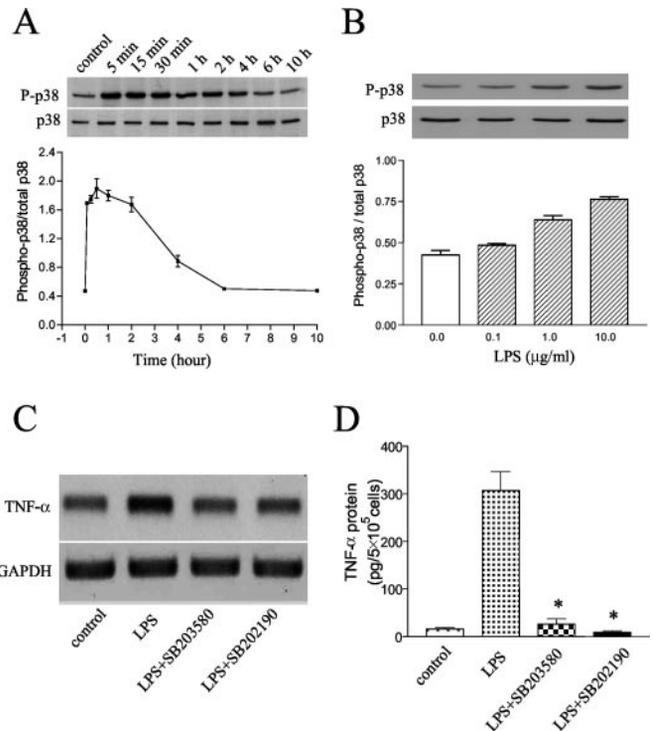


FIG. 8. Requirement of p38 MAPK activation for LPS-induced TNF- α expression in wild-type neonatal cardiomyocytes. *A*, time course of LPS-induced phosphorylation of p38 MAPK. Phosphorylation of p38 MAPK was increased within 5 min, reaching the maximal levels within 15 min and returning to basal levels after 6 h of LPS treatment (10 μ g/ml). *B*, LPS (0.1–10 μ g/ml) dose-dependently induced phosphorylation of p38 MAPK in cardiomyocytes after 4 h of treatment. Incubation with a p38 MAPK-specific inhibitor SB203580 or SB202190 (5 μ M) abrogated TNF- α mRNA (*C*) and protein expression (*D*) in response to LPS (10 μ g/ml). Measurements were made in triplicate. Data are means \pm S.D. from 3 to 5 independent experiments. *, $p < 0.05$ versus LPS alone.

eNOS resulted in decreased cAMP levels, leading to attenuated TNF- α expression in response to LPS in cardiomyocytes. Consistent with this hypothesis, our data showed that intracellular cAMP levels were decreased in eNOS^{-/-} cardiomyocytes compared with wild-type cardiomyocytes (Fig. 6). DETA-NO (2 μ M) treatment elevated cAMP levels in eNOS^{-/-} cardiomyocytes (Fig. 6). Furthermore, LPS-induced TNF- α protein was decreased by inhibition of cAMP production using a selective AC inhibitor SQ22536 in a dose-dependent manner and by a specific PKA inhibitor H89 in wild-type cardiomyocytes (Fig. 7*A*). A membrane-permeable cAMP analogue 8-Br-cAMP (10–100 pM) enhanced LPS-induced TNF- α expression in eNOS^{-/-} cardiomyocytes (Fig. 7*B*). These results demonstrated that NO enhances LPS-induced TNF- α expression via cAMP-dependent pathway in cardiomyocytes.

Essential Role of p38 MAPK in LPS-stimulated TNF- α Expression—It has been shown that p38 MAPK is required for LPS-stimulated TNF- α expression in neutrophils (16). However, the role of p38 MAPK in LPS-induced TNF- α expression in cardiomyocytes is not clear. In the present study, we demonstrated that LPS induced activation of p38 MAPK. A time course response to LPS showed that activation of p38 MAPK in cardiomyocytes occurred within 5 min, reaching the maximal response at 30 min, and returned to basal level after 6 h of LPS challenge (Fig. 8*A*). The activation of p38 MAPK preceded the production of TNF- α protein in cardiomyocytes (Fig. 2*B*). LPS treatment dose-dependently increased phosphorylation of p38 MAPK in cardiomyocytes (Fig. 8*B*). Moreover, inhibition of p38 MAPK by using a selective inhibitor, SB203580 or SB202190

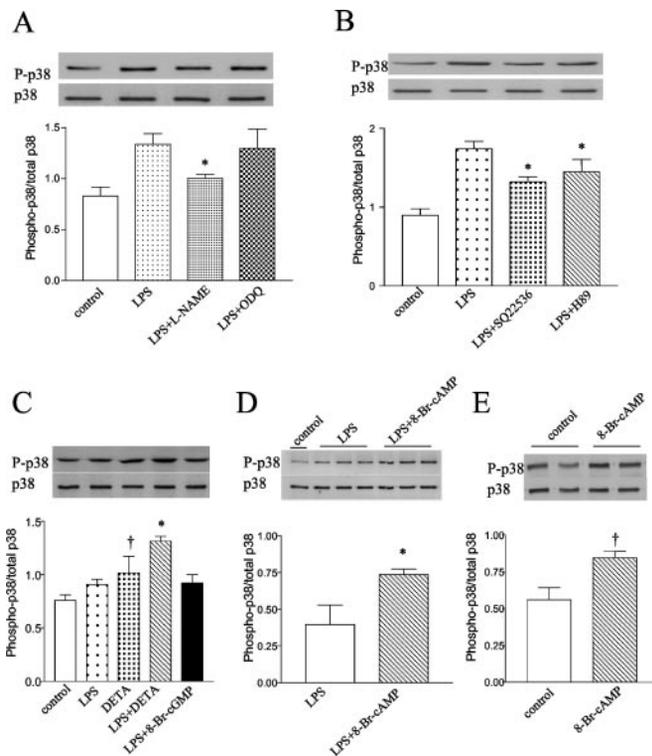


FIG. 9. NO enhances LPS-induced p38 MAPK activation via cAMP/PKA-dependent pathway. *A*, pretreatment with a NOS inhibitor L-NAME (500 μ M) for 30 min but not a selective guanylate cyclase inhibitor ODQ (100 μ M) attenuated phosphorylation of p38 MAPK induced by LPS (10 μ g/ml) in wild-type cardiomyocytes. *B*, pretreatment with a selective adenylate cyclase inhibitor SQ22536 (200 μ M) or a specific PKA inhibitor H89 (20 μ M) for 30 min attenuated phosphorylation of p38 MAPK induced by LPS (10 μ g/ml) in wild-type cardiomyocytes. *C*, NO donor DETA-NO (2 μ M) increased phosphorylation of p38 MAPK and enhanced the effects of LPS (10 μ g/ml) on p38 phosphorylation in eNOS^{-/-} cardiomyocytes. However, 8-Br-cGMP (10 μ M) had no effect. *D* and *E*, 8-Br-cAMP (100 μ M) alone or in the presence of LPS enhanced p38 MAPK phosphorylation in eNOS^{-/-} cardiomyocytes, mimicking the effect of NO. Measurements were made in triplicate. Data are mean \pm S.D. from 3 to 6 independent experiments. *, $p < 0.05$ versus LPS alone; †, $p < 0.05$ versus control.

(5 μ M), abrogated TNF- α mRNA (Fig. 8C) and protein expression (Fig. 8D) induced by LPS in wild-type cardiomyocytes, indicating that LPS-induced TNF- α expression is mediated by activation of p38 MAPK in cardiomyocytes.

NO Enhances TNF- α Expression via cAMP-mediated p38 MAPK Pathway—To demonstrate further whether the effects of NO on LPS-induced TNF- α expression were mediated by p38 MAPK activation via cAMP-dependent pathway, the following experiments were performed. 1) Inhibition of NOS by L-NAME (500 μ M, Fig. 9A), inhibition of AC by SQ22536 (200 μ M, Fig. 9B), or inhibition of PKA by H89 (20 μ M, Fig. 9B) attenuated LPS-induced phosphorylation of p38 MAPK in wild-type cardiomyocytes ($p < 0.05$). 2) NO donor DETA-NO (2 μ M) enhanced the effects of LPS on phosphorylation of p38 MAPK in eNOS^{-/-} cardiomyocytes ($p < 0.05$, Fig. 9C). 3) Neither ODQ nor 8-Br-cGMP had any effect on LPS-induced activation of p38 MAPK (Fig. 9, A and C). 4) A cAMP analogue 8-Br-cAMP (100 μ M) increased phosphorylation of p38 MAPK in the presence or absence of LPS in eNOS^{-/-} mouse cardiomyocytes ($p < 0.05$, Fig. 9, D and E), mimicking the effect of NO (Fig. 9C). The results demonstrate that NO enhances phosphorylation of p38 MAPK via cAMP-dependent pathway in response to LPS.

DISCUSSION

The major findings of the present study are that endogenous NO production from eNOS enhanced basal and LPS-stimulated

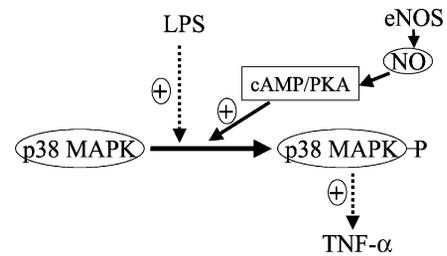


FIG. 10. Proposed model of signaling pathways leading to TNF- α expression during LPS stimulation in neonatal mouse cardiomyocytes. Activation of p38 MAPK is required for LPS-induced TNF- α expression. NO produced from eNOS enhances LPS-stimulated TNF- α expression through activation of adenylate cyclase and cAMP/PKA-dependent p38 MAPK pathway.

TNF- α expression in cultured neonatal mouse cardiomyocytes. TNF- α expression was preceded by increased phosphorylation of p38 MAPK, and selective inhibition of p38 MAPK abrogated TNF- α expression stimulated by LPS, suggesting an important role of p38 MAPK in LPS-induced TNF- α expression. We further demonstrated that the effects of NO were mediated through the cAMP/PKA-dependent p38 MAPK pathway. Our study demonstrated a novel signaling mechanism by which endogenously produced NO from eNOS modulates LPS-stimulated TNF- α expression in cardiomyocytes.

NO from eNOS Enhances LPS-induced TNF- α Expression via cAMP-dependent Pathway—Cardiomyocytes constitutively express eNOS, which plays an important role in the regulation of myocardial function (21). In the present study we demonstrated that eNOS-derived NO enhanced LPS-stimulated TNF- α expression in neonatal mouse cardiomyocytes. This conclusion was based on the following three lines of evidence. First, deficiency in eNOS decreased both basal and LPS-stimulated TNF- α mRNA and protein expression in mouse cardiomyocytes. Second, treatment of NOS inhibitor L-NAME in wild-type cardiomyocytes decreased LPS-induced TNF- α expression to the level similar to that of eNOS^{-/-} cardiomyocytes. Third, either NO donor DETA-NO or Adv-eNOS infection was able to restore LPS-stimulated TNF- α production in eNOS^{-/-} cardiomyocytes to a similar level of wild-type cardiomyocytes. In the present study, TNF- α was measured at 4 h after LPS treatment when iNOS was not detectable. This is consistent with previous demonstrations that induction of iNOS in cardiomyocytes or heart occurs at late stages of LPS administration (4, 15). In addition, deficiency in iNOS did not influence the response to LPS in terms of TNF- α production in cardiomyocytes. Thus, the contribution of iNOS in LPS-induced TNF- α expression is negligible in the present study.

Signal transductions of NO are mediated by both GC/cGMP-dependent and -independent pathways (23). A recent study (24) showed that exogenous NO donors induced TNF- α expression in cardiomyocytes via the GC/cGMP-dependent pathway. However, in the present study 8-Br-cGMP failed to mimic the effects of eNOS-derived NO on TNF- α expression in eNOS^{-/-} cardiomyocytes. ODQ abrogated NO donor-stimulated cGMP production but did not affect TNF- α biosynthesis in wild-type cardiomyocytes in response to LPS. The results indicate that the effect of eNOS-derived NO on LPS-stimulated TNF- α expression is independent of cGMP in cardiomyocytes. Indeed, cGMP-independent effects of NO on LPS-induced TNF- α expression have been demonstrated in non-cardiomyocytes such as neutrophils (28). Studies have also shown that low levels of NO activate AC and increase cAMP by a cGMP-independent mechanism in rat adult cardiomyocytes (23). Consistent with this notion, our data demonstrated that DETA-NO at low concentrations increased intracellular cAMP levels in eNOS^{-/-}

cardiomyocytes. Moreover, deficiency in eNOS decreased cAMP levels in cardiomyocytes. We further demonstrated that the effects of eNOS-derived NO on LPS-induced TNF- α production were mediated through AC/cAMP-dependent pathways in cardiomyocytes. Inhibition of cAMP production attenuated LPS-induced TNF- α expression in wild-type cardiomyocytes. cAMP analogue 8-Br-cAMP mimicked the effects of NO by increasing TNF- α production in eNOS^{-/-} cardiomyocytes. These results were somewhat unexpected as previous studies (29) have shown that cAMP analogues at 10 μ mol/liter concentrations inhibited LPS-induced TNF- α expression in cardiomyocytes. It is important to note that the physiological level of cAMP in cardiomyocytes is \sim 6 pmol/mg protein (23). To mimic the endogenous level of cAMP, we used low concentrations of cAMP analogue (10 and 100 pmol/liter) in the present study. Our results demonstrated that low levels of cAMP enhance basal as well as LPS-induced TNF- α production. Thus, cAMP may have completely different effects depending on its intracellular concentrations in cardiomyocytes. Whereas high concentrations of cAMP analogue (at micromolar range) inhibit LPS-induced TNF- α production, low levels of cAMP enhance LPS-induced TNF- α expression in cardiomyocytes.

Activation of p38 MAPK Is Required for LPS-induced TNF- α Expression—An important finding in the present study is that LPS-stimulated TNF- α expression is mediated through p38 MAPK activation in neonatal cardiomyocytes. LPS-induced phosphorylation of p38 MAPK was less than 5 min, reaching a maximum within 30 min, which preceded the release of TNF- α production. More importantly, inhibition of p38 MAPK abrogated TNF- α expression induced by LPS. The mechanism by which LPS induces phosphorylation of p38 MAPK in cardiomyocytes remains elusive. It is possible that LPS stimulates p38 MAPK by activating protein tyrosine kinases through CD14 (30) and TLRs (13), and by inducing oxidative stress through Rac1-dependent pathway (31).

NO from eNOS Promotes LPS-stimulated p38 MAPK Activation via cAMP/PKA-dependent Pathway—Recent studies (32) have shown that cAMP activates p38 MAPK via a PKA-dependent mechanism in adult mouse cardiomyocytes. PKA inhibits Ser/Thr protein phosphatases and increases p38 MAPK activity (33, 34). However, it is not known if cAMP/PKA-dependent p38 MAPK activation is responsible for the effects of NO on LPS-induced TNF- α expression. In the present study, inhibition of either NOS or AC activities attenuated LPS-induced phosphorylation of p38 MAPK and TNF- α release in cardiomyocytes. Addition of a NO donor or a cAMP analogue at low concentrations increased phosphorylation of p38 MAPK and TNF- α release induced by LPS in eNOS^{-/-} cardiomyocytes. Furthermore, inhibition of PKA decreased phosphorylation of p38 MAPK and TNF- α expression induced by LPS. Our results demonstrated that eNOS-derived NO enhances LPS-induced p38 MAPK and TNF- α expression via a cAMP/PKA-dependent pathway.

In summary, the present study demonstrated for the first time that endogenously produced NO from eNOS enhances LPS-stimulated TNF- α expression in cultured neonatal mouse cardiomyocytes. Activation of p38 MAPK is essential in LPS-stimulated TNF- α expression. Moreover, the effects of NO on LPS-stimulated TNF- α expression are mediated through cAMP/PKA-dependent p38 MAPK mechanisms (Fig. 10). These findings provide novel pathways leading to LPS induction of TNF- α in cardiomyocytes. The significance of the finding that eNOS-derived NO enhances LPS-induced TNF- α expression in cardiomyocytes in sepsis is presently not clear. High levels of TNF- α have been demonstrated to have detrimental effects on myocardial function. However, TNF- α is required for innate resistance to invading pathogens (2). It is possible, albeit spec-

ulative, that increased expression of TNF- α by eNOS leads to an enhancement of innate immune response in the heart, which in turn promotes the recruitment of neutrophils, monocytes, and natural killer cells to the areas of the myocardium where the pathogens are located. Increased TNF- α expression has also been shown to induce manganese superoxide dismutase expression (35) which may reduce oxidative stress and tissue injury during sepsis. Therefore, eNOS enhancement of TNF- α production may confer some beneficial effects. However, the pathophysiological significance of the eNOS/cAMP/p38 MAPK pathway in sepsis requires further investigation.

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