Pivotal Role of gp91<sub>phox</sub>-Containing NADH Oxidase in Lipopolysaccharide-Induced Tumor Necrosis Factor-α Expression and Myocardial Depression

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**Background**—Lipopolysaccharide (LPS) induces cardiomyocyte tumor necrosis factor-α (TNF-α) production, which is responsible for myocardial depression during sepsis. The aim of this study was to investigate the role of gp91<sub>phox</sub>-containing NADH oxidase signaling in cardiomyocyte TNF-α expression and myocardial dysfunction induced by LPS.

**Methods and Results**—In cultured mouse neonatal cardiomyocytes, LPS increased NADH oxidase (gp91<sub>phox</sub> subunit) expression and superoxide generation. Deficiency of gp91<sub>phox</sub> or inhibition of NADH oxidase blocked TNF-α expression stimulated by LPS. TNF-α induction was also inhibited by tempol, N-acetylcysteine, or 1,3-dimethyl-2-thiourea. NADH oxidase activation by LPS increased ERK1/2 and p38 phosphorylation, and inhibition of ERK1/2 and p38 phosphorylation blocked the effect of NADH oxidase on TNF-α expression. Isolated mouse hearts were perfused with LPS (5 µg/mL) alone or in the presence of apocynin for 1 hour. Myocardial TNF-α production was decreased in gp91<sub>phox</sub>-deficient or apocynin-treated hearts compared with those of wild type (P<0.05). To investigate the role of gp91<sub>phox</sub>-containing NADH oxidase in endotoxemia, mice were treated with LPS (4 mg/kg IP) for 4 and 24 hours, and their heart function was measured with a Langendorff system. Deficiency of gp91<sub>phox</sub> significantly attenuated LPS-induced myocardial depression (P<0.05).

**Conclusions**—gp91<sub>phox</sub>-Containing NADH oxidase is pivotal in LPS-induced TNF-α expression and cardiac depression. Effects of NADH oxidase activation are mediated by ERK1/2 and p38 MAPK pathway. The present results suggest that gp91<sub>phox</sub>-containing NADH oxidase may represent a potential therapeutic target for myocardial dysfunction in sepsis.

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**Key Words:** lipids ■ hormones ■ cardiomyocytes ■ myocardial infarction

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Lipopolysaccharide (LPS) of Gram-negative bacteria induces expression of tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, in cardiomyocytes. Previous studies have demonstrated that cardiomyocytes are the major local source of TNF-α in the myocardium during sepsis. TNF-α is responsible for myocardial depression induced by endotoxemia. Thus, modulation of local myocardial TNF-α levels produced by cardiomyocytes may be of therapeutic significance in sepsis-induced myocardial dysfunction. Mechanisms by which LPS induces TNF-α expression in cardiomyocytes are not fully understood. It is well established that the effects of LPS are mediated via toll-like receptors. We have recently demonstrated that activation of p38 mitogen-activated protein kinase (MAPK) is required for LPS-induced TNF-α expression in cardiomyocytes; however, LPS signaling downstream of toll-like receptors and upstream of MAPK remains largely unknown in cardiomyocytes.

NADPH or NADH oxidase is an inducible electron transport system found in cells that transfer reducing equivalents from NADPH or NADH to oxygen, which results in superoxide anion (O<sub>2</sub><sup>-</sup>) generation. The enzyme complex comprises 2 membrane subunits (gp91<sub>phox</sub> and p22<sub>phox</sub>) and 4 cytosolic proteins (p40<sub>phox</sub>, p47<sub>phox</sub>, p67<sub>phox</sub>, and Rac1/2). The oxidase complex is a highly regulated enzyme. In the resting cells, the cytosolic complex is separated from the membrane-bound catalytic core. On stimulation, the cytosolic component p47<sub>phox</sub> becomes phosphorylated, and the cytosolic complex migrates to the membrane, where it binds to cytochrome b558 to assemble into an active oxidase. NAD(P)H oxidase expression has also been demonstrated in cardiomyocytes and has been considered as a critical determinant of the redox state of the myocardium. In response to LPS, NAD(P)H oxidase activity and O<sub>2</sub><sup>-</sup> production are markedly increased in the heart; however, the contribution of the...
NADH oxidase to TNF-α expression in LPS-stimulated cardiomyocytes remains unknown, and the role of NADH oxidase in septic myocardial depression has not been investigated. In addition, the membrane subunit of NADH oxidase, gp91<sub>phox</sub> (Nox2), has at least 3 other homologs, Nox1, Nox3, and Nox4. Both gp91<sub>phox</sub> and Nox4 are expressed in cardiomyocytes. A recent study suggested a differential response of the cardiac Nox isoforms, gp91<sub>phox</sub> and Nox4, to different pathological stimuli for cardiac hypertrophy; however, the role of gp91<sub>phox</sub> in sepsis-induced cytokine induction and myocardial dysfunction has not been investigated.

In the present study, we aimed to investigate the role of gp91<sub>phox</sub>-containing NADH oxidase signaling in LPS-induced TNF-α expression and myocardial dysfunction induced by endotoxemia. To achieve this goal, experiments were performed on gp91<sub>phox</sub>-deficient (gp91<sub>phox</sub>−/−) mice and wild-type mice with cardiomyocyte culture, isolated-heart, and whole-animal approaches. Our results showed that gp91<sub>phox</sub>-containing NADH oxidase plays an important role in cardiomyocyte TNF-α expression and cardiac dysfunction during LPS stimulation.

**Methods**

**Animals and Preparation of Neonatal Mouse Cardiomyocytes**

The breeding pairs of C57BL/6 wild-type and gp91<sub>phox</sub>−/− mice (C57BL/6 background) were purchased from Jackson Laboratory. A breeding program was performed 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 and cultured according to methods we described previously.

**Transfections**

Small interfering RNAs (siRNAs) for ERK1 and p38 MAPK were purchased from Santa Cruz Biotechnology, and a universal control siRNA was obtained from Qiagen. The transfection of siRNA was performed in cardiomyocytes with TransMessenger transfection reagent (Qiagen) according to the manufacturer’s instructions. One microliter of siRNA (10 μmol/L) was used for each well of cells (48-well plate). After transfection, cells were maintained in normal culture medium for another 48 hours before LPS treatment.

**Reagents**

LPS (Salmonella typhosa), diphenyleneiodonium (DPI), apocynin, N-acetylcysteine (NAC), tempol, 1,3-dimethyl-2-thiourea (DMTU), etc.
H9252-NADH, lucigenin, PD98059, and SB203580 were purchased from Sigma.

Measurement of TNF-α Protein

Concentrations of TNF-α protein were determined with a mouse TNF-α ELISA kit (ALPCO Diagnostics) as in our previous reports.3,4

Measurement of Superoxide Generation (O2−)

NADH-dependent O2− generation was measured in cell lysates by lucigenin-enhanced chemiluminescence (20 μmol/L NADH, 5 μmol/L lucigenin) with a multilabel counter (Victor3 Wallac).8 Some experiments were performed in the presence of superoxide dismutase (SOD; Sigma). The light signal was monitored for 5 seconds, and counts per second (CPS) were presented as NADH oxidase activity that was SOD inhibitable.

Analysis of TNF-α, gp91phox, and MAPK mRNA by Reverse-Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from cardiomyocytes with the TriZol reagent (Gibco-BRL) according to the manufacturer’s instructions. Semi-quantitative reverse-transcriptase–polymerase chain reaction (RT-PCR) for TNF-α was performed as described previously.3 The DNA oligonucleotide primers for gp91phox, ERK1, and p38α MAPK were as follows: gp91phox (Accession No. U43384), 5′-ACGGCCCTT-TGCTTCATTCT 3′ (sense) and 5′-GGCTCAGGGCCACACAGGAA 3′ (antisense); ERK1 (Accession No. BC029712), 5′-TCC AACG GC TAC ACC AAA TC-3′ (sense) and 5′-GCT CCA TGT CGA AGG TGA AT-3′ (antisense); and p38α (Accession No. NM_011951), 5′-TAC CAC GAC CCT GAT GAT GA-3′ (sense) and 5′-GCC AAG GAC CAT TCA CAA CT-3′ (antisense).

Western Blot Analysis

A total of 30 μg of protein in each sample was subjected to SDS-PAGE with 10% gels, followed by electrotransfer to nitrocellulose membranes. Expression of gp91phox protein was determined by probing the blots with specific antibodies against gp91phox (Signal Transduction, 1/2000), followed by enhanced chemiluminescence detection. Gp91phox protein was detected as a 67-kDa band.

Measurement of ERK1/2 and p38 MAPK Phosphorylation

Assessment of the phosphorylation status of ERK1/2 and p38 MAPK in cardiomyocytes was accomplished by Western blotting (see Figure 3. Effects of DPI and apocynin on TNF-α expression in LPS-stimulated cardiomyocytes. Cardiomyocytes were incubated with LPS (10 μg/mL) in presence or absence of DPI (20 and 50 μmol/L) or apocynin (1 mmol/L) for 4 hours. A, Representative RT-PCR amplification for TNF-α mRNA and GAPDH mRNA expression from 3 independent experiments. DPI (20 μmol/L) inhibited LPS-induced TNF-α mRNA expression. B, DPI inhibited LPS-induced TNF-α protein levels in dose-dependent manner. C, Apocynin inhibited LPS-induced TNF-α protein levels. TNF-α protein levels in B and C were determined in culture medium by ELISA. Data are mean±SD of 3 to 5 independent experiments. *P<0.05 vs LPS alone.

Figure 3. Effects of DPI and apocynin on TNF-α expression in LPS-stimulated cardiomyocytes. Cardiomyocytes were incubated with LPS (10 μg/mL) in presence or absence of DPI (20 and 50 μmol/L) or apocynin (1 mmol/L) for 4 hours. A, Representative RT-PCR amplification for TNF-α mRNA and GAPDH mRNA expression from 3 independent experiments. DPI (20 μmol/L) inhibited LPS-induced TNF-α mRNA expression. B, DPI inhibited LPS-induced TNF-α protein levels in dose-dependent manner. C, Apocynin inhibited LPS-induced TNF-α protein levels. TNF-α protein levels in B and C were determined in culture medium by ELISA. Data are mean±SD of 3 to 5 independent experiments. *P<0.05 vs LPS alone.

β-NADH, lucigenin, PD98059, and SB203580 were purchased from Sigma.
Effects of LPS on NADH Oxidase Expression and NADH Oxidase Activity in Cardiomyocytes

To examine the role of gp91phox-containing NADH oxidase activity in LPS-induced oxidative stress, we measured gp91phox expression and NADH oxidase activity in cardiomyocytes. As shown in Figure 1, LPS treatment increased gp91phox mRNA and protein expression. The upregulation of NADH oxidase expression induced by LPS was associated with an increase in O$_2^-$ generation, which was SOD-inhibitable. These data showed that LPS increases NADH oxidase activity in cardiomyocytes.

LPS-Induced TNF-α Expression in gp91phox-/- Cardiomyocytes

To investigate the role of gp91phox-containing NADH oxidase in TNF-α expression in LPS-stimulated cardiomyocytes, cardiomyocytes from gp91phox-/- mice were used. In response to LPS, TNF-α protein and mRNA expression in gp91phox-/- cardiomyocytes were decreased by 49% and 39%, respectively, compared with wild-type cardiomyocytes (P<0.05; Figures 2A and 2B). This was associated with a significant decrease in O$_2^-$ generation in gp91phox-/- cardiomyocytes (P<0.05; Figure 2C).

Effects of NADH Oxidase Inhibitor on LPS-Induced TNF-α Expression in Cardiomyocytes

The contribution of NADH oxidase to TNF-α expression in response to LPS was also examined by using its pharmaco-
logical inhibitors, DPI and apocynin. The inhibitory effects of DPI and apocynin on NADH oxidase were confirmed by measuring NADH-dependent $O_2^-$ generation (data not shown). As shown in Figure 3, either DPI or apocynin abolished LPS-induced TNF-α protein and mRNA expression. These data suggest that LPS induces TNF-α expression via NADH oxidase activation in cardiomyocytes.

We further determined the contribution of $O_2^-$ in LPS-induced TNF-α expression. A scavenger of $O_2^-$, the membrane-permeable SOD mimetic tempol, was used in LPS-stimulated cardiomyocytes. Tempol decreased LPS-induced TNF-α production in a dose-dependent manner (Figure 4A). To confirm this finding, another antioxidant, NAC, was used. Similarly, NAC dose-dependently suppressed TNF-α expression in LPS-stimulated cardiomyocytes (Figure 4B). Superoxide can form hydroxyl radicals via the Haber-Weiss reaction. To examine whether hydroxyl radicals played a role in LPS-induced TNF-α expression, a hydroxyl radical scavenger, DMTU, was used. As shown in Figure 4C, incubation with DMTU dose-dependently blocked LPS-induced TNF-α expression. Thus, NADH oxidase-produced $O_2^-$, hydroxyl radicals, and oxidative stress contributed to TNF-α expression in LPS-stimulated cardiomyocytes.

**Role of NADH Oxidase in LPS-Induced ERK1/2 and p38 MAPK in Cardiomyocytes**

We have shown that p38 MAPK activation is essential for LPS-induced TNF-α expression in cardiomyocytes. This was also demonstrated in the present study in which treatment with the p38 inhibitor SB203580 completely blocked LPS-induced TNF-α production (Figure 5A). To further investigate the role of ERK1/2 activation in LPS-induced TNF-α expression, cardiomyocytes were treated with PD98059, which blocks ERK1/2 signaling. PD98059 abrogated TNF-α expression in LPS-stimulated cardiomyocytes (Figure 5A). The effect of p38 and ERK1/2 MAPK on TNF-α expression was further confirmed by specific downregulation of MAPK with siRNAs against p38α and ERK1. siRNA treatment selectively suppressed p38α and ERK1 expression, as demonstrated by semiquantitative RT-PCR (data not shown), and decreased LPS-induced TNF-α expression by 24% and 50.8%, respectively (Figure 5B). The data showed that both p38 and ERK activation are required for TNF-α expression. To determine whether NADH oxidase activation leads to p38 and ERK1/2 activation in response to LPS, phosphorylation of p38 and ERK1/2 MAPK was measured after inhibition of NADH oxidase by DPI. As shown in Figure 5C, DPI...
treatment significantly blunted phosphorylation of p38 and ERK1/2 stimulated by LPS. These results suggest that NADH oxidase signaling-induced TNF-α expression is mediated through ERK1/2 and p38 MAPK activation.

Role of NADH Oxidase in LPS-Induced TNF-α Expression in Isolated Hearts
To demonstrate the role of gp91(phox)-containing NADH oxidase in LPS-induced TNF-α expression at the organ level, mouse hearts isolated from gp91(phox)/H11002/H11002 and wild-type mice were perfused with LPS in the presence or absence of apocynin for 1 hour. The perfusates were collected and assayed for TNF-α production. Deficiency of gp91(phox) and apocynin treatment significantly decreased TNF-α production by 53% and 74% (Figure 6), respectively, which indicates that gp91(phox)-containing NADH oxidase also played an important role in adult hearts in terms of TNF-α production induced by LPS.

Role of NADH Oxidase in Myocardial Dysfunction of Endotoxemia
To explore the role of gp91(phox)-containing NADH oxidase in myocardial depression induced by endotoxemia, gp91(phox)/H11002/H11002 and wild-type mice were given vehicle or LPS (4 mg/kg IP). Four or 24 hours later, cardiac function was assessed in isolated hearts to avoid systemic reflex influences. Although there was no change in heart rate, heart work and rate of contraction were significantly reduced in endotoxemic mice compared with sham animals at both time points (Figures 7 and 8), which indicates myocardial depression. Lack of gp91(phox) restored heart work and rate of contraction and relaxation without affecting heart rate in endotoxemic mice (Figures 7 and 8). These results demonstrated that gp91(phox) contributed to myocardial dysfunction at both early (4 hours) and late (24 hours) stages of endotoxemia in mice.

Discussion
The present study used cardiomyocytes, isolated hearts, and an in vivo model of endotoxemia to investigate the role of NADH oxidase in LPS-induced TNF-α expression. We demonstrated for the first time that gp91(phox), a subunit of NADH oxidase, plays an important role in myocardial depression induced by endotoxemia and that gp91(phox)-containing NADH oxidase signaling contributes to LPS-stimulated TNF-α expression in cardiomyocytes. The effect of gp91(phox)-containing NADH oxidase was mediated through p38 and ERK1/2 MAPK signaling pathway. The present study provides definitive evidence that LPS-induced myocardial dysfunction is mediated by gp91(phox)-containing NADH oxidase. Activation of NADH oxidase represents a novel signaling pathway by which LPS induces p38 and ERK1/2 MAPK, leading to TNF-α expression in cardiomyocytes.
NADH Oxidase Activation in Sepsis

NAD(P)H oxidase has been shown to be a major source of oxidative stress in the myocardium. The upregulation of NAD(P)H oxidase activity has been observed in human failing hearts. NAD(P)H oxidase-derived O$_2^-$ production has been demonstrated to contribute to various cardiovascular diseases, including cardiac hypertrophy, hypertension, and atherosclerosis. In sepsis, NAD(P)H oxidase activation in neutrophils plays a major role in mediating sepsis-induced acute lung injury. O$_2^-$ production by NAD(P)H oxidase is involved in the pathogenesis of endothelial dysfunction, platelet-endothelial cell adhesion in intestinal venules, and production of peroxynitrite and prostaglandin E$_2$ in activated rat microglia. Recent studies have shown that NAD(P)H oxidase plays an important role in LPS-induced TNF-α expression and neurotoxicity in microglia and astrocytes. In the heart, NAD(P)H oxidase activity and O$_2^-$ production are increased in response to LPS stimulation. However, a causal relationship between NAD(P)H oxidase activation and myocardial dysfunction during sepsis has not been reported. In the present study, LPS-induced myocardial dysfunction was restored in gp91$^{phox/-/-}$ mice. The present results demonstrated that gp91$^{phox}$-containing NADH oxidase activity contributes to myocardial depression in endotoxemia. Mechanisms by which gp91$^{phox}$-containing NADH oxidase mediates myocardial dysfunction are not fully understood. Data from the present study suggest that LPS activates gp91$^{phox}$-containing NADH oxidase, which increases oxidative stress and TNF-α production in cardiomyocytes, leading to myocardial dysfunction.

NADH Oxidase Signaling in TNF-α Expression in LPS-Stimulated Cardiomyocytes

TNF-α has been shown to be a major factor responsible for myocardial depression during endotoxemia; however, mechanisms by which LPS induces TNF-α expression in cardiomyocytes are not fully understood. The present study aimed to investigate the role of NADH oxidase in LPS-induced TNF-α expression in cardiomyocytes. Using cultured neonatal mouse cardiomyocytes and isolated heart preparations, we demonstrated the following. First, LPS increased gp91$^{phox}$ expression and O$_2^-$ generation. Second, deficiency of gp91$^{phox}$ blunted TNF-α production in response to LPS. Third, the blocking of NADH oxidase activity abrogated LPS-induced TNF-α expression. The effect of NADH oxidase was mediated through O$_2^-$ generation, hydroxyl radicals, and related oxidative stress, because either the O$_2^-$ scavenger tempol, the hydroxyl radical scavenger DMTU, or the antioxidant NAC dose-dependently inhibited LPS-induced TNF-α expression. These results strongly support a pivotal role of gp91$^{phox}$-containing NADH oxidase signaling in TNF-α expression in LPS-stimulated cardiomyocytes.

A deficiency of gp91$^{phox}$ blunted LPS-induced TNF-α production, whereas NADH oxidase inhibitors abrogated TNF-α production in cardiomyocytes, which suggests the presence of an alternative Nox isofrom. Indeed, a previous study showed that the heart expresses at least 2 Nox isoforms, gp91$^{phox}$ and Nox4. Nox4 isoform was also detected in both wild-type and gp91$^{phox/-/-}$ cardiomyocytes in the present study (data not shown). Further studies are required to investigate the contribution of different Nox isoforms in myocardial TNF-α expression during LPS stimulation.

NADH Oxidase in MAPK Activation by LPS

We have demonstrated that p38 MAPK is required for TNF-α expression in LPS-stimulated cardiomyocytes. MAPK is sensitive to oxidative stress. In the present study, our working hypothesis was that the signaling pathway downstream of NADH oxidase involves ERK1/2 and p38 MAPK activation. Consistent with this hypothesis, we showed that inhibition of NADH oxidase attenuated ERK1/2 and p38 MAPK phosphorylation in LPS-stimulated cardiomyocytes. Selective inhibition of ERK1 and p38α with their respective siRNAs significantly attenuated LPS-induced TNF-α expression. The present study suggests that LPS increases NADH oxidase activity, which increases oxidative stress and hydroxyl radicals through O$_2^-$ generation. Increased oxidative stress and hydroxyl radicals subsequently lead to activation of ERK1/2 and p38 MAPK, which upregulates TNF-α expression in cardiomyocytes (Figure 9).

In summary, the present study provided definitive evidence that gp91$^{phox}$-containing NADH oxidase activity is pivotal in LPS-induced TNF-α expression in the heart. This signaling pathway involves O$_2^-$ generation and activation of ERK1/2 and p38 MAPK. Moreover, gp91$^{phox}$-containing NADH oxi-
Thrombin contributes to myocardial dysfunction during endotoxemia (Figure 9). The present study suggests that NADH oxidase may represent a novel therapeutic target for TNF-α expression and myocardial dysfunction in sepsis.

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