NADH oxidase signaling induces cyclooxygenase-2 expression during lipopolysaccharide stimulation in cardiomyocytes

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SPECIFIC AIMS

The aim of the present study was to investigate NADH oxidase signaling mechanisms leading to cyclooxygenas-2 (COX-2) expression in cardiomyocytes during lipopolysaccharide (LPS) stimulation. The roles of gp91^{phox}-containing NADH oxidase, mitogen-activated protein kinases (MAPK), and nuclear factor (NF) -κB in LPS-induced COX-2 expression were studied in cultured neonatal mouse cardiomyocytes.

PRINCIPAL FINDINGS

1. Basal COX-2 expression in cultured neonatal cardiomyocytes

Under normal culture conditions, COX-2 mRNA was detected by RT-PCR in cardiomyocytes. PGE$_2$, an end product of COX-2, was detectable the culture medium, which was almost completely inhibited by a selective COX-2 inhibitor NS398 (20 μM). These data suggest that cardiomyocytes express basal levels of COX-2 and produce PGE$_2$.

2. LPS-induced COX-2 expression does not require de novo protein synthesis

In response to LPS (10 μg/mL), up-regulation of COX-2 mRNA occurred in < 30 min and reached a maximum at ~2–4 h. To determine whether increased COX-2 expression resulted from the effect of LPS per se, cardiomyocytes were treated with LPS in the presence or absence of cycloheximide (10 μM), an inhibitor of protein translation. Results showed that LPS-increased COX-2 mRNA levels were not decreased by cycloheximide, suggesting that LPS induces COX-2 mRNA expression by a mechanism independent of de novo protein synthesis in cardiomyocytes.

3. NADH oxidase signaling contributes to COX-2 expression in LPS-stimulated cardiomyocytes

LPS treatment (10 μg/mL) increased O$_2^-$ production by >2-fold as well as gp91^{phox} and p47^{phox} mRNA expression, subunits of NADH oxidase. Cardiomyocytes were treated with LPS in the presence or absence of a selective NADH oxidase specific inhibitor diphenyleneiodonium (DPI, 20 μM) or apocynin (1 mM). LPS-induced COX-2 mRNA expression and PGE$_2$ release were measured 4 h after LPS treatment. Results showed that inhibition of NADH oxidase activity with either DPI or apocynin prevented increases in COX-2 mRNA expression and abolished PGE$_2$ release in response to LPS.

To further investigate the role of NADH oxidase in COX-2 expression induced by LPS, cardiomyocytes from gp91$^{phox-/-}$ mice were used. Deficiency of gp91^{phox} significantly decreased basal NADH oxidase activity and PGE$_2$ release ($P<0.05$, **Fig. 1**). In response to LPS, NADH oxidase activity, COX-2 expression and PGE$_2$ release were all significantly decreased in gp91$^{phox-/-}$ compared with wild-type cardiomyocytes ($P<0.05$, Fig. 1). The contribution of NADH oxidase to COX-2 expression was further confirmed by using p22^{phox} antisense oligonucleotides. Incubation with the antisense oligonucleotide of p22^{phox} decreased p22^{phox} mRNA expression and blocked the increase in COX-2 mRNA expression in response to LPS. Thus, data from pharmacological and antisense specific inhibition and from knockout mice support the notion that NADH oxidase signaling plays an important role in COX-2 expression during LPS challenge in cardiomyocytes.

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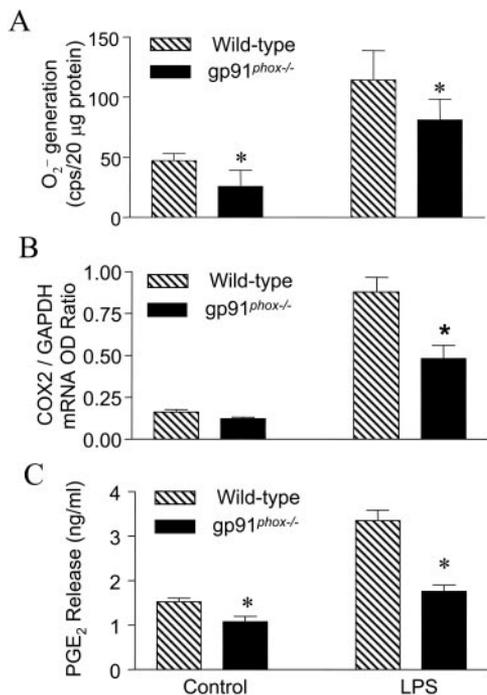
Figure 1. Deficiency of gp91^{phox} on O$_2^-$ generation, COX-2 expression, and PGE$_2$ release in LPS-stimulated cardiomyocytes. *A)* O$_2^-$ generation determined by lucigenin-enhanced chemiluminescence (cps, counts/s) in cardiomyocytes from wild-type or gp91$^{phox-/-}$ mice after LPS challenge. *B, C)* Cardiomyocytes from wild-type or gp91$^{phox-/-}$ mice were treated with vehicle or LPS (0.2 µg/mL) for 4 h. Total RNA was isolated from cardiomyocytes. COX-2 and GAPDH mRNA was determined by RT-PCR. PGE$_2$ release was determined in the supernatant by ELISA. *B)* Quantitative analysis for basal and LPS-stimulated COX-2 mRNA expression from 3 independent experiments in wild-type and gp91$^{phox-/-}$ cardiomyocytes. Basal and LPS-stimulated COX-2 mRNA expression was significantly decreased in gp91$^{phox-/-}$ compared with wild-type cardiomyocytes (*P*<0.05). *C)* PGE$_2$ release in wild-type and gp91$^{phox-/-}$ cardiomyocytes. Data are means ± SD of 3–5 independent experiments (*P<0.05 vs. wild-type).

4. MAPK-dependent pathway is required for COX-2 expression and inhibition of NADH oxidase attenuates MAPK activation

LPS (10 µg/mL) treatment rapidly increased phosphorylation of all three subfamilies of MAPK: ERK1/2, p38, and JNK1/2 in cardiomyocytes. Increased phosphorylation of MAPK attains maximum within 30 min and returns to basal levels after 4 h. To examine the role of MAPK in LPS-induced COX-2 expression, cardiomyocytes were treated with LPS in the presence or absence of selective inhibitors of MEK1/2 (PD98059), p38 (SB203580), or JNK1/2 (SP600125), then COX-2 mRNA and PGE$_2$ release was determined. Inhibition of ERK1/2, p38, or JNK1/2 with PD98059 (20 µM), SB203580 (10 µM), or SP600125 (10 µM) decreased COX-2 mRNA expression by 60%, 93%, and 69%, respectively, and abrogated PGE$_2$ release in response to LPS. These data indicate that LPS-induced COX-2 expression is mediated through an MAPK-dependent pathway.

To further investigate the role of MAPK in COX-2 expression induced by LPS, siRNAs or adenoviral vectors containing dominant negative mutant were used for specific inhibition of ERK1/2, p38 MAPK, and JNK1. Transfection with ERK1, ERK2, or p38 siRNAs suppressed ERK1, ERK2, and p38α mRNA expression, respectively, and decreased COX-2 mRNA expression in LPS-stimulated cardiomyocytes (**Fig. 2A, B**). Infection with adenoviral vectors containing dominant negative mutant of JNK1 blocked the increase in COX-2 mRNA expression induced by LPS (Fig. 2C). These data indicate that LPS-induced COX-2 expression is mediated through an MAPK-dependent pathway.

To explore the role of NADH oxidase signaling in LPS-induced MAPK activation, cardiomyocytes were treated with LPS in the presence or absence of DPI (20 µM). After 30 min coincubation, phosphorylated levels of ERK1/2, p38, and JNK1/2 were determined. Inhibition of NADH oxidase activity attenuated phosphorylation of ERK1/2, p38, and JNK1/2 by 50%, 57%, and 80%, respectively, suggesting that MAPK activation depends on NADH oxidase signaling in LPS-stimulated cardiomyocytes.

5. Inhibition of NADH oxidase and MAPK pathway suppresses NF-κB activation

After LPS treatment (10 µg/mL), NF-κB was activated within 30 min and peaked at 2–4 h. Cardiomyocytes were treated with DPI (20 µM) for 30 min followed by LPS challenge for 4 h. DPI decreased NF-κB/DNA binding by 89%. Cardiomyocytes were treated with SP600125 (10 µM) PD98059 (20 µM), or SB203580 (10 µM) for 30 min followed by LPS challenge for 4 h. Inhibition of JNK 1/2 and ERK 1/2 by SP600125 and PD98059 attenuated LPS-increased NF-κB activation by 53% and 32%, respectively, but inhibition of p38 by SB203580 had no effect. These data suggest that NF-κB activation depends on NADH oxidase signaling-mediated JNK1/2 and ERK1/2 pathway during LPS stimulation.

CONCLUSIONS AND SIGNIFICANCE

COX-2 is generally considered an inducible isoform of COX. In the present study, neonatal cardiomyocytes express basal COX-2 mRNA and activity. Basal levels of PGE$_2$ were decreased by inhibition of COX-2 activity. COX-2 mRNA and activity increased by 4- and 2-fold, respectively, in response to LPS. Our data suggest that COX-2 is constitutively expressed in cardiomyocytes and LPS up-regulates its expression and activity.

Earlier studies have shown that activation of MAPK plays an important role in COX-2 expression and PGE$_2$ production in monocytes and macrophages in response to LPS. We demonstrated here that LPS induces activation of all three subfamilies of MAPK: ERK1/2, p38, and JNK1/2 in cardiomyocytes. More important, inhibiting each one prevented COX-2 expression and abol-
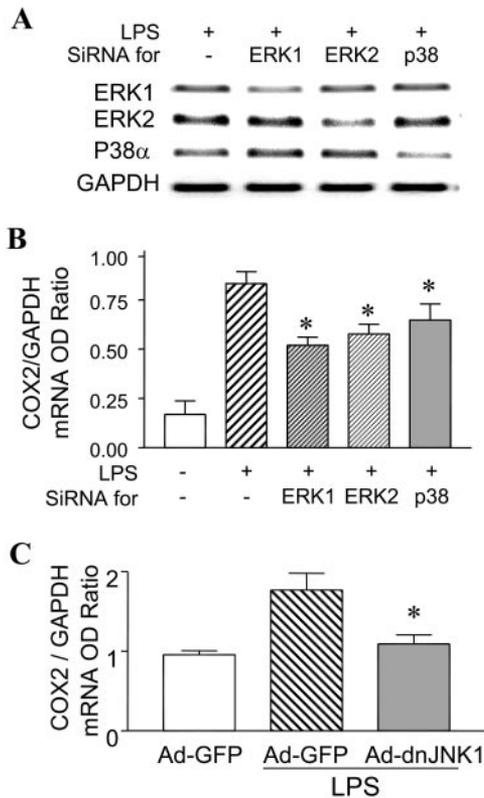
Figure 2. Effects of MAPK knock-down on LPS-induced COX-2 expression. *A, B)* Cardiomyocytes were transfected with siRNAs for ERK1, ERK2, and p38 MAPK. After siRNA treatment, cardiomyocytes were incubated with vehicle or LPS (10 μg/mL) for 4 h. *A)* Representative RT-PCR for MAPK mRNA expression from 3 different experiments shows specific down-regulation of ERK1, ERK2, and p38α MAPK mRNA expression by respective siRNA. *B)* Effects of ERK1, ERK2, and p38 MAPK siRNA on LPS-induced COX-2 mRNA expression by semiquantitative RT-PCR. *C)* Cardiomyocytes were infected with adenoviral vectors containing dnJNK1 (Ad-dnJNK1) or GFP (Ad-GFP). LPS (10 μg/mL) was added 48 h later and incubated another 4 h. Total RNA was extracted from cardiomyocytes and semiquantitative RT-PCR was performed to analyze COX-2 and GAPDH mRNA. Infection with Ad-dnJNK1 abrogated LPS-induced COX-2 mRNA expression. Data are means ± SD of 3–4 independent experiments (*$P<0.05$ vs. LPS or Ad-GFP+LPS).

ished PGE$_2$ release in LPS-stimulated cardiomyocytes. LPS increased NF-κB activity and inhibition of JNK1/2 or ERK1/2, but p38 activation decreased NF-κB activity in response to LPS. These data suggest that NF-κB activation and downstream COX-2 expression are mediated by activation of JNK1/2 and ERK1/2 MAPK in LPS-stimulated cardiomyocytes.

NADH oxidase has been shown to be a major source of oxidative stress in the myocardium. Recent studies have demonstrated that NADH oxidase activity and O$_2^-$ production are markedly increased in LPS-stimulated hearts. The present study showed that gp91^{phox}-containing NADH oxidase signaling is pivotal in LPS-induced COX-2 expression in cardiomyocytes. This conclusion was drawn from the following data: *1)*

LPS-induced NADH oxidase activation as demonstrated by increases in gp91^{phox} and p47^{phox} expression and NADH oxidase activity; *2)* deficiency of gp91^{phox} or down-regulation of p22^{phox}, blocking NADH oxidase activity, or scavenging O$_2^-$ production abrogated COX-2 mRNA expression and PGE$_2$ production in response to LPS; *3)* activation of ERK1/2, p38, and JNK1/2 MAPK was prevented in LPS-stimulated cardiomyocytes after inhibition of NADH oxidase activity; and *4)* LPS-induced NF-κB activation was blocked by NADH oxidase inhibition. One may argue the selectivity of DPI and apocynin on NADH oxidase activity. However, the same results obtained from gp91$^{phox-/-}$ mice and selective down-regulation of p22^{phox} provided definitive evidence on the role of NADH oxidase in COX-2 expression.

LPS via toll-like receptor (TLR) -4 increases NADH oxidase activity, which in turn induces reactive oxygen species (ROS) formation through O$_2^-$ generation. Increased ROS levels lead to activation of MAPK and NF-κB, which promotes COX-2 expression and PGE$_2$ production in cardiomyocytes (**Fig. 3**). Since LPS-induced COX-2 expression is considered detrimental to cardiovascular function during sepsis, the discovery of the NADH oxidase/MAPK/NF-κB/COX-2 pathway in cardiomyocytes may have therapeutic implications in sepsis. FJ
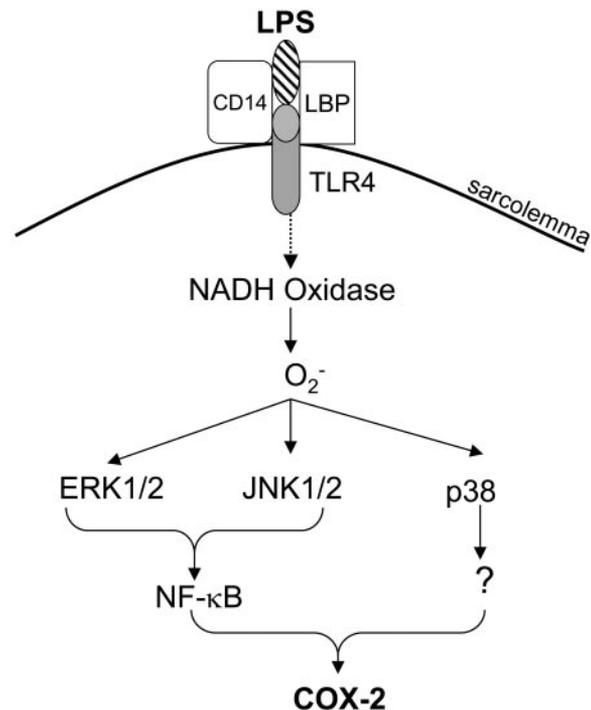

Figure 3. Schematic NADH oxidase signaling pathway leading to COX-2 expression during LPS stimulation in cardiomyocytes. LPS activates NADH oxidase via TLR-4 and produces O$_2^-$, which increases phosphorylation of ERK1/2, JNK1/2, and p38 MAPK. Activation of MAPK results in COX-2 expression. While effects of ERK1/2 and JNK1/2 are mediated by NF-κB activation, the effect of p38 on COX-2 expression is independent of NF-κB signaling.