Resistance to Endotoxic Shock in Endothelial Nitric-oxide Synthase (eNOS) Knock-out Mice

A PRO-INFLAMMATORY ROLE FOR eNOS-DERIVED NO IN VIVO

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The expression of inducible nitric-oxide synthase (iNOS) and subsequent “high-output” nitric oxide (NO) production underlies the systemic hypotension, inadequate tissue perfusion, and organ failure associated with septic shock. Therefore, modulators of iNOS expression and activity, both endogenous and exogenous, are important in determining the magnitude and time course of this condition. We have shown previously that NO from the constitutive endothelial NOS (eNOS) is necessary to obtain maximal iNOS expression and activity following exposure of murine macrophages to lipopolysaccharide (LPS). Thus, eNOS represents an important regulator of iNOS expression in vitro. Herein, we validate this hypothesis in vivo using a murine model of sepsis. A temporal reduction in iNOS expression and activity was observed in LPS-treated eNOS knock-out (KO) mice as compared with wild-type animals; this was reflected in a more stable hemodynamic profile in eNOS KO mice during endotoxemia. Furthermore, in human umbilical vein endothelial cells, LPS leads to the activation of eNOS through phosphoinositide 3-kinase- and Akt/protein kinase B-dependent enzyme phosphorylation. These data indicate that the pathogenesis of sepsis is characterized by an initial eNOS activation, with the resultant NO acting as a co-stimulus for the expression of iNOS, and therefore highlight a novel pro-inflammatory role for eNOS.

The systemic hypotension, organ failure, and morbidity that occur during bacterial sepsis are associated with the expression of inducible nitric-oxide synthase (iNOS) and excessive production of nitric oxide (NO). This is evidenced by the increased levels of nitrite (NO₂⁻) and nitrate (NO₃⁻); stable metabolites of NO) measured in the plasma of septic patients (1, 2), inflammation-induced iNOS expression (3, 4), and by the ability of selective iNOS inhibitors to restore blood pressure in experimental models of sepsis and reverse hypotension in human endotoxaemia (5–10).

Inducible NOS is effectively absent under physiological conditions but is expressed in many cell types in response to pro-inflammatory cytokines and lipopolysaccharides (LPS); in accord, induction of this NOS isoform is necessary for “high output” NO production and cytostatic and cytotoxic effects that facilitate host defense (11). While iNOS expression is essential to combat bacterial infection, the sustained overproduction of NO is deleterious to the host, as exemplified by the cardiovascular dysfunction during sepsis. Since iNOS is regulated primarily at a transcriptional level (12–14), a better understanding of the mechanisms involved in iNOS mRNA and protein expression should lead to improved treatment for endotoxemia and other inflammatory cardiovascular disorders.

In contrast to iNOS, a potential role for endothelial NOS (eNOS) in the pathogenesis of sepsis is unsubstantiated. Initial studies suggested that eNOS knock-out (KO) animals respond to endotoxin in an identical manner to wild-type (WT) littermates (15), and more recently, mice overexpressing eNOS were shown to express equivalent levels of iNOS mRNA and generate similar levels of plasma NO₂⁻ and NO₃⁻ (an index of iNOS activity) to control animals (16) in response to LPS. However, we have recently demonstrated that eNOS acts in a pro-inflammatory manner in immune cells by facilitating iNOS expression in response to endotoxin, such that in bone marrow-derived macrophages from eNOS KO mice, iNOS expression and activity are less than 50% of WT controls (17). This pro-inflammatory effect of eNOS-derived NO occurs, at least in part, via a modulation of the activity of the transcription factor, nuclear factor κB (NF-κB) (18). Further studies give support to a potential pro-inflammatory role for eNOS. For instance, a reduction in both basal and LPS-induced tumor necrosis factor-α (TNF-α) production occurs in neonatal mouse cardiomyocytes from eNOS KO mice (19) and in HeLa and microvascular endothelial cells, TNF-α has been shown to activate eNOS (20, 21). These studies do not only demonstrate the importance of eNOS for the up-regulation of pro-inflammatory protein expression but also illustrate the auto-regulation of NOS expression by NO, since iNOS-derived NO is known to feedback and inhibit the expression and activity of eNOS (22, 23). In combination, these data give rise to the hypothesis that eNOS plays a key role in the expression of iNOS and the pathogenesis of sepsis.

In accord, in the present study we have employed eNOS KO mice to examine the role of this NOS isoform in an in vivo conscious animal model of sepsis (10). Moreover, we have used human umbilical vein endothelial cells (HUVECs) and murine bone marrow-derived macrophages (BMMDMs) (from WT and eNOS KO mice) to dissect the mechanisms underlying LPS-stimulated eNOS activity. Herein, we demonstrate that eNOS plays an important role in facilitating iNOS expression in vivo.
during endotoxaemia with a marked reduction in iNOS protein observed in the liver, lung, heart, and aorta of eNOS KO mice as compared with WT, in addition to an accompanying reduction in plasma levels of NO₂⁻ and NO₃⁻. The impaired iNOS expression and activity are accompanied by a more stable hemodynamic profile and reduced mortality in response to LPS. We also show that in HUVECs and BMDMØs, LPS activates eNOS via a phosphoinositide 3-kinase (PI3K)- and Akt/protein kinase B-dependent mechanism.

MATERIALS AND METHODS

Reagents—Deguelin was purchased from Alexis (Nottingham, UK). 3-Isobutyl-1-methylxanthine and LY294002 were purchased from Calbiochem (Nottingham, UK). All drugs were resuspended in dimethyl sulfoxide (Me₂SO) such that the final concentration of Me₂SO in culture did not exceed 0.001%. *Salmonella typhosa* LPS was purchased from Sigma. All other reagents were obtained from Sigma unless stated otherwise.

**LPS Treatment and Tissue Homogenization—** *S. typhosa* LPS (12.5 mg/kg, intravenously) was administered to 6–8-week-old male WT (C57/B16; 15–25 g) and eNOS KO mice via the tail vein. After the 15-min time interval the animals were euthanized and tissues and/or blood collected. Tissues were snap frozen in liquid nitrogen and stored at −80 °C before transfer into whole-cell homogenization buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 8 mM EGTA, and 1 μg/ml benzamidine, leupeptin, antipain, and aprotinin). Tissues were homogenized, centrifuged (13,793 × g, 4 °C, 15 min), and the supernatants retained for subsequent analysis.

**Measurement of Mean Arterial Blood Pressure—** Mice (WT and eNOS KO; male; 15–25 g) were anesthetized briefly with isoflurane (2%). A cannula (internal diameter: 0.28 mm, outer diameter: 0.61 mm) was implanted through the axillary artery and guided into the aorta and catheterized and connected to a swivel/tether system secured to the mouse using four silk sutures. This enabled the mouse, on recovery, to have unimpeded movement around the cage with free access to food and water. Both lines were flushed continuously with 0.1 ml/h heparinized-saline (1:1,000,000).

**Reagents—** LPS was purchased from *Salmonella typhosa* (C57/BL6; 15–25 g) and eNOS KO mice via the tail vein. After the 15-min time interval the animals were euthanized and tissues and/or blood collected. Tissues were snap frozen in liquid nitrogen and stored at −80 °C before transfer into whole-cell homogenization buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 8 mM EGTA, and 1 μg/ml benzamidine, leupeptin, antipain, and aprotinin). Tissues were homogenized, centrifuged (13,793 × g, 4 °C, 15 min), and the supernatants retained for subsequent analysis.

**Western Blot Analysis—** Protein concentrations were determined by densitometry (NIH image).

**Cyclic GMP Assay—** BMDMØs were seeded into 6-well culture plates (4 × 10⁶ cells/well) in 2 ml of medium and incubated overnight at 37 °C in a humidified incubator containing 5% CO₂ in air. After activation with LPS (100 ng/ml) for appropriate times, the medium was removed, and cells were washed with PBS before addition of phospho-homogenization buffer (as described above). For experiments to study the effect of deguelin or LY294002 on Akt phosphorylation, cells were preincubated with deguelin (1 μM), LY294002 (10 μM), or Me₆SO (control) for 30 min before addition of LPS (100 ng/ml). Homogenates were scraped into 1.5-ml tubes and then centrifuged (13,793 × g, 5 min, 4 °C) and the supernatants retained for subsequent analysis.

**Plasma Cyclic GMP Assays—** Plasma samples were analyzed for NO₂⁻ and NO₃⁻ using chemiluminescence as described previously (24). Briefly, samples and standards containing NO₂⁻ and NO₃⁻ were first reduced to NO, which was then quantified after reaction with ozone using a nitric oxide analyzer (NOA 280, Sievers). To determine total NO₂⁻ and NO₃⁻ concentrations, collectively termed “NO” samples were added to 0.1 M vanadium (III) chloride in 1 M hydrochloric acid refilling at 90 °C under nitrogen.

**Data Analysis—** All statistical analysis was performed using Graph Pad Prism (Graph Pad Software Inc., San Diego, CA). Densitometric analyses were performed using NIH Image. All data are plotted graphically as mean values with vertical bars representing standard error of the mean (S.E.). A Student’s t test was used to assess differences between individual experimental conditions. Analysis of variance was...
used to compare temporal changes in MABP in WT and eNOS KO animals. A probability ($p$) value of $<0.05$ was taken as an appropriate level of significance.

**RESULTS**

**Hemodynamic Profile in WT and eNOS KO Mice in Response to LPS**—The administration of LPS (12.5 mg/kg) led to a biphasic effect on the MABP of WT mice ($n = 6$). There was an immediate but transient drop in MABP following administration of LPS (~10 mm Hg) that recovered to basal levels after 4–5 h. At this time point, the MABP fell again and continued to decline for the duration of the experiment (18 h) or until the animal died (two out of six; Fig. 1). In contrast, in eNOS KO mice the blood pressure remained stable immediately after injection of LPS, and while at 6 h the MABP began to fall slightly, this only deviated from basal levels by $<20\%$ (Fig. 1).

**Plasma NOx Accumulation in Response to LPS**—Resting plasma [NO$_x$] were higher in WT compared with eNOS KO animals, as would be expected in animals lacking eNOS-derived NO. Administration of LPS (12.5 mg/kg) resulted in an increase in plasma [NO$_x$] in both WT and eNOS KO mice (Fig. 2). The increases in plasma [NO$_x$] closely mirrored the sustained fall in MABP observed in WT animals. However, the plasma NO$_x$ levels in eNOS KOs were significantly reduced in comparison with WT animals, such that at 12 h there was an approximate 50% reduction in plasma [NO$_x$] in eNOS KO animals (Fig. 2).

**Effect of eNOS Deletion on iNOS Expression in Response to LPS**—There was no detectable iNOS expression in tissues from control mice either WT or eNOS KO (saline-treated). Administration of LPS (12.5 mg/kg) led to a time-dependent increase in the expression of iNOS protein in the liver, lung, heart, and aorta of WT and eNOS KO mice (Fig. 3). However, in tissues from eNOS KO mice there was a significantly lower level of iNOS protein expression after treatment with LPS, with the maximum differential effect observed between 9 and 12 h (Fig. 3). The peak iNOS protein levels were $69.1 \pm 10.5\%$ in the liver, $51.3 \pm 22.9\%$ in the lung, $52.6 \pm 30.0\%$ in the heart, and $58.1 \pm 1.8\%$ in the aorta of those observed in WT animals ($p < 0.05$ for each).

**FIG. 2.** NO$_x$ production is reduced in eNOS KO mice. Accumulation of NO$_x$ (NO$_2 +$ NO$_3$) in the plasma of WT and eNOS KO mice injected with LPS (12.5 mg/kg; intravenously) is shown. Data are represented as mean ± S.E. plasma [NO$_x$] with background (basal NO$_x$ concentration in eNOS KO mice) subtracted. $p < 0.05$, WT versus eNOS KO ($n = 4$).

**FIG. 3.** Expression of iNOS is reduced in tissues from eNOS KO mice. A, Western blot depicting iNOS expression in the liver, lung, heart, and aorta of WT and eNOS KO mice 12 h following administration of LPS (12.5 mg/kg; intravenously). B–E, expression of iNOS protein in liver (B), lung (C), heart (D), and aorta (E) from WT (open circles) and eNOS KO (closed circles) mice injected with LPS (12.5 mg/kg; intravenously). Protein expression was analyzed by Western blot 3, 6, 9, and 12 h after LPS administration, and bands were quantified by densitometry. Data are represented as mean ± S.E. density expressed as a percentage of peak protein expression in WT animals. ($n = 4$).
Akt and eNOS Phosphorylation in Response to LPS in HUVECs—Since our in vivo investigations had revealed a key role for eNOS in facilitating iNOS expression, we investigated whether the PI3K/Akt pathway was responsible for activation of eNOS in response to LPS in HUVECs.

HUVECs were incubated in basal medium without growth factors for 4 h (to minimize basal Akt and eNOS phosphorylation) followed by stimulation with LPS (1 μg/ml) for 60 min and Akt phosphorylation assessed by Western blot. LPS caused a transient Akt phosphorylation that peaked at ~15 min and returned to basal levels within 60 min (Fig. 4).

Since Akt was phosphorylated in response to LPS stimulation, the downstream phosphorylation of eNOS was also studied under the same conditions. After 4-h incubation in basal medium, cells were stimulated with LPS (1 μg/ml) for 60 min and eNOS phosphorylation assessed by Western blot. In a pattern that mirrored Akt activation, eNOS was transiently phosphorylated after LPS treatment with peak levels of phosphorylation occurring between 15 and 30 min after activation (Fig. 5).

Akt and eNOS Phosphorylation in Response to LPS in Murine BMDMØs—Since HUVECs do not express iNOS in response to inflammatory stimuli in vitro, and so that it was possible to exploit KO technology, we used BMDMØs to demonstrate a link between LPS-induced PI3K/Akt and eNOS phosphorylation with iNOS expression. Cells were treated with LPS (100 ng/ml) and phosphorylation of Akt monitored over 60 min by Western blot. A transient increase in phosphorylated Akt was observed in response to LPS treatment, with a peak effect observed between 15 and 30 min (Fig. 6). Since BMDMØs express very low levels of eNOS (some 100-fold less than endothelial cells (17)), it was not possible to determine whether eNOS phosphorylation occurred as a result of LPS-stimulated Akt activation in these cells. However, a similar pattern of Akt phosphorylation was observed in eNOS KO cells in response to LPS (data not shown) confirming that the activation of Akt occurs upstream of eNOS.

To investigate whether the phosphorylation and activation of Akt was linked with eNOS activation and the expression of iNOS, WT and eNOS KO BMDMØs were activated with LPS (100 ng/ml) for 9 h in the presence and absence of the PI3K/Akt inhibitors deguelin (1 μM) (25) or LY294002 (10 μM) and iNOS expression determined by Western blot after 15 min. In the presence of deguelin or LY294002, iNOS expression in WT cells was 32.4 ± 8.2% (Fig. 7) and 64.9 ± 5.4% (Fig. 7) of the expression levels observed with LPS only (control). However, in eNOS KO cells, both deguelin and LY294002 failed to cause a significant reduction in LPS-stimulated iNOS expression (Fig. 7). Indeed, in the presence of deguelin and LY294002, iNOS expression in response to LPS was essentially identical in cells from WT and eNOS KO mice (Fig. 7), confirming the importance of eNOS-derived NO to iNOS expression.

Effect of Deguelin and LY294002 on Akt Phosphorylation in Response to LPS in WT and eNOS KO BMDMØs—To confirm that the effects of deguelin and LY294002 on iNOS expression were due specifically to inhibition of Akt activity, BMDMØs were activated with LPS (100 ng/ml) in the presence and absence of deguelin (1 μM) or LY294002 (10 μM) and Akt phosphorylation determined by Western blot after 15 min. In the presence of deguelin, Akt phosphorylation was reduced to 60.5 ± 10.9% of the level observed with LPS only (control) in WT cells and 35.9 ± 11.3% of control levels in eNOS KO cells (Fig. 8). Accordingly, in the presence of LY294002, Akt phosphorylation was reduced to 49.0 ± 10.3% of the level observed with LPS only (control) in WT cells and 32.3 ± 6.5% of control levels in eNOS KO cells (Fig. 8). Thus, while both deguelin and LY294002 have an inhibitory effect on Akt phosphorylation in

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**Fig. 4. Phosphorylation of Akt in HUVECs in response to LPS.** Levels of Akt and phospho-Akt (P-Akt) in HUVECs following stimulation with LPS (1 μg/ml) over 60 min. Akt and phospho-Akt were analyzed by Western blot (upper panels) and bands quantified by densitometry (lower panel). Data are represented as mean ± S.E. density expressed as a percentage of peak Akt phosphorylation. Background phosphorylation has been subtracted from each sample and phospho-Akt levels normalized to Akt expression (n = 3).

**Fig. 5. Phosphorylation of eNOS in HUVECs in response to LPS.** Levels of eNOS and phospho-eNOS in HUVECs following stimulation with LPS (1 μg/ml) over 60 min. Endothelial NOS and phospho-eNOS were analyzed by Western blot (upper panels) and bands quantified by densitometry (lower panel). Data are represented as mean ± S.E. density expressed as a percentage of peak eNOS phosphorylation. Background phosphorylation has been subtracted from each sample and phospho-eNOS levels normalized to eNOS expression (n = 3).

**Fig. 6. Phosphorylation of Akt in BMDMØs in response to LPS.** Levels of Akt and phospho-Akt in WT BMDMØs after stimulation with LPS (100 ng/ml) over 60 min. Akt and phosphorlated Akt were analyzed by Western blot (upper panels) and bands quantified by densitometry (lower panel). Data are represented as mean ± S.E. density expressed as a percentage of peak Akt phosphorylation. Phospho-Akt levels normalized to Akt expression (n = 3).
both WT and eNOS KO, this was only manifested as an inhibition of iNOS activity in cells from WT animals.

Effect of Deguelin and LY294002 on cGMP Accumulation in Response to LPS in WT and eNOS KO BMDMØs—Since the concentrations of NO₂⁻ and NO₃⁻ generated by BMDMØ eNOS are too low to be detected by chemiluminescence analysis, we measured cGMP accumulation as an index of eNOS activity to further demonstrate that stimulation of the PI3K/Akt pathway underlies eNOS activation by LPS. We have previously demonstrated that stimulation of BMDMØs with LPS (100 ng/ml) leads to an accumulation of intracellular cGMP in WT but not eNOS KO cells (17). In this case, cGMP accumulation in response to LPS stimulation was measured in the presence or absence of the PI3K/Akt inhibitors deguelin (1 μM) and LY294002 (10 μM). The stimulation of cells with LPS led to an ~40% increase in intracellular cGMP concentration as compared with background (Fig. 9); this increase was reversed in the presence of deguelin (1 μM) or LY294002 (10 μM; Fig. 9).

DISCUSSION

This study demonstrates that eNOS-derived NO plays a key role in facilitating iNOS expression in LPS-induced endotoxemia in mice in vivo. In eNOS KO animals, the systemic hypotension and mortality associated with sepsis were markedly reduced, and this was mirrored by a reduction in iNOS expression in the heart, lung, liver, and aorta and a significantly smaller production of NO (as assessed by NOₓ accumulation). Furthermore, the activation of eNOS by LPS to enable maximal iNOS expression is triggered by the PI3K/Akt pathway, as has been identified previously to couple shear stress to NO production by the endothelium (26, 27). These observations extend our previous finding that eNOS-derived NO is necessary to achieve peak iNOS expression in LPS-treated murine bone marrow-derived macrophages (17) and suggest that this constitutive NOS isoform plays an important role in the pathogenesis of sepsis. Moreover, the findings assign a potential pro-inflammatory role to eNOS, an enzyme that has been classically considered to act in an anti-inflammatory manner via NO-mediated inhibition of smooth muscle proliferation, leukocyte recruitment, and platelet aggregation (28).

To determine whether eNOS is important for the hemodynamic changes (and consequent aberrations in tissue perfusion) observed during septic shock, conscious WT and eNOS KO animals were exposed to LPS for 18 h. During this time, WT animals displayed a rapid, transient reduction in systemic blood pressure (within 1 h) that recovered almost entirely by 5 h and then dropped steadily throughout the remainder of the
investigation. In marked contrast, the eNOS KO animals did not show an initial hypotensive response to LPS administration, and while systemic blood pressure in these animals waned over the length of the experiment, this was considerably more stable than that observed in WT animals and did not deviate by more than ~20% from baseline values. These data establish two important principles with respect to eNOS activity in response to LPS. First, endotoxin causes an immediate up-regulation of eNOS activity, which is manifested as a hypotensive effect that is both rapid in onset and short-lived; such a hypotensive effect of LPS has been demonstrated previously in WT animals (10, 29) but not linked to eNOS activation. Second, a lack of eNOS-derived NO conveys resistance against endotoxemia.

To assess whether the hemodynamic responses observed in response to LPS were paralleled by iNOS expression and high output NO production, these parameters were assessed and compared in WT and eNOS KO animals. In all tissues studied (liver, lung, heart, and aorta) a reduction in iNOS expression was evident in eNOS KO mice as compared with WT. This was accompanied by reduced NO production, as evidenced by lower plasma levels of nitrite and nitrate, in eNOS KO animals. Interestingly, the time course of iNOS expression differed markedly between the tissues studies. In the liver and heart, there was detectable iNOS expression as early as 3 h following LPS administration, whereas in the lung and aorta, significant iNOS expression was not apparent until 6 h. Thus, the increase in plasma [NOx] in the first 3 h following exposure to LPS, which was more substantial in the WT animals, is likely to stem from a combination of eNOS and iNOS-derived NO; how which was more substantial in the WT animals, is likely to in plasma [NOx].

Marked between the tissues studies. In the liver and heart, there was detectable iNOS expression as early as 3 h following LPS administration, whereas in the lung and aorta, significant iNOS expression was not apparent until 6 h. Thus, the increase in plasma [NOx] in the first 3 h following exposure to LPS, which was more substantial in the WT animals, is likely to stem from a combination of eNOS and iNOS-derived NO; however, since initial iNOS expression did not differ substantially between WT and eNOS KO animals in the first 3 h, the significantly larger increase in plasma [NOx] in WT animals is likely to signify eNOS activation. Nonetheless, the extent of iNOS expression and NO production closely matched the severity of the hemodynamic perturbations following induction of endotoxaemia and confirm that eNOS KO animals exhibit a significance resistance to septic shock. Moreover, these observations establish a pivotal cross-talk between these two NOS isoforms, which is reciprocated following high-output NO production by iNOS, which inhibits the expression and activity of eNOS (22, 23).

Endothelial NOS is constitutively expressed and regulated predominantly via modulation of enzyme activity in response to changes in intracellular calcium concentration (30). This NOS isoform produces low concentrations of NO that are key under physiological conditions in the vasculature to regulation of smooth muscle tone and proliferation, leukocyte recruitment, and platelet aggregation (31). However, it has recently become apparent that both the regulation of eNOS expression and activity are more complex and extensive than was previously thought. For example, the expression of eNOS can be increased severalfold by multiple factors including shear stress, vascular endothelial growth factor, and estrogen (32–34). In addition, phosphorylation via the PI3K/Akt pathway, subcellular targeting to caveolae and interactions with proteins such as Hsp90 (26, 27, 35, 36) have also been shown to regulate enzyme activity. Since we had identified an important role for eNOS in facilitating iNOS expression in response to endotoxin in vivo, we determined whether LPS might lead directly to eNOS activation in HUVECs in vitro. In HUVECs, treatment with LPS caused a transient phosphorylation of the kinase Akt, which peaked after ~15 min. This was accompanied by phosphorylation of eNOS with a slight lag period, as would be expected if eNOS phosphorylation is downstream to Akt activation. Thus, in human endothelial cells, LPS induces eNOS activation via phosphorylation and the PI3K/Akt pathway, similar to that recently described to signal eNOS activation in response to shear stress (26, 27); this also fits with a previous finding that LPS activates Akt in vascular smooth muscle cells (37). Thus, facilitation of iNOS expression and activity by eNOS is likely to stem via a PI3K/Akt-dependent phosphorylation of the constitutive isoform, which results in increased NO production and, at least in part, facilitates iNOS expression by augmenting the activity of NF-κB, as we have demonstrated previously (17, 18). This is supported by the observation that LPS treatment of endothelial cells results in inhibition of platelet aggregation, suggesting production of NO by eNOS (38, 39). Moreover, phosphorylation of eNOS, accompanied by increases in urinary cGMP accumulation, occurs in the bladder 1 h after LPS treatment in mice (40), and in cardiomyocytes from eNOS KO mice there is a reduced accumulation of TNF-α in response to LPS as compared with WT cells (19). This later observation also provides indirect evidence of the involvement of NF-κB in the facilitatory effect of eNOS-derived NO, since this, a common transcription factor, is key in the expression of both iNOS and TNF-α.

To link the activation of eNOS by LPS, via Akt, with a modulatory effect on iNOS expression, we employed murine BMDMØs from WT and eNOS KO animals, as we have characterized previously (17). BMDMØs were exposed to LPS in the presence and absence of the PI3K/Akt inhibitors, deguelin and LY294002. LPS alone resulted in transient phosphorylation of Akt, which followed an essentially identical profile to that observed in HUVECs, peaking at ~15–30 min; this effect was significantly attenuated by deguelin or LY294002 in cells from both WT and eNOS KO mice. However, in the presence of deguelin or LY294002, a strong inhibition of LPS-stimulated iNOS expression was only observed in macrophages from WT mice; iNOS expression in eNOS KO BMDMØs was similar in the presence or absence of PI3K/Akt inhibition. Importantly, iNOS expression in BMDMØs from WT animals in the presence of deguelin or LY294002 was equivalent to iNOS expression in eNOS KO BMDMØs in the absence of PI3K/Akt inhibition; this intimates that PI3K/Akt inhibition (and consequently abolition of eNOS phosphorylation and activation) accounts for the significant difference between iNOS expression in WT versus eNOS KO. It should be noted that in a parallel series of experiments conducted with the PI3K inhibitor wortmannin, we observed a clear blockade of Akt and eNOS phosphorylation in response to LPS and yet a corresponding increase in iNOS expression. Importantly, however, this enhancement occurred in both WT and eNOS KO cells, indicating that wortmannin exerts a nonspecific (i.e. eNOS-independent) effect on iNOS expression per se. As such, it cannot be used to delineate the role of eNOS in iNOS expression.

We have demonstrated previously that the potentiation of iNOS expression by eNOS is dependent, at least in part, on the activation of sGC and the production of cGMP (17). Since the activation of eNOS by LPS in BMDMØs is not detectable using chemiluminescence analysis of NOx and NO3− production, the generation of cGMP was measured to demonstrate a direct link between phosphorylation of Akt and the activation of eNOS. Stimulation of cells with LPS alone led to an increase in intracellular cGMP accumulation, and this was blocked in the presence of deguelin or LY294002. Since cGMP accumulation in response to LPS only occurs in WT cells (18), this confirms that stimulation of PI3K/Akt phosphorylation by LPS results in eNOS activation.

The present study, in concert with previous observations, establishes a key reciprocal regulation of eNOS and iNOS.
expression during sepsis. This study provides clear evidence that eNOS-derived NO is vital for maximal iNOS expression, but in addition, iNOS-derived NO appears to feedback and inhibit the expression and activity of eNOS. For instance, a down-regulation of eNOS protein expression is observed in human endothelial cells after 72 h of treatment with either LPS or TNF-α (41), and eNOS expression is reduced 15 h after exposure to LPS in WT but not iNOS KO mice (22). Endothelial NOS activity is also very sensitive to inhibition by NO (23). Thus, while eNOS initially promotes iNOS expression, it appears to be followed by a reciprocal feedback whereby iNOS diminishes the production of NO from eNOS by reducing protein expression and activity. This would prevent prolonged and perhaps deleterious potentiation of iNOS expression by eNOS-derived NO but is also manifested as endothelial dysfunction, which is thought to contribute to the pathogenesis of sepsis and underlie the dysregulation of tissue/organ perfusion.

In conclusion, this study has extended our observations of a crucial role for eNOS derived NO in facilitating iNOS expression to an in vivo model of sepsis. Furthermore, we have demonstrated that phosphorylation and activation of both Akt and eNOS by LPS underlies this phenomenon. This pathway is critical to the initiation and prolonged expression of iNOS observed in sepsis and suggests that the activation of eNOS and subsequent NO production is an important regulatory factor in terms of pathogenesis and mortality.

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