Nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) is responsible for sepsis-induced hypotension and plays a major contributory role in the ensuing multiorgan failure. The present study aimed to elucidate the role of endothelial NO in lipopolysaccharide (LPS)-induced iNOS expression, in isolated rat aortic rings. Exposure to LPS (1 μg/ml, 5 h) resulted in a reversal of phenylephrine precontracted tone in aortic rings (70.7 ± 3.2%). This relaxation was associated with iNOS expression and NF-κB activation. Positive immunoreactivity for iNOS protein was localized in medial and adventitial layers of LPS-treated aortic rings. Removal of the endothelium rendered aortic rings resistant to LPS-induced relaxation (8.9 ± 4.5%). Western blotting of these rings demonstrated an absence of iNOS expression. However, treatment of endothelium-denuded rings with the NO donor, diethylamine-NONOate (0.1 mM), restored LPS-induced relaxation (61.6 ± 6.6%) and iNOS expression to levels comparable with arteries with intact endothelium. Blockade of endothelial NOS (eNOS) activation using geldanamycin and radicicol, inhibitors of heat shock protein 90, in endothelium-intact arteries suppressed both LPS-induced relaxation and LPS-induced iNOS expression (9.0 ± 8.0% and 2.0 ± 6.2%, respectively). Moreover, LPS treatment (12.5 mg/kg, intravenous, 15 h) of wild-type mice resulted in profound elevation of plasma [NO$_3$] measurements that were reduced by ~50% in eNOS knock-out animals. Furthermore, LPS-induced changes in vascular reactivity and iNOS expression evident in wild-type tissues were profoundly suppressed in tissues taken from eNOS knock-out animals. Together, these data suggest that eNOS-derived NO, in part via activation of NF-κB, regulates iNOS-induction by LPS. This study provides the first demonstration of a proinflammatory role of vascular eNOS in sepsis.

Bacterial sepsis is a systemic inflammatory state characterized by vascular smooth muscle dysfunction, leading to hypotension, inadequate tissue perfusion, and multiple organ failure (1). A key molecule believed to be responsible for several aspects of the septic condition is inducible nitric oxide synthase (iNOS)-derived nitric oxide (NO). In response to systemic bacterial infection, iNOS is expressed in several diverse cell types, resulting in a high output of NO production. This high concentration of NO is thought to have dual functionality; i.e. it is protective in terms of its bactericidal actions in host defense (2) but also conversely pathogenic in terms of contributing to some of the deleterious effects of endotoxemia. In particular, the impaired contractile function of smooth muscle and resultant decreased blood pressure have been attributed to increased NO-mediated dilatation, secondary to the induction and activity of iNOS (3–5), primarily in the smooth muscle and adventitial fibroblasts of the blood vessel wall (6).

Endotoxemia-associated vascular iNOS expression is induced by a variety of cytokines, including interleukin-1β and tumor necrosis factor-α, and lipopolysaccharide (LPS), an abundant glycolipid present on the outer membrane of Gram-negative bacteria (for a review, see Ref. 7). The molecular pathways involved in the regulation of iNOS expression occur largely at a transcriptional level and appear to be immensely heterogeneous, with particular mechanisms invoked in specific cell types (for a review, see Ref. 7). However, a common signaling molecule involved in these diverse pathways is the ubiquitous inflammatory transcription factor, nuclear factor (NF)-κB. Activation of toll-like receptors directly by components of infectious organisms, such as LPS, will elevate NF-κB levels in vascular cells, as also will cytokines expressed in response to the microorganism, by binding to specific cytokine receptors (for a review, see Ref. 8). NF-κB then translocates to the nucleus, where it binds to the promoter regions of various inflammatory genes including iNOS (9, 10). Interestingly, recent reports have identified an autoregulatory role for NO mediated by regulation of NF-κB levels: at low concentrations of NO, NF-κB levels are raised, and conversely, at high NO concentrations, NF-κB levels and DNA binding activity are suppressed (11, 12). These studies, largely conducted using NO donors as the source of NO, indicate a dual nature of NO dependent on concentration and imply tight autoregulation by NO of its own expression.

There is good evidence to support the thesis that high...
concentrations of NO produced consequent to iNOS induction suppress constitutive NOS activity i.e. endothelial NOS (eNOS) and neuronal NOS activity (13). Indeed, our own recent studies, using iNOS knock-out mice, demonstrate that this autoregulation underlies the endothelial dysfunction evident in sepsis (14). However, recent work using bone marrow-derived macrophages generated from eNOS knock-out and wild-type mice indicate a reciprocal relationship between eNOS-derived NO and NO produced by iNOS, such that constitutive low level NO production is essential for maximal iNOS expression in response to LPS in these cells (15). Whether such a reciprocal interaction exists between vascular eNOS-derived NO and iNOS activity in terms of the vascular effects of NO in sepsis is unknown. Therefore, in the present study, we investigated whether endothelial NO played a regulatory role on LPS-induced iNOS expression and function in rat isolated aortic rings, *ex vivo*, and in thoracic aortae from wild-type (WT) and eNOS knock-out (KO) mice, *in vivo*.

**EXPERIMENTAL PROCEDURES**

**Materials**—All compounds used were obtained from Sigma except diethylamine-NONOate (DETA-NO), which was obtained from Cayman Chemical. Drugs were prepared using saline (0.9% w/v) except geldanamycin (GA), radicicol (RAD), wortmannin, and LY294002, which were dissolved in Me2SO and diluted with saline.

**Organ Bath Studies**—All experiments were conducted according to the Animals (Scientific Procedures) Act 1986, UK.

**Rat Aortic Rings**—Sprague-Dawley rats (200–250 g) were killed by cervical dislocation. The thoracic aorta was removed and placed in cold physiological salt solution of composition (in mM): 119 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 25 NaHCO3, 1.2 KH2PO4, and 5.5 glucose. Aortae were cleaned of extraneous tissue, and rings were (2–3 mm) cut and mounted in 10-ml organ baths, under 1 g of tension, for online isometric tension recording using Chart™ v 4.2 software (AD Instruments). Tissues were bathed in physiological salt solution maintained at 37 °C and bubbled with 5% CO2 in O2.

Following a 45-min washing period, rings were subjected to repeated administration of phenylephrine (PE; 10 μM) until responses were reproducible. Following this, rings were precontracted with PE to an EC80, and the relaxant response to acetylcholine (10 μM) was measured to determine endothelial integrity; those vessels in which reversal of PE-induced tone was >50% were deemed endothelium-intact. In endothelium-denuded rings, tissues were deemed denuded if the relaxant response to acetylcholine was <10%. Arteries were then precontracted with PE to an EC80, by titration, and LPS (*Escherichia coli* 055:B5; 1 μg/ml) was added to the organ baths. Thereafter, tissue tension was monitored for 5 h, and at the end of this time, tissues were collected, flash-frozen in liquid nitrogen, and stored at −80 °C for biochemical analyses. To investigate the role of eNOS in LPS-induced relaxation and iNOS expression, some rings were pretreated with the NO donor DETA-NO (0.1–1 μM) and inhibitors of eNOS activation: the heat shock protein 90 (HSP90) inhibitors GA (10 μM) and RAD (1 μM) (16) or the phosphatidylinositol 3-kinase (PI3-K)/Akt inhibitors wortmannin (0.5 μM) and LY294002 (10 μM) (17, 18). All drugs were administered 20 min prior to the addition of LPS. Since PE was titrated for precontraction, the level of precontraction between groups was maintained, irrespective of drug treatment.

**Murine Aortic Rings**—Male (20–40 g) WT (C57BL/6J) and eNOS gene knock-out mice (19) that were bred in-house were pretreated with saline or LPS (12.5 mg/kg, intravenous, 15 h) prior to culling by cervical dislocation. The thoracic aortae were cleaned of surrounding fat and mounted in organ baths, as above, under a basal tension of 0.3 g. Rings were equilibrated with KCl (48 mM) as above, and endothelial integrity was determined in aortic rings of WT animals only. Following this, rings were precontracted with phenylephrine (to produce a level of contraction equivalent to 80% of the maximal response to KCl), and relaxation response curves to the NO donor spermine-NONOate (Sper-NO; 0.001–3 μM) were constructed.

**Western Blot Analysis**—Aortic tissues from rat and mice were pulverized at −80 °C by using a stainless steel pestle and mortar and resuspended in homogenization buffer of composition (in mM): 0.1 PIPES, 5 MgCl2, 5 EGTA, 0.5% Triton X-100 (v/v), 20% glycerol (v/v), and protease inhibitor mixture (Complete®, Roche Applied Science). Samples were centrifuged, and the supernatant was collected and assayed for total protein concentrations using the Bradford method (Bio-Rad). Equal amounts of proteins were resolved by 7.5% (w/v) SDS-
Bonferroni’s multiple comparison test. or LPS-treated (†) aortic rings using one-way ANOVA followed by

tion. The thoracic aortae were removed and stored at

mice were pretreated with LPS or saline and killed by cervical disloca-

for nitrite (NO2

rpm for 10 min, and the plasma was collected. Samples were analyzed

treated WT and eNOS KO animals. Samples were centrifuged at 13,000

radicicol (RAD, 1

centrations of NO3

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image of the page.
Comparison of concentration response curves, two-way ANOVA was used (GraphPad Prism 3). The n values indicate the number of experiments and animals used.

RESULTS

LPS-induced Relaxation in Rat Aortic Rings—PE (EC80) produced sustained contraction of saline-treated endothelium-intact or -denuded rat aortic rings over a 5-h period (Figs. 1A and 2A, p < 0.001). In contrast, the addition of LPS induced a gradual and significant reversal of PE-precontracted tension over the same time period (Figs. 1B and 2A, p < 0.001). This response was dependent on the endothelium since endothelial denudation rendered aortic rings resistant to the vasorelaxant effects of LPS (Figs. 1C and 2B). However, treatment of endothelium-denuded rings with the NO donor DETA-NO at 0.1 μM, a concentration that showed little or no vasorelaxant activity per se (Figs. 1E and 2B), restored LPS-induced relaxation (Figs. 1D and 2B, p < 0.001) to levels observed in endothelium-intact rings. Higher concentrations of the NO donor (1 μM) caused relaxation in endothelium-denuded rings per se (18.0 ± 5.1%, n = 8) that was not significantly different from the relaxation observed in the presence of LPS and, therefore, were not used in further experimentation since it was difficult to determine whether responses seen were due to DETA-NO directly or to LPS.

In endothelium-intact rings, LPS-induced relaxation was blocked by the NOS inhibitor, L-nitroarginine methyl ester, and HSP90 inhibitors GA and RAD, whereas inhibitors of PI3-K/Akt, wortmannin, and LY294002 did not affect the relaxation (Fig. 2A, p < 0.001). However, in endothelium-denuded aortic rings, HSP90 inhibitors GA and RAD had no effect on the restoration of LPS-induced relaxation by DETA-NO (Fig. 2B, p < 0.001).

Expression of iNOS Protein in LPS-treated Aortae—Expression of iNOS protein, as assessed by Western blotting, was evident in tissue homogenates of LPS-treated rat aortic rings with intact endothelium (Fig. 3A and B). In contrast, no iNOS immunoreactivity was detected in LPS-treated endothelium-denuded rings (Fig. 3A) and nor in tissue homogenates of control (saline-treated) tissues with or without the endothelium (Fig. 3A). In homogenates of endothelium-denuded rings pretreated with DETA-NO (0.1 and 1 μM) and LPS, immunoreactivity for iNOS protein was restored (Fig. 3B). Treatment of tissues with DETA-NO alone produced no detectable iNOS protein (Fig. 3B). Similar to the functional response to LPS (Fig. 2A), HSP90 inhibitors GA and RAD profoundly suppressed iNOS protein expression (Fig. 3C).

To further confirm the essential role of eNOS activity in the induction of iNOS protein, we assessed iNOS protein expres-
in eNOS KO and WT mice. In vivo administration of LPS (12.5 mg/kg, intravenous, 15 h) induced iNOS expression in aortae from WT mice but not in tissues of saline-treated animals (Fig. 4A). However, in comparison, the levels of iNOS expression in aortae of eNOS KO LPS-treated mice were significantly reduced to the extent that they were not significantly different from those observed in tissues of eNOS KO saline-treated animals (Fig. 4A, p < 0.01).

Plasma $\text{NO}_x$ Measurements in LPS-treated eNOS KO Animals—Fig. 4B shows that LPS treatment of WT mice causes a profound increase in plasma $\text{NO}_x$ levels. This elevation in plasma $\text{NO}_x$ is significantly suppressed in LPS-treated eNOS KO mice. In accord with this difference in plasma $\text{NO}_x$, LPS-induced changes in vasoactive responses to SPER-NO were likewise abrogated in tissues of eNOS KO when compared with WT animals. In aortic rings of WT animals, LPS treatment causes a small but significant rightward shift of the relaxation concentration-response curve to SPER-NO when compared with saline-treated WT mice (Fig. 4C). This shift was absent in rings taken from eNOS KO mice (Fig. 4D).

NF-κB Activation in LPS-treated Rat Aortic Rings—In some experiments, DNA binding activity of NF-κB was measured in tissue homogenates from control and LPS-treated rat aortic rings by electrophoretic mobility shift assay. NF-κB activation was markedly higher in endothelium-intact, LPS-treated rings when compared with control tissues (Fig. 5, A and B). This DNA binding activity was severely reduced in endothelium-denuded rings either with or without LPS treatment (Fig. 5, A and B). Interestingly, replacement of constitutive NO with the NO donor DETA-NO (0.1 μM) restored DNA binding activity of NF-κB in LPS-treated denuded rings to levels observed in LPS-treated endothelium-intact rings (Fig. 5A). At this concentration, DETA-NO had no significant effect on NF-κB activation (Fig. 5A).

To demonstrate that NO-induced activation of NF-κB DNA binding led to transcriptional activity, HEK 293T cells transfected transiently with pBIIx NF-κB-luciferase reporter plasmid were stimulated with DETA-NO for 5 h. Under these conditions, the luciferase activity was increased in a concentration-dependent manner, with a maxi-
conclusion of the vascular reactivity studies, expression of cross-sections (8 μm thick) of rat aortic rings. Positive immunoreactivity was observed staining was visible in cross-sections from LPS-treated (1 μg/ml, 5 h) (C) but not in untreated aortic rings (A). Strong staining for iNOS was localized in the adventitial layer (arrowhead), and moderate staining was visible in the medial (arrow) layer. B, control sections with nonspecific immune serum shows no positive staining with autofluorescence of elastic laminae. D, hematoxylin and eosin stained cross-sections of rat aortic ring showing the adventitia (Adv), media (Med), and the endothelium (arrow).

mal increase of ~2-fold at 1 μM DETA-NO (Fig. 5C).

Localization of iNOS Expression in Rat Aortic Ring—At the conclusion of the vascular reactivity studies, expression of iNOS protein was also assessed by immunocytochemistry in cross-sections (8 μm thick) of rat aortic rings from control and LPS-treated rings. Positive immunoreactivity was observed predominantly in cross-sections from LPS-treated (1 μg/ml, 5 h) aortic rings (Fig. 6C) when compared with untreated rings (Fig. 6A). The staining was localized to the medial and adventitial layers, with moderate staining of the adventitial layer and no evidence of immunoreactivity in the endothelium (Fig. 6C, arrowheads). Immunoreactivity was totally absent from LPS-treated rings, where the antibody against iNOS had been omitted from the procedure (Fig. 6B).

DISCUSSION

Using LPS, ex vivo, to mimic aspects of sepsis, herein we demonstrate that activation of constitutive eNOS activity is an essential prerequisite for efficient arterial expression and function of vascular iNOS. LPS induced a reversal of PE-precontracted, endothelium-intact rat thoracic aortic rings that was temporally associated with induction of iNOS protein in the walls of these arteries, specifically in the media and adventitia. In contrast, LPS treatment of endothelium-denuded rings did not relax precontracted tissues, and similarly, no expression of iNOS was detectable. Furthermore, in endothelium-intact tissues, inhibition of HSP90-dependent eNOS activity blocked both LPS-induced iNOS expression and vasorelaxation. However, in endothelium-denuded rings, HSP90 inhibitors affected neither the LPS-induced iNOS expression nor vasorelaxation. Together, these data demonstrate an implicit dependence of LPS-induced iNOS expression and vascular reactivity on the presence of functional endothelial eNOS. In addition, these observations intimate an essential, hitherto unknown, role of eNOS in the pathologic processes involved in the vascular response to LPS and highlight a novel pathway that might prove useful in the treatment of sepsis.

Application of LPS to precontracted rat aortic rings produced a profound, near maximal, vasorelaxation of these tissues by 5 h that was associated with iNOS expression as assessed using Western blotting. In contrast, removal of the endothelium rendered aortic rings resistant to the vasorelaxant effects of LPS, and Western blotting analysis confirmed the absence of detectable iNOS expression. This absence of iNOS expression was not due simply to the possibility that, in endothelium-intact arteries, the endothelial cell is the site of LPS-induced iNOS expression since immunohistochemical analysis of these arteries clearly indicates LPS-induced iNOS expression in the adventitia and media only. This is in agreement with previous findings (22) in which vascular iNOS expression was found to be localized to the smooth muscle of the media and the fibroblasts of the adventitia. Together, the data suggest an essential role of the endothelium in facilitating the induction of iNOS and its vasoactive functions in endotoxemia.

The endothelium dependence of this LPS response appeared to be related to provision of constitutive amounts of NO since administration of a concentration of DETA-NO (0.1 μM), which had little or no effect on vascular tone or iNOS expression over the 5-h incubation period, per se, resulted in a restoration of sensitivity to LPS with respect to both parameters. In these experiments, DETA-NO was administered prior to the precontraction of aortic rings, and the level of precontraction was set identical to that in endothelium-intact controls by titration of phenylephrine to give an EC_{50} for PE. Therefore, the vasorelaxant response in these arteries was not due simply to NO donor-induced relaxation. However, experiments with a 10-fold higher concentration of DETA-NO (1 μM) gave rise to a progressive drop in tone over 5 h in endothelium-denuded arteries in the absence of LPS, and therefore, this concentration was not used for studies with LPS. Since eNOS is the predominant isoform of NOS in the endothelium, we hypothesized that the dependence of LPS-induced iNOS expression and function on low level NO production related to basal and/or up-regulation of eNOS activity.

The enzyme eNOS is constitutively active, however, it is now clear that its activity can be up-regulated by specific stimuli in essentially two distinct ways, either through protein-protein interactions or through protein phosphorylation. In particular, it is evident that phosphorylation of specific serine residues by the PI3-K/Akt (17, 18) and formation of HSP90-eNOS multiprotein complexes (23) enhance eNOS activity. HSP90 is a member of the heat shock family of proteins involved in restoring cellular homeostasis, subcellular trafficking, and folding of several signaling proteins (for a review, see Ref. 24). The interaction between HSP90 and eNOS enhances the activation of this enzyme in cells as well as in intact blood vessels (25–27). Inhibition of HSP90 by geldanamycin, a member of the benzoquinone ansamycins, which binds specifically to the unique ATP binding pocket of HSP90 in a stable and pharmacologically specific fashion (16), suppresses endothelium-dependent relaxations of various arteries including rat aorta (25, 27, 28). In the present study, the HSP90 inhibitor geldanamycin suppressed LPS-induced reversal of precontraction of endothelium-intact aortic rings. Furthermore, in accord with this loss of function, LPS-induced iNOS expression was not detected in these aortic rings. This action of geldanamycin is unlikely to be due to a nonspecific action of the inhibitor since another structurally unrelated HSP90 inhibitor, radicicol (29), likewise blocked both LPS-induced relaxation and iNOS expression. Our findings with geldanamycin are in agreement with those of Joly et al. (30), who likewise demonstrated block of hyporeactivity of PE in rat aorta; however, this study suggested that this effect of geldanamycin was due to a direct action on iNOS, although iNOS protein expression was not analyzed. More recently, it has been demonstrated that HSP90...
association with iNOS, as with eNOS, enhances activity of this enzyme in murine macrophages (31). However, it is unlikely that a direct effect on iNOS accounts for the effects of the HSP90 inhibitors in this study since neither geldanamycin nor radicicol attenuated the DETA-NO-induced restoration of iNOS expression and thus LPS-induced relaxation (Fig. 2B). The reason for this difference is uncertain but may be related to the difference in cell type and warrants further investigation. In addition, a recent report suggested that HSP90 also complexes with soluble guanylate cyclase and serves as a stabilizer of the enzyme in bovine aortic endothelial cells (32). However, in vascular preparations including rat aortic rings, it is unlikely that this is the mechanism of action since geldanamycin blocks acetylcholine-induced relaxation but not the relaxant effects of the NO donors sodium nitroprusside and nitroglycerin (25, 33).

In contrast, inhibition of the main alternative mechanism for up-regulation of eNOS activity using wortmannin or LY294002, i.e. post-translational phosphorylation of specific amino acid residues by PI3 kinase, had no effect on LPS-induced iNOS expression in rat aorta. These results suggest that it is unlikely that eNOS phosphorylation was responsible for the increase in eNOS activity induced by LPS in the current study. That eNOS was the source of the NO responsible for the endothelium dependence was supported by studies using eNOS knock-out mice. At a time point associated with maximal LPS-induced NO production attributed to iNOS expression and activity in wild-type mice (14, 34), we demonstrated a profoundly suppressed expression of this NOS isoform in aorta of eNOS knockouts. This was accompanied by an associated attenuation in plasma NO levels in LPS-treated eNOS KO animals when compared with WT animals. This suppression of iNOS expression and parallel decrease in NO production was associated with an absence of the changes in vasoreactivity associated with LPS treatment in mice. In addition, we demonstrate that relaxation responses to NO donors in aortic rings that are suppressed in LPS-treated WT animals, as we have reported previously (14), are unaffected in rings of eNOS KO animals. This depression in sensitivity of smooth muscle to NO is consistent with a down-regulation of soluble guanylate cyclase activity in the vascular smooth muscle (14) in response to iNOS-derived

**Fig. 7. Schematic diagram of the mechanism of regulation of LPS-induced iNOS expression by endothelial NO.** In sepsis, activation of the transcription factor NF-κB leads to the transcription of the gene encoding for iNOS. Consequently, increased NO-mediated dilatation contributes to impaired contractile function of smooth muscle and decreased blood pressure, ultimately leading to organ failure. The presence of functional eNOS activity is essential to this process. Blockade of eNOS activity by specific inhibitors of HSP90, geldanamycin, and radicicol, but not inhibitors of Akt-dependent phosphorylation, wortmannin, and LY294002, suppresses LPS-induced iNOS expression, and thus, delays the associated vascular effects in endotoxemia. CaM = calmodulin.
The absence of this change in responses to SPER-NO, in tissues of eNOS KO mice, suggests that eNOS activation is an essential step in iNOS-induced repression of soluble guanylate cyclase activity. Thus, together our data support a facilitatory role for eNOS in LPS-induced iNOS expression and function.

Although the precise signal transduction pathway for iNOS induction in endotoxemia remains to be established, it is now evident that interactions between LPS and its cellular receptor(s) lead to the activation of the transcription factor, NF-κB, resulting in the transcription of downstream elements, e.g. the gene encoding for iNOS (35). Thus, suppression of NF-κB activation by calpain inhibitor I (inhibitor of the proteolysis of 1kβ) attenuates the circulatory failure, multiple organ dysfunction (liver and pancreatic dysfunction/injury, lactic acidosis, hypoglycemia), as well as induction of iNOS and COX-2 protein and activity in rats with endotoxemic shock (36). In addition, there is increasing evidence supporting the concept that NO, itself, is an important regulator of the NF-κB pathway. This thesis is supported by a reduced NF-κB activation when compared with WT cells (15). In the present study, sublethal LPS-stimulated NF-κB activation in endotoxemia remains to be established, it is now clear that the essential step in iNOS-induced repression of soluble guanylate cyclase activity, thus lending support to our thesis that NO directly regulates NF-κB activity, thus providing a self-perpetuating system that contributes to the progression of inflammatory reactions. In the present study, we observed that iNOS expression in LPS-treated endothelium-intact aortic rings was temporally associated with NF-κB activation. In endothelium-denuded rings, where there was no significant relaxation to LPS and a lack of iNOS expression, the absence of functional eNOS, the vascular effects of iNOS are abolished via the up-regulation and activation of eNOS, and that in the absence of functional eNOS, the vascular effects of iNOS are delayed (Fig. 7). The findings from this study lend support to the growing numbers of reports demonstrating a regulatory role of NO in gene expression of inflammatory mediators. Moreover, our data provide the first demonstration of a proinflammatory role for eNOS in regulating vascular function in endotoxemia.

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*Autoregulation of Nitric Oxide Production in Sepsis*