Bacterial nitric oxide detoxification prevents host cell \(S\)-nitrosothiol formation: a novel mechanism of bacterial pathogenesis

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ABSTRACT \(S\)-nitrosylation is an important mediator of multiple nitric oxide-dependent biological processes, including eukaryotic cellular events such as macrophage apoptosis and proinflammatory signaling. Many pathogenic bacteria possess NO detoxification mechanisms, such as the nitric oxide reductase (NorB) of Neisseria meningitidis and the flavohemoglobins (Hmp) of Salmonella enterica and Escherichia coli, which serve to protect the microorganism from nitrosative stress within the intracellular environment. In this study, we demonstrate that expression of meningococcal NorB increases the rate at which low-molecular-weight \(S\)-nitrosothiol (SNO) decomposes \textit{in vitro}. To determine whether this effect occurs in cells during infection by bacteria, we induced SNO formation in murine macrophages by activation with lipopolysaccharide and \(\gamma\)-interferon and observed a reduced abundance of SNO during coincubation with \(N\). meningitidis, \(S\). enterica, or \(E\). coli. In each case, this effect was shown to be dependent on bacterial NO detoxification genes, which act to prevent SNO formation through the removal of NO. This may represent a novel mechanism of host cell injury by bacteria.—Laver, J. R., Stevanin, T. M., Messenger, S. L., Dehn Lunn, A., Lee, M. E., Moir, J. W. B., Poole, R. K., Read, R. C. Bacterial nitric oxide detoxification prevents host cell \(S\)-nitrosothiol formation: a novel mechanism of bacterial pathogenesis. \textit{FASEB} J. 24, 286–295 (2010). www.fasebj.org

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Nitric oxide (NO) is a ubiquitous, biologically potent signaling molecule with functionally pleiotropic effects. NO, in the presence of a strongly oxidizing cofactor, can bond covalently with the thiol residues of proteins to form \(S\)-nitrosothiols (SNOs). The formation of SNOs, called \(S\)-nitrosylation, represents a form of reversible posttranslational modification of cellular proteins (1) that is critically involved in a number of cell signaling events, akin to the effect of tyrosine phosphorylation (2). Thus, \(S\)-nitrosylation has been implicated in regulating apoptosis (3–6), control of intracellular signaling cascades (7), neurotransmission (8), and regulation of gene expression (9–11). \(S\)-nitrosylation meets the criteria for validation as a cell signaling mechanism: it is stimulus evoked (12, 13), precisely targeted (14), reversible (15), spatiotemporally restricted (16, 17), and necessary for specific cell responses (18). The reverse process of \(S\)-nitrosylation is termed denitrosylation and involves the release of the NO moiety from the SNO bond. The rate of SNO decomposition in solution is affected by factors such as light, temperature, pH, and the presence of contaminating transition metal ions (19). Recent studies demonstrated that denitrosylation is also an enzymatically mediated process. In addition to \(S\)-nitrosoglutathione reductase (GSNOR), a ubiquitous and well-conserved SNO-metabolizing enzyme (20), denitrosylation has also been demonstrated as a function of the thioredoxin/thioredoxin reductase system (21).

Many bacteria can metabolize NO, for example, by reduction in denitrification and NO detoxification for defense against phagocyte-induced nitrosative stress. One example occurs in \textit{Neisseria meningitidis} (the meningococcus), which causes meningococcal disease after colonization of its sole biological niche, the NO-rich human nasopharynx (22–24). Although \textit{N. meningitidis} is incapable of anaerobic respiration (25), it can also grow under oxygen-limited conditions (26) and supplement its growth by partial denitrification, using the gene products of \textit{aniA} (NMB1622) and \textit{norB} (NMB1623) (26). The \textit{norB} gene encodes an NO-reducing quinol oxidase
(NorB, qNOR), which confers resistance to NO-mediated killing by monocyte-derived macrophages (MDMs) and enhances survival within human nasopharyngeal tissue (27). In addition, NorB exerts effects on the host response, modulating the cytokine and chemokine output of infected MDMs (28) and inhibiting MDM apoptosis (29). Expression of norB is controlled transcriptionally by the nitric oxide-sensitive protein, NsrR (30).

However, the best-characterized bacterial NO detoxification mechanism is that catalyzed by flavohemoglobins (Hmp), which have properties similar to erythrocyte hemoglobin and associated flavin-containing methemoglobin reductases (31, 32). Hmp consists of an N-terminal heme-binding domain integrated with a flavin-binding domain, both of which are required to provide protection against nitrosative stress (33). The noncovalently bound FAD transfers electrons from NAD(P)H to the heme and thence to oxygen (34) to form an oxyhemoglobin species. However, in the presence of NO and physiological oxygen concentrations, Hmp preferentially binds NO (35) and reduces it to NO\(^{-}\), which aerobically forms nontoxic nitrate anion (NO\(_3^{-}\)) (36). Thus, Hmp catalyzes a denitrosylase, not a dioxygenase, reaction.

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We postulated that NorB-mediated alterations in host cell physiology are driven by bacterial perturbation of S-nitrosylation. If so, this would represent a mechanism of host cell septic injury and have far-reaching implications for all pathogens that consume NO. As proof of this principle, we tested the hypothesis that bacterial NO detoxification mechanisms can affect the abundance of host cell SNO, using a murine macrophage infection model. In response to cellular agonists, murine macrophage cell lines have a high natural output of NO and contain relatively high concentrations of SNO (44–46).

### MATERIALS AND METHODS

#### Reagents and apparatus

Unless otherwise indicated, all chemicals were purchased either from Sigma-Aldrich (St. Louis, MO, USA) or BDH Laboratories (Poole, UK). Lipopolysaccharide (LPS) from *E. coli* 0127:B8 was purchased as a filter-sterilized stock solution (1 mg/ml) from Sigma-Aldrich, as were N-[(3-aminomethyl)phenyl]methyl]ethanaminamido dilydrochloride (1400W) and diethylene triamine pentaacetic acid (DTPA). Recombinant mouse interferon-γ (rmIFN-γ) was purchased from R&D Systems (Minneapolis, MN, USA). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Lonza (Basel, Switzerland). S-nitrosoglutathione (GSNO) was synthesized in-house, as described previously (47). Chemiluminescence measurements of nitrogen oxides were performed using a Bo 280i Nitric Oxide Analyzer (NOA; Sievers, Boulder, CO, USA) with output data analyzed using either the LIQUID program or OriginPro 8.0.

#### Bacterial strains and growth conditions

The bacterial strains used in this study are detailed in Table 1. Frozen stocks of viable bacteria were maintained in cryopreservation fluid (Protect™; TSC, Heywood, UK) at −80°C. Aliquots of frozen *N. meningitidis* stocks were streaked onto GC agar supplemented with 1% Vitox supplement (Oxoid, Basingstoke, UK) and spectinomycin (50 μg/ml) where appropriate. Plates were incubated overnight at 37°C, 5% CO\(_2\) and propagated daily on fresh medium. Cultures were produced using ≥3 meningococcal colonies from fresh overnight plates, to avoid problems of phase variability of surface structures in *Neisseria*. Meningococcal cultures were grown in 10 ml Mueller Hinton broth in 25 ml universal tubes at 37°C, 5% CO\(_2\) with agitation (<90 rpm) provided by a plate mixer. For infecting activated J774.2 macrophage cells, cultures were grown to midlog phase (OD\(_{600}\)=0.25). For those experiments requiring a heat-killed wild-type control for meningococcal NorB activity, a suspension of wild-type bacteria, containing the same number of cells as the wild-type inoculum, was incubated at 80°C for 20 min.

*Salmonella* strains were propagated from frozen stocks onto Columbia agar supplemented with horse blood (Oxoid) and kanamycin (50 μg/ml) where appropriate. Plates were incubated overnight at 37°C, 5% CO\(_2\) to produce single colonies. Cultures of *Salmonella* were produced by inoculating 100 ml of brain heart infusion broth in a 250 ml flask with 3 *Salmonella* colonies from fresh, overnight plates and incubating at 37°C.

| Strain          | Relevant genotype | Source/ref.                  |
|-----------------|-------------------|------------------------------|
| *Neisseria meningitidis* |                  |                              |
| MC58            | Wild-type group B strain | McGuinness et al. (70)       |
| norB            | MC58, norB::Ω       | Anjum et al. (26)            |
| nsrR            | MC58, nsrR::Ω       | Rock et al. (51)             |
| *Salmonella enterica* spp. |            |                              |
| Typhimurium     | Wild type          | Crawford & Goldberg (71)     |
| ATCC 14028s     | ATCC 14028s, hmp::kan\(^R\) | Crawford & Goldberg (71)   |
| 14028Δhmp       |                  |                              |
| *Escherichia coli* |                |                              |
| MG1665         | Wild type          | Laboratory collection (R.K.P.) |
| MG1665Δhmp     | MG1665, hmp::Tn5   | Laboratory collection (R.K.P.) |
with shaking at ~200 rpm. For infecting activated J774.2 cells, cultures were grown to midlog phase (OD₆₀₀=0.35).

Single colonies of each E. coli strain were grown on Luria-Bertani (LB) agar containing kanamycin (25 μg/ml) where appropriate. An overnight culture of the appropriate strain was used to inoculate (1:1000 v/v) 100 ml of fresh LB medium in a 250-ml flask, which was then incubated at 37°C with shaking at 200 rpm. For infection of activated J774.2 cells, cultures were grown to midlog phase (OD₆₀₀=0.35). In experiments investigating the effect of NorB activity on the decomposition of GSNO, DMEM + 10% HI-FCS was supplemented with 100 μM GSNO before being inoculated with meningococci.

Tissue culture

The mouse [BALB/c] tumor monocyte-macrophage cell line, J774.2 (ECACC 85011128) was maintained in DMEM plus 10% heat-inactivated fetal calf serum (HI-FCS), in suspensions between 3 and 9 × 10⁵ cells/ml at 37°C, 5% CO₂. For use in the measurement of endogenous SNO, J774.2 cells were seeded at 1 × 10⁶ cells/ml in 6-well plates and incubated at 37°C, 5% CO₂ for 24 h before their use.

Activation of murine macrophages

A sterile preparation of LPS from E. coli 0127:B8 was added to DMEM + 10% HI-FCS at a final concentration of 1 μg/ml. rmIFN-γ was added to DMEM + 10% HI-FCS at a final concentration of 1000 U/ml. Where used, the specific inducible nitric oxide synthase (iNOS) inhibitor 1400W was added at a concentration of 1000 U/ml. Where NO synthesis was inhibited at the same time as bacterial infection, suspensions were produced in medium containing 100 μM 1400W. Inocula were determined by 10-fold serial dilution followed by viable counting according to the Miles-Misra method (48). Infected macrophage cells were incubated at 37°C, 5% CO₂ for either 2 h (Salmonella and E. coli) or 4 h (N. meningitidis). At the end of each infection, the number of viable bacteria in each supernatant was determined before removal of the supernatant.

Preparation of cell lysates

Washed, postinfection J774.2 cells were lysed using 300 μl SNO-compatible lysis buffer (50 mM phosphate buffer, pH 7.4+1 mM DTPA+protease inhibitor cocktail+50 mM NEM). For measurements using tri-iodide (I₃⁻)-dependent chemiluminescence, lysis buffer also contained 2% saponin (from Quillaja bark). Plates were incubated at 37°C, 5% CO₂ for 15 min to allow the alkylation of free thiol. Cell debris was scraped off each well and transferred to a fresh tube, where they were kept on ice and in the dark. In saponized lysates, cell debris and bacterial cells were removed immediately by centrifugation at 10,000 g for 5 min; otherwise, samples were first subjected to 3 freeze-thaw cycles using liquid N₂. Supernatants were transferred carefully to fresh tubes, and where appropriate, aliquots were pretreated for measurement of SNO. The remainder of each lysate was stored for protein assay at −20°C. In some experiments, lysates were dialyzed (2000 Da cutoff) overnight against 50 mM phosphate buffer + 1 mM DTPA at 4°C, prior to measurement of SNO concentration.

Measurement of SNO in liquid samples

Measurement of SNO was performed using ozone-based chemiluminescence. To release NO from &nitratosothiol, the purging vessel contained either I₃⁻ solution, as described previously (47), or a buffered solution of Cu(I) and cysteine, as described by Gaston and co-workers (49). Tri-iodide-dependent SNO measurements required samples to be treated with either 10% (v/v) 100 mM sulfanilamide in 2 N HCl or 10% (v/v) HgCl₂, followed by 10 min incubation on ice and in the dark. Hg²⁺-treated samples were then treated with 10% (v/v) acidified sulfanilamide and incubated on ice and in the dark for another 10 min. No Hg²⁺ treatment was performed on GSNO-supplemented DMEM. SNO was measured by duplicate injection into the appropriate solution at either 30°C (I₃⁻) or 50°C (Cu(I)/Cys). The mean AUC for duplicate injections was used to determine the SNO concentration, with reference to a GSNO standard curve. SNO-obliterated (Hg²⁺-treated) signal was corrected for extra dilution (×1.1) and the adjusted concentration was deducted from each sulfanilamide-treated sample. SNO concentrations of lysates were normalized to the protein concentration of each lysate (pmol SNO/mg protein).

Protein assay

Determination of the protein concentration in lysates was performed using either the RCDC Protein Assay Kit (Bio-Rad, Hercules, CA, USA) or Bio-Rad Protein Assay Reagent, according to the manufacturer’s instructions.

Statistical analysis

Data were analyzed for skewness using SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA). Data were considered significantly skewed—and therefore nonparametrically distributed—if the skewness was equal to or exceeded double the value of the SE of the skewness. Values presented in the text are means ± SD of parametrically distributed data or the median and interquartile range of nonparametrically distributed data, unless otherwise specified. Details of specific statistical tests are detailed in the figure legends. Results of statistical tests were considered significant at derived values of P ≤ 0.05.

RESULTS

NorB accelerates the decomposition of SNO in vitro

DMEM + 10% HI-FCS was spiked with the nitrosating compound GSNO to a final concentration of 100 μ
and used to grow either wild-type \textit{N. meningitidis} or the \textit{norB}-mutant derivative. Inoculation of DMEM + 10% HI-FCS supplemented with 100 μM GSNO (circles; solid line) determined at intervals by duplicate injection of acidified sulfanilamide-treated medium into I₃⁻ reaction mixture and quantified by ozone-based chemiluminescence. Data are percentages of the starting SNO concentration. Individual points, representing means ± se, were compared using unpaired samples \( t \) test. Area under curve for the three groups was compared by unpaired samples \( t \) test. \( n > 5 \). Error bars fall within the points where not visible.

Exposure to LPS and rmIFN-γ generates a prolonged enrichment of endogenously produced SNO in J774.2 murine macrophage cells by activating expression of iNOS

Stimulation of J774.2 murine macrophage cells with 1 μg/ml LPS + 1000 U/ml rmIFN-γ in DMEM for 18 h increased the level of SNO in cell lysates (median 128.5 pmol/mg; interquartile range 101.7–136.3 pmol/mg), \( cf. \) lysates from untreated cells (median 0.00 pmol/mg; interquartile range 0.00–0.94 pmol/mg) (Fig. 2A). The iNOS inhibitor 1400 W 1400 W (100 μM) was also added to medium (stim. +1400W). Horizontal bars denote medians; \( n > 6 \). ** \( P < 0.001 \); Mann Whitney \( U \) test. The iNOS inhibitor 1400 W removed this effect (median 22.41 pmol/mg; interquartile range 11.7–72.3 pmol/mg). The high SNO concentration of stimulated J774.2 cell lysates remained detectable for 4 h after withdrawal of LPS and rmIFN-γ (Fig. 2B). Throughout this period, we found no significant change in the SNO signal.

Infection of activated J774.2 murine macrophage cells with wild-type \textit{N. meningitidis} results in a reduced abundance of endogenous SNO

At 4 h after infection of J774.2 cells with \textit{N. meningitidis} (Fig. 3B), the SNO concentration in stimulated but uninfected J774.2 cells (139.7±52.2 pmol/mg) was reduced significantly by coinoculation with wild-type bacteria. Macrophages were infected at either MOI 10 or 100, but after 4 h, lysed macrophages contained similar concentrations of SNO (~57 pmol/mg), in each case significantly lower than those of uninfected cells. After overnight dialysis of lysates produced from wild-type infected cells, the distribution of SNO was determined to be 35% low-molecular-weight SNO (<2000 Da) (data not shown). At 2 h after infection, while the sizes of the two wild-type populations (MOI 10 and 100) were similar (data not shown), cells infected...
different concentrations of macrophage cell SNO. Cells (heat-killed wild-type MC58) did not result in significantly derivative) or the metabolic inactivity of the bacterium effective deletion of the cells with bacteria lacking functional NorB, either due to respectively (Fig. 4A). In keeping with previous findings that the growth of the norB-mutant strain is attenuated in human MDMs (27), viable counts of bacteria at 4 h postinfection showed that significantly fewer of these bacteria were present per J774.2 cell than of either the wild-type or nsrR-mutant strain (data not shown). To overcome this confounding factor, we exposed cells to the f-actin polymerization inhibitor cytochalasin D, which itself had no effect on the concentration of cellular SNO, compared to uninfected cells without cytochalasin D treatment (data not shown). Inhibition of phagocytosis in this manner eliminated the difference in numbers of bacteria per J774.2 cell (data not shown). The concentration of SNO in uninfected, cytochalasin-treated cells was 106.8 pmol/mg (median; interquartile range 68.57–184.5 pmol/mg). Infection of cytochalasin-treated cells with wild-type or mutant meningococci resulted in a similar pattern of SNO abundance to that observed in untreated cells (Fig. 4B). From these findings, it is concluded that the differences in SNO depletion between NorB-expressing wild-type and the mutant are not due to intracellular attenuation of bacterial growth.

In an identical series of experiments using cytochalasin D, we corroborated our initial findings in I3 using a phosphate-buffered solution (pH 7.0) containing a saturating concentration of Cu(I) ion and 1 mM L-cysteine (Fig. 4C). Wild-type-infected and nsrR-infected macrophage cells contained a reduced abundance of SNO compared to uninfected controls (62.0±20.4 and 73.8±28.6%, respectively), while cells infected with strains unable to detoxify NO contained SNO concentrations exceeding those of control cells (norB, 101.9±37.4%; heat-killed wild-type, 103.4±41.4%).

Bacterial NO detoxification prevents new SNO formation

To determine whether NorB activity degrades preformed SNO or prevents new SNO formation, we added the iNOS inhibitor 1400W to the macrophage cells at the point of infection (t=18 h). Exposure of stimulated J774.2 cells to 1400W resulted in a significant reduction in cellular SNO concentrations over 4 h cf. uninfected cells not exposed to the inhibitor (data not shown). The SNO concentration in 1400W-treated, uninfected cells was 32.5±10.1 pmol/mg. Infection of 1400W-treated cells with any strain of N. meningitidis had no effect on the abundance of SNO in these cells (Fig. 5).

Hmp is also associated with reduced SNO abundance in infected J774.2 murine macrophage cells

Infection of stimulated J774.2 cells with the gram-negative enteric bacteria Salmonella enterica spp. Typhimurium (strain 14028s) or E. coli (strain MG1655) also caused a

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**Figure 3.** Effect of infection by wild type N. meningitidis on endogenously produced SNO in activated J774.2 cells. At t = 0 h, duplicate wells of J774.2 cells were activated with 1 μg/ml LPS and 1000 U/ml rmIFN-γ for 18 h and then infected with a suspension of log-phase wild-type N. meningitidis in fresh medium at MOI 10 or 100 for 2 h (t=20 h) (A) or 4 h (t=22 h) (B). Lysates were produced using SNO-compatible lysis buffer plus 2% saponin. SNO content was determined by duplicate injection into I3 reaction mixture linked to ozone-based chemiluminescence and normalized to the protein concentration of the lysate. Data are presented as SNO concentration (pmol SNO/mg protein). Bars denote means ± se. *P<0.05, ***P<0.001; ANOVA with Tukey’s multiple comparisons test.

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at MOI 100 contained significantly lower concentrations of SNO (64.5±13.9 pmol/mg) than uninfected cells (126.0±47.4 pmol/mg), in contrast to cells infected at MOI 10 (77.5±39.5 pmol/mg) (Fig. 3A).

**NorB of N. meningitidis is associated with reduced abundance of SNO following infection of J774.2 murine macrophage cells**

Infection of stimulated J774.2 cells with N. meningitidis strains expressing functional NorB (i.e., wild-type MC58 and the nsrR-mutant derivative) resulted in reduced abundance of host cell SNO (Fig. 4A). Stimulated but uninfected cells contained 139.7±52.2 pmol/mg SNO (100%), whereas wild-type infected and nsR-infected cells contained only 40.5±18.5 and 60.2±28.6% as much SNO, respectively (Fig. 4A). Conversely, infection of stimulated cells with bacteria lacking functional NorB, either due to the effective deletion of the norB gene (i.e., norB-mutant derivative) or the metabolic inactivity of the bacterium (heat-killed wild-type MC58) did not result in significantly different concentrations of macrophage cell SNO. Cells infected with norB-mutant bacteria produced lysates containing 78.7±22.3% of the SNO concentration measured in uninfected cells, while cells infected with heat-killed wild-type bacteria contained 91.6±25.6% as much SNO as uninfected cells (Fig. 4A). In keeping with previous findings that the growth of the norB-mutant strain is attenuated in human MDMs (27), viable counts of bacteria at 4 h postinfection showed that significantly fewer of these bacteria were present per J774.2 cell than of either the wild-type or nsrR-mutant strain (data not shown). To overcome this confounding factor, we exposed cells to the f-actin polymerization inhibitor cytochalasin D, which itself had no effect on the concentration of cellular SNO, compared to uninfected cells without cytochalasin D treatment (data not shown). Inhibition of phagocytosis in this manner eliminated the difference in numbers of bacteria per J774.2 cell (data not shown). The concentration of SNO in uninfected, cytochalasin-treated cells was 106.8 pmol/mg (median; interquartile range 68.57–184.5 pmol/mg). Infection of cytochalasin-treated cells with wild-type or mutant meningococci resulted in a similar pattern of SNO abundance to that observed in untreated cells (Fig. 4B). From these findings, it is concluded that the differences in SNO depletion between NorB-expressing wild-type and the mutant are not due to intracellular attenuation of bacterial growth.

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reduced abundance of macrophage SNO (Fig. 6). 14028s-infected cells produced lysates containing only 26.4 ± 15.2% of the SNO concentrations measured in uninfected cells, which in this experimental series was determined to be 53.7 ± 19.2 pmol/mg (Fig. 6A). This activity was Hmp dependent; although 14028sΔhmp-infected cells yielded significantly less SNO than uninfected cells (58.5±36.2% of controls); these concentrations were significantly higher than those of cells infected with 14028s (Fig. 6A).

A different pattern was observed following infection of stimulated J774.2 cells with wild-type and mutant E. coli strains (Fig. 6B). Deletion of the hmp gene from strain MG1655 removed the ability of E. coli to influence macrophage SNO concentrations significantly. MG1655-infected cells produced lysates containing only 27.3 ± 20.2% of the SNO in uninfected cells, which were measured as 62.57 ± 27.97 pmol/mg in these experiments. However, no significant difference was found between the SNO concentration of lysates produced from uninfected and MG1665Δhmp-infected cells (Fig. 6B).

**DISCUSSION**

This work has shown that the process of bacterial NO detoxification, vital to the survival of pathogenic organisms under conditions of nitrosative stress, can prevent the formation of host cell SNO. Both the NorB of N. meningitidis and the Hmp of the enteric pathogens S. enterica and E. coli, which detoxify NO by different mechanisms in different cellular environments, have been demonstrated to inhibit the formation of SNO in infected macrophage cells significantly. To our knowledge, this represents the first observation of its kind: that bacterial energy metabolism can disrupt the formation of a specific chemical adduct inside host cells, one that is regulatory for a number of diverse host cell processes.

This study has demonstrated the capacity for a combination of LPS and IFN-γ to induce the formation of
high concentrations of endogenously formed SNO in murine macrophage-like cells, through the expression of iNOS. Although the chemical species responsible for S-nitrosylation per se remains contentious, NO—as a precursor molecule—is necessary for the formation of these adducts. In this simplistic view, NO represents the precursor molecule—is necessary for the formation of SNO per se. The differences measured in this study might, therefore, be due to differences in the relative expression levels of Hmp, the availability of oxygen for the denitrosylase reaction, or perhaps different contributions of alternative NO detoxification mechanisms, such as the NO reductase NorB (41).

For both strains of enteric bacteria and for *N. meningitidis*, the association of NO detoxification machinery with a reduced abundance of host cell SNO suggests that the effect takes place through the detoxification of NO per se rather than through direct protein–protein interactions, akin to the mechanism shown for the thioredoxin/thioredoxin reductase system (21) and GSNO reductase (20). It is unlikely that these bacterial proteins physically interact with host cell SNO, given their subcellular localization to either the inner bacterial membrane (NorB) or the cytoplasm (Hmp). While NO is itself freely diffusible in biological systems, SNO requires transport across membranes (53).
By simultaneously inhibiting iNOS activity and infecting stimulated J774.2 cells with meningococci, we have shown that bacterial mechanisms for NO detoxification act to prevent de novo SNO formation rather than accelerate the decomposition of preexisting SNO (Fig. 5). Addition of 1400W at the start of the infection significantly impaired NO synthesis and removed one of the substrates necessary for SNO formation. In uninfected cells, the inhibition of NO synthesis prevented the replenishment of SNO, which continued to degrade and be degraded by cellular denitrosylation mechanisms. The result was a gradual decline in cellular SNO concentration, similar to observations made by Zhang and Hogg in RAW264.7 cells (46). If the activity of NorB were increasing the rate of degradation of preexisting SNO, then infection of cells with NorB-expressing strains would have caused further reduction in SNO concentrations, over and above the normal rate. Therefore, we would expect infection with the wild-type and nsrR mutant strains to result in far less host cell SNO compared to the other samples. Instead, Fig. 5 shows no significant difference between the cellular SNO concentrations of J774.2 cells infected with any strain of N. meningitidis. This observation is consistent with the proposal that, because the rate of NO diffusion exceeds any rate of NO oxidation, cellular SNO formation involves NO that has first diffused out of, then back into a given cell (54). Addition of extracellular hemoglobin to LPS-stimulated RAW cells resulted in a reduced abundance of SNO (46), which was attributed to the failure of NO to return from the extracellular milieu after having been effectively scavenged (54). The activity of meningococcal NorB may be acting analogously to hemoglobin in our infection model, preventing NO that has diffused out of the cell from returning and forming SNO, by way of its reduction to N2O.

An alternative mechanism, not addressed experimentally in this work, may be that the SNO-depletion phenomenon is due to some effect of NO detoxification on amino acid transport. Since NO donation can enhance cystine transport across mammalian cell membranes (55), it is conceivable that bacterial modulation of NO concentration results in selective loss of SNO-associated amino acids (i.e., S-nitrosocysteine). Transmembrane movement of S-nitroso-L-cysteine is mediated by system L amino acid transporters (56). However, in a rat model of bacterial sepsis, no significant difference was observed in the activity of amino acid transport system L between septic and control animals (57).

According to the data reported in Fig. 3, infection with wild-type meningococci results in the attainment of a lower, steady-state concentration of host cell SNO, the formation of which is not prevented by bacteria (Fig. 3). A lower inoculum of wild-type bacteria could not prevent a rise in host cell SNO over 2 h (Fig. 3A; MOI 10) but after 4 h had replicated to a population size sufficient to maintain this basal SNO concentration (Fig. 3B; MOI 10). The reduction in SNO concentration observed between 2 and 4 h represents the continued degradation/denitrosylation of SNO in the absence of new SNO formation, and a gradual return to basal levels. Larger inocula were sufficient to prevent any significant rises in SNO concentration (Fig. 3; MOI 100), but a detectable concentration of SNO remained in these cells (~57 pmol/mg). This steady state may be evidence of a core subset of stable S-nitrosylated thiol, which corroborates the findings of Paige et al. (58). In a recent proteomic study, this group identified a subset of 10 proteins containing thiol-insensitive SNO, which they hypothesize to be the most likely mediators of the persistent cellular effects of NO (58). Their work divides SNOs into stable and unstable groups, the former of which shields the NO-cysteine bond from degradation. These observations are difficult to reconcile with the idea that bacteria metabolize NO before SNO formation can take place, especially given the demonstrated colocalization of NO synthesis with SNO formation (17) and the evidence that $\text{S}^1$ nitrosylation is compartmentalized (59). While we have postulated that bacterial NO detoxification prevents the formation of new SNO, it is possible that there is limited $\text{S}^1$ nitrosylation in the immediate vicinity of NOS to form so-called stable SNO. This is plausible if we assume that the qualities that stabilize the $\text{S}^1$ nitrosothiol bond also encourage its formation (60). In this scenario, therefore, bacterial NO detoxification is preventing the formation of “unstable” SNO.

Rapidly accumulating evidence indicates the critical importance of homeostatic regulation of SNO concentration in health and disease. Indeed, S$\text{S}^1$ nitrosylation and its dysregulation are implicated in a number of chronic diseases, including amyotrophic lateral sclerosis (61), Parkinson’s disease (62, 63), multiple sclerosis (64), and Alzheimer’s disease (65). Deletion of GSNOR in mice is associated with higher systemic SNO concentrations and is protective against pathologies such as airway hyperresponsivity (66) but also increases mortality in endotoxemia (67). Conversely, humans with asthma have higher concentrations of GSNOR in bronchoalveolar lavage (BAL) fluid compared to nonasthmatic individuals and reduced abundance of pulmonary SNO (68). Our findings suggest a potential role for $\text{S}^1$ nitrosylation, or at least its dysregulation, in sepsis. Rather than regulated denitrosylation, the effect we have described results in wholesale SNO dysregulation, which we predict will have important consequences for the behavior of host cells, and therefore the natural history of infectious disease. Fine dissection of the physiological consequences of this phenomenon is likely to be difficult, given the apparent ubiquity of $\text{S}^1$ nitrosylation. While the $\text{S}^1$ nitrosylation of one protein may positively influence or stimulate a particular cellular process, the same process may be affected adversely or inhibited by $\text{S}^1$ nitrosylation of a different target. However, the evidence that SNO depletion is mediated by at least two NO detoxification mechanisms in three different mammalian pathogens supports the hypothesis that this phenomenon is a previously uncharacterized mechanism of septic disease. The bacterial burdens used in our system are physiologically relevant, given that studies...
of meningococcal septicemia, for example, have shown titers in the region of 10^8 bacterial genome copies/ml blood, occurring commonly in infected people (69). Similarly, the abundance of enteric bacteria in the intestine make it likely that, in these oxygen-poor, NO-rich environments, cells in intimate contact with bacteria under these conditions (i.e., endothelial cells and macrophages) are likely to experience SNO depletion and that soluble SNOs, such as plasma S-nitrosoalbumin, are also likely to be depleted.

We posit that bacterial proteins capable of detoxifying NO can remove freely diffusible NO from the cellular environment at a rate sufficient to prevent the formation of unstable SNO inside host cells. The potential pathological consequences of this phenomenon are considerable and are currently under investigation.

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