Activation of Inducible Nitric Oxide Synthase/Nitric Oxide by Curli Fibers Leads to a Fall in Blood Pressure during Systemic Escherichia coli Infection in Mice

Zhao Bian,1 Zhong-Qun Yan,2 Göran K. Hansson,2 Peter Thorén,3 and Staffan Normark1

Septic shock, a major cause of death, is characterized by a pathophysiological increased production of nitric oxide (NO), which leads to vasodilation and myocardial toxicity. Septic Escherichia coli frequently express proteinaceous curli fibers. In this study, curliated E. coli induced high levels of NO by directly inducing type 2 nitric oxide synthase (NOS2) both in vitro and in vivo. More severe hypotension and higher plasma nitrite/nitrate levels were seen in wild type mice systemically infected with curliated E. coli than in animals infected with E. coli mutants that lacked curli proteins. Blood pressure remained stable in NOS2-deficient mice with curliated bacteria. Increased heart rates, transient hypothermia, and loss of gross activity were seen in all mice, regardless of curli expression. Study results suggest that expression of curli fibers by E. coli activates the NO/NOS2 arm of the innate immune system, which leads to a significant fall in blood pressure.

Sepsis is defined as the systemic inflammatory response to infection [1] manifested by ≥2 symptoms, including fever (>38°C) or hypothermia (<36°C) and tachycardia or tachypnea. Septic shock refers to sepsis associated with cardiovascular collapse, such as hypotension, with a reduction of >40 mm Hg from baseline. Recent reports calculate that, in the United States alone, >100,000 deaths annually are caused by severe gram-negative sepsis [2].

Hypotension caused by nitric oxide (NO) production via inducible type 2 nitric oxide synthase (NOS2) is a major contributing factor for patient death with bacterial septic shock [3]. Inhibitors of NO synthesis, such as N(G)-monomethyl-L-arginine, have been tried as a potential treatment of septic shock in humans and in animal models [4, 5]. NO radicals are important antibacterial effectors [6]. Hence, deficiency in NOS2 also results in increased susceptibility to various bacterial pathogens in rodents [7]. NOS2 induction depends on the recognition of so-called “pattern molecules” on the bacterium that directly or indirectly interact with Toll-like receptors (TLR), leading to the activation of phosphorylation cascades, including mitogen-activated protein kinase pathways and transcriptional factors, such as NF-κB [8–10].

Lipopolysaccharide (LPS) is believed to constitute the major pattern recognition molecule that leads to septic shock [11, 12]. Purified LPS, when injected systemically into mice, elicits an NOS2-dependent fall in central arterial blood pressure similar to that seen in gram-negative septic shock [7]. However, several clinical studies found no significant increase in survival when LPS was blocked in septic patients [13–15], which suggests that other components of gram-negative bacteria may participate in rendering the pathology seen in septic shock.

Curli are conserved fibrillar surface structures expressed in both Escherichia coli and Salmonella organisms [16, 17] and are composed of a major subunit protein, CsgA, and a minor component, CsgB, that acts as a nucleator in the formation of curli organelles [18]. In the absence of CsgB, CsgA is secreted in a soluble form. Although in vitro curli can bind a number of different human serum proteins and interact with major histocompatibility complex class I molecules [19–22], the exact role of curli in pathogenesis remains unknown. It was recently reported that curli are capable of activating the contact phase system in vitro, allowing an anticoagulation effect and generation of bradykinin [23]. Furthermore, curliated E. coli are potent inducers of proinflammatory cytokines in human macrophages, in contrast to mutants lacking expression of both CsgA and/or CsgB, and antibodies to CsgA have been found in serum samples from patients with E. coli sepsis [24]. We therefore hypothesized that expression of these highly interactive and structurally conserved fibers might contribute to some of the symptoms seen during overwhelming E. coli sepsis.
Materials and Methods

Bacterial cell manipulation. MC4100, a derivative of E. coli K12 [25], expresses curli organelles. MHR222, MHR204, and MHR261 are isogenic curli-deficient mutant strains generated by transposon insertional mutagenesis of the csgA or csgB genes in MC4100 [26]. We grew bacteria on colonization factor agent (CFA) agar [27] at 28°C for 48 h for optimal curli expression. Bacterial cells collected from the agar plate were used for NO induction in vascular smooth muscle (VSM) cells and for intraperitoneal (ip) infection in mice. We estimated the number of bacterial cells from absorbancy at OD600 by using a predetermined calibration curve.

NO induction in VSM cells. VSM cells were isolated from rat thoracic aorta [28]. Cell cultures were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Gibco BRL), 1 mM l-glutamine (Gibco BRL), 50 U/mL penicillin, and 50 μg/mL streptomycin (Sigma Chemical). Cells were grown at 37°C in a humidified 5% CO2/95% air atmosphere in a culture flask (Nunc). Cells from passages 5–10 were seeded into each well at 105 cells in 0.5 mL of DMEM with 1 mM l-glutamine and 50 μg/mL gentamicin on a 24-well culture plate (Costar) for 24 h. The cultures were prepared in duplicate and were infected with 105 cfu of E. coli MC4100, MHR261, MHR204, and MHR222. As a positive control, cells were treated with 10 μg/mL purified E. coli LPS (O55:B5; Sigma). The cell-free supernatants were analyzed 8, 16, and 24 h after stimulation.

Reverse transcription-polymerase chain reaction (RT-PCR). We pretreated VSM cells (4 × 104 cells/well) with cycloheximide (CHX) for 15 min before exposure to LPS (10 μg/mL), 4 × 107 cfu of curliated E. coli (MC4100), and noncurliated mutant (MHR222) for 20 h. Total RNA was extracted with RNAzol (Biotecx Laboratories). The first strand of cDNA was prepared by use of SuperScript RNase H RT (Gibco BRL) and random hexamer primers. The primers [29] used to target NOS2 mRNA were 5'-GCC- TTCCGAAGTTTCTGGCAGCAG-3' and 5'-GGGCTCTCA- AGGTGTTGGCCC-3'. As a control for cDNA synthesis, PCR amplification was done with the gene for hypoxanthine phosphoribosyl transferase (HPRT). An oligonucleotide primer set specific for HPRT was 5'-CTGCGCTCTGATTAAGG-3' and 5'-CCA- ACACCTCGTGGATT-3'. PCR was done in a final volume of 40 μL (containing 2 μL of cDNA) for 31 cycles.

Western blot analysis. VSM cells or tissue samples were lysed with Laemmli sample buffer (Bio-Rad Laboratories) and were boiled for 5 min. In total, 10 μg of protein from each sample was separated on 7.5% SDS-PAGE gels and was transferred to a hydrophobic polyvinylidene difluoride membrane (Amersham). The membrane was soaked in 5% nonfat milk in Tris-buffered saline (pH 7.4) plus 0.1% Tween 20 (Bio-Rad Laboratories) and was subsequently probed with an anti-NOS2 monoclonal antibody for 1 h at room temperature (1:2000 dilution; Transduction Laboratory). Immunoreactive signals were visualized with horseradish peroxidase–conjugated anti–mouse immunoglobulin F(ab')2 fragments (1:4000 dilution) with an ECL detection kit (Amersham).

Animal preparation. For our experiments, we used 25–29 g C57BL/6J (NOS2+/+; B&K) normal mice and 23–26 g C57BL/6J NOS2tm1Lau (NOS2+/−; Jackson Laboratory) NOS2-deficient mice [30]. Mice were acclimated in a 12-h day-night cycle at 21–22°C at 55%–60% humidity. They were given free water and standard diet (B&K). Mice were anesthetized with 2% isoflurane (Abbott Laboratories). A cannula was implanted in the right carotid artery and was placed subcutaneously to exit at the top of the back, where it was connected to a custom-made swivel tether system for continuous monitoring of blood pressure (recorded twice per minute; Grass polygraph model 7). Sterile saline (Baxter) was infused continuously via the cannula (0.2 mL/h) to maintain patency of the blood pressure cannula line. Blood pressure was measured for 24 h after surgery. Only animals with a normal basal pressure (>90 mm Hg) during this period were entered into the study. The telemetry device and its sensors for electrocardiography (EKG), body temperature, and gross activity (Data Sciences) were implanted as described elsewhere [31]. Monitoring of physiologic parameters (EKG, body temperature, and gross activity) was initiated 1 week after surgery. Recordings (every 2 min) were done continuously for 48 h in freely moving mice. C57BL/6J mice that underwent surgery but had no further treatments served as sham controls.

Induction of ip sepsis. E. coli MC4100 and MHR222 were harvested from CFA agar plates and were suspended in saline. Either live bacteria (5 × 107 cfu in 0.5 mL of saline) or 0.5 mL of saline alone was administrated ip to the mice. Blood pressure was monitored for 8 h after infection, and heart rate, body temperature, and gross activity were monitored for 24 h after infection. Blood samples were obtained at relevant time points by exsanguination via the carotid artery after terminal gaseous anesthesia with isoflurane. Organs were observed and preserved at −80°C.

Assays for nitrite/nitrate concentrations in culture medium and plasma. The accumulation of nitrite, a stable end product of NO, in conditioned medium was measured as an indicator of NO production by VSM cells [28]. We incubated 100 μL of cell-free conditioned medium for 10 min with 100 μL of Griess reagent (Calbiochem) at room temperature and measured absorbancy at OD540. Nitrite in the samples was calculated from a standard curve of sodium nitrite. Nitrite/nitrate levels in plasma were measured as nitrite by use of the Griess reaction after enzymatic conversion by nitrate reductase (0.25 U/mL), as described elsewhere [32].

Statistical analysis. Results are reported as mean ± SE. We used factorial analysis of variance analysis and 2-tailed Student’s t test to evaluate statistical differences between means of controls and of experimental groups. P < .05 was considered statistically significant.

Results

Curli proteins of E. coli induce NO production in VSM cells. The expression of NOS2 from activated VSM cells is thought to play a detrimental role in the pathogenesis of septic shock by overproduction of vasodilating NO [33, 34]. To determine whether NO production is associated with E. coli–expressing curli proteins, VSM cells were infected with 105 cfu of wild-type (wt) curli-expressing E. coli MC4100 or its noncurliated mutant derivatives: MHR261 (csgB), MHR204 (csgA), and MHR222 (csgBA). The accumulation of nitrite, an NO end product, in the culture supernatants was determined 8, 16, and 24 h after infection (figure 1A).

VSM cells infected with mutant MHR222 had constant low
Figure 1. Kinetics of nitric oxide (NO) production (A) and NO synthase (NOS2) expression (B) in vascular smooth muscle (VSM) cells exposed to curliated *Escherichia coli* and its isogenic mutants. Nitrite levels were quantified 8, 16, and 24 h after treatment (see Materials and Methods). Data are mean ± SE of 5–7 independent experiments. 

B. For immunoblotting, protein lysates from untreated VSM cells and VSM cells treated with lipopolysaccharide (LPS) or MC4100 and MHR222 were probed with anti-NOS2 antibody (see Materials and Methods); left, molecular mass marker (kDa). +, Addition of the components (CsgA, CsgB, or curli); −, no addition of the components.

nitrite levels 8, 16, and 24 h after infection (*P* > .05) that was only slightly higher than that in the uninfected control cells, in contrast to the other *E. coli* strains, which all showed a time-dependent increase of NO production. Nitrite levels were much higher in cells infected with wt *E. coli* (MC4100; *P* < .0001) or with bacteria secreting CsgA but lacking CsgB (MHR261; *P* < .001), compared with infection with mutant MHR204, which lacks CsgA but expresses CsgB on its surface. However, the nitrite level in MHR204-infected cells was higher (*P* < .001) than in untreated cultures or in cultures infected with MHR222, which lack both curli proteins.

Taken together, the data demonstrated a striking difference, depending on the presence or absence of curli organelles and the expression of CsgA and CsgB. Hence, both subunits of curli organelles, CsgA or CsgB, appear to play a role for the in vitro production of NO in VSM cells by *E. coli* infection. As a positive control, VSM cells were treated with 10 μg/mL purified *E. coli* LPS. At 16 and 24 h after treatment, almost the same level of nitrite (*P* > .05) in the culture supernatant was recovered as from cells infected with MC4100. Importantly, the total LPS content from the 10⁷ cfu of intact *E. coli* used for infection was only ~100 ng [35] (i.e., 100 times less than the applied purified LPS). Such amounts of purified LPS (100 ng) were unable to induce in vitro NO production in VSM cells 24 h after infection (data not shown). However, the biologic effects of LPS in “solution” may differ from that of LPS in its native form when present in the bacterial envelope.

Curliated *E. coli* induced NOS2 induction in VSM cells. We followed NOS2 expression after infection by the 4 isogenic *E. coli* strains by immunoblot assay of cell homogenates with an anti-NOS2 antibody (figure 1B). A 130-kDa immunoreactive band was detected in cells infected for 24 h with *E. coli* MC4100, MHR261, MHR204, and MHR222 and in cells treated with LPS. Control cells lacked the signal. The intensity of the band was considerably stronger in MC4100-infected cells than the corresponding signal in MHR222-infected cells. Strains MHR261 and MHR204 produced signals with intermediate intensity. Hence, NOS2 in VSM cells is silent under resting conditions and is induced and expressed at higher levels by *E. coli* that express curli proteins than in *E. coli* mutants that cannot express CsgA and/or CsgB. This explains NO accumulation differences in the culture supernatants.

NOS2 mRNA in VSM cells in the absence of de novo protein synthesis. Proinflammatory cytokines, such as interferon-γ and tumor necrosis factor (TNF-α), induce NOS2 expression [36]. Because curliated *E. coli* induce synthesis of TNF-α [24], we tested whether the induction of NOS2 in VSM cells by curliated *E. coli* depends on the synthesis of second mediator proteins. NOS2 mRNA was induced by curliated *E. coli*...
Figure 3. Plasma levels of nitrite/nitrate in wild-type (wt), nitric oxide synthase (NOS2)-deficient, and sham control mice after intra-peritoneal challenge with curliated *Escherichia coli* MC4100 and *csgBA* mutant MHR222 (5 × 10⁹ cfu) 8 h after infection (see Materials and Methods). Data are mean ± SE of 4 independent experiments.

(MC4100) and by LPS in the presence or absence of CHX, an inhibitor of protein synthesis (figure 2). Hence, NOS2 mRNA induction by curliated *E. coli* does not require induction of proinflammatory cytokines, which suggests a direct signal transduction pathway similar to that for LPS. A weak mRNA band was detectable in VSM cells treated with CHX alone, indicating that the NOS2 gene is under negative regulation by an inhibitor protein [37].

Plasma NO level in mice systemically infected with curliated *E. coli*. Because systemic infection with curliated *E. coli* induced NOS2 expression (figure 4) and elevated plasma NO levels (figure 3), we tested whether infection in conscious mice with curliated *E. coli* could cause NO-mediated hypotension, a characteristic response in septic shock. Figure 5 illustrates the an indicator of septic shock depends on renal function [38, 39], we assessed the serum levels of lactate dehydrogenase, aspartate aminotransferase, bilirubin, and creatinine in serum samples 8 h after infection. We found no differences between bacteria-challenged animals and the sham controls (data not shown). However, these serum samples may have been obtained too early to see an elevation in enzymes released from damaged liver or kidney cells. To determine whether the elevated serum levels of NO were directly due to the activation of the NOS2 pathway, we monitored the expression of NOS2 protein in different organs from wt mice treated with MC4100 (*n* = 2), MHR222 (*n* = 2), and saline (*n* = 2) by immunoblot assay (figure 4). The NOS2 protein was strongly expressed in liver, lung, kidney, and spleen tissue of curliated *E. coli* MC4100-infected mice (figure 4, rows 1 and 2). NOS2 was also induced in the noncurliated mutant MHR222-infected mice (rows 3 and 4), but there was less expression. The NOS2 signals in the tissues of the 2 mice treated with saline (rows 5 and 6) and in heart tissue of all 6 treated mice (data not shown) were below the detection limit. These data suggest that the overproduction of NO in our septic model resulted from induced expression of NOS2 in different organs and was triggered by curliated *E. coli* systemic infection.

Blood pressure in mice systemically infected with curliated *E. coli*. Because systemic infection with curliated *E. coli* induced NOS2 expression (figure 4) and elevated plasma NO levels (figure 3), we tested whether infection in conscious mice with curliated *E. coli* could cause NO-mediated hypotension, a characteristic response in septic shock. Figure 5 illustrates the

![Figure 4](image-url)

Figure 4. Nitric oxide synthase (NOS2) expression in organs of mice treated by curliated *Escherichia coli* MC4100, noncurliated mutant MHR222, and saline. Tissue lysates of organs from individual animals (rows 1 and 2, infected with MC4100; rows 3 and 4, infected with MHR222; rows 5 and 6, saline treated) were probed with anti-NOS2 antibody (see Materials and Methods). Li, liver; Lu, lung; Ki, kidney; Sp, spleen. Protein bands were 130 kDa.
Heart rate, body temperature, and gross activity in mice sequentially infected with curliated E. coli. Because the heart rate and the body temperature are both affected during sepsis, we investigated these 2 parameters and the gross activity in mice after systemic E. coli infection. Figure 6 (left panel) demonstrates the pathophysiologic alterations of these 3 parameters during 23 h before and after infection in MC4100-challenged (n = 9) and MHR222-challenged (n = 8) wt mice, in MC4100-challenged NOS2-deficient mice (n = 4), and in sham controls. Statistical analysis of data collected for 10 h after infection (figure 6, right panel) showed that heart rate (beats per minute [bpm]), body temperature, and gross activity (counts per minute [cpm]) were significantly higher (P < .001) in MC4100-challenged wt mice (635 ± 15 bpm, 36.0 ± 0.3°C, and 0.46 ± 0.22 cpm), MHR222-challenged wt mice (628 ± 13 bpm, 36.1 ± 0.3°C, and 0.83 ± 0.38 cpm), and MC4100-challenged NOS2-deficient mice (644 ± 15 bpm, 36.1 ± 0.3°C, and 0.23 ± 0.21 cpm) than in sham controls (555 ± 24 bpm, 36.9 ± 0.3°C, and 4.1 ± 1.5 cpm), respectively. The transient decrease in body temperature in MC4100- and MHR222-challenged wt mice and in MC4100-challenged NOS2-deficient mice suggests that at least the major component in this temperature drop was not related to the hypotension and occurs independent of NOS2 induction. Normal EKG recordings were obtained from the 3 groups of mice before and after bacterial administration (data not shown).

Discussion

Normal VSM cells produce no NO in the resting state. After stimulation, however, NOS2 is expressed, and excessive NO is produced, which leads to vasorelaxation and hypotension [33, 36]. This has been confirmed in human studies [40, 41]. Studies that documented an increased resistance to hypotension and mortality in animals that are genetically NOS2 deficient or treated with NOS blocker show that NO is an important mediator of septic shock after LPS or live microbial challenge [7, 42, 43].

To date, several bacterial products other than LPS have been reported to activate, directly or indirectly, NOS2 and NO production by macrophages [44]. However, the intracellular pathways involved in the induction of NOS2 by these agents are not well characterized. Recently, a number of related TLR were identified in mammals and were shown to be required for intracellular signaling leading to the activation of transcriptional factors, such as NF-κB, activating promoters for NOS, costimulatory molecule B7.1, and proinflammatory cytokines [8, 9]. TLR4 responds to LPS [11, 12].

Our results show that curliated E. coli are potent inducers of NOS2 and cause an overproduction of NO, which leads to a marked hypotension in conscious mice. We previously showed that curliated and CsgA-secreting E. coli can stimulate production of proinflammatory cytokines (interleukin [IL]-6, IL-8, and TNF-α) in human macrophages in vitro [24]. It is there-
fore possible that the effect of curli proteins on NOS2 induction is related to their ability to stimulate proinflammatory cytokines. However, because curliated E. coli were also potent inducers of NOS2 in VSM cells in vitro and induced NOS2 mRNA in the absence of de novo protein synthesis, we prefer the idea that curli proteins have a direct role in activating the signaling pathway leading to NOS2 induction. Recently, constitutive expression of TLR4 was found in cardiac myocytes, coronary microvascular endothelial cells [45]. We found that TLRs are expressed by VSM cells (authors’ unpublished data). Whether curli are dependent on TLR-mediated induction of NOS2 signaling remains to be shown. Because CsgA-secreting but noncurliated E. coli also induce NO production in VSM cells, albeit at a lower level than curliated E. coli, it is thought that host recognition is not dependent on bacterial binding to cells via associated curli organelles.

It is possible that curli also activate the constitutive form of NO synthase (NOS3), because curli can activate the contact phase system that leads to generation of the cellular mediator bradykinin [23]. This is an important endogenous activator of NOS3 that binds to and stimulates Ca\(^{2+}\)-mobilizing B2 receptor in the vascular wall [46, 47]. Activation of the B2 receptor can cause pronounced hypotension and bronchoconstriction [48]. However, because curli proteins induce NOS2 expression and because bradykinin has no reported effects on NOS2, we favor the hypothesis that curli proteins cause hypotension independently of bradykinin release by directly acting as potent inducers of the innate host defense system and by leading to overexpression of NOS2.

Septic shock is characterized by cardiovascular collapse (hypotension and tachycardia), hyperthermia or fever, and multiple organ dysfunction. Gram-negative bacteria, especially E. coli and other gram-negative rods, are the principal pathogens isolated from blood culture in septic patients [49]. The systemic E. coli infection model in mice that we used reproduces some of the above features, although all animals eventually clear the
infection. Our data indicate that hypotension depends on NOS2 induction, whereas the tachycardia and the transient decrease in body temperature do not, suggesting that ≥2 different pathways may produce septic shock symptoms. A transient hypothermia is an indicator of acute effects of LPS and can also be evoked by the direct administration of IL-1β [50, 51]. In a recent study, mice treated with IL-10 had transient decreases in body temperature and resistance to fever induced by low dose of LPS, whereas IL-10 knockout mice developed exacerbated and prolonged fever [52]. Thus, curli expression may primarily stimulate the NOS2 pathway, whereas other bacterial components such as LPS may be dominant in stimulating a separate pathway involving IL-10.

We believe that our results are the first to show a physiologic role of curli in a sepsis model. Further experiments using specific antibodies to curli (CsgA and CsgB) in combination with LPS antibodies and antibodies to IL-10 and other cytokines in wt mice and in mice deficient in various genes required for innate immune responses will be done to inhibit the circulatory collapse seen in this sepsis model.

Acknowledgments

We thank Lilian Sundberg (Dept. of Physiology and Pharmacology, Karolinska Institute, Stockholm) for skillful technical help with part of the animal work.

References