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N_2O_3 enhances the nitrosative potential of IFNγ-primed macrophages in response to *Salmonella*

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Abstract

We show here that the nitric oxide (NO)-detoxifying Hmp flavohemoprotein increases by 3-fold the transcription of the *Salmonella* pathogenicity island 2 (SPI2) in macrophages expressing a functional inducible NO synthase (iNOS). However, Hmp does not prevent NO-related repression of SPI2 transcription in IFNγ-primed phagocytes, despite preserving intracellular transcription of *sdhA sdhB* subunits of *Salmonella* succinate dehydrogenase within both control and IFNγ-primed phagocytes. To shed light into the seemingly paradoxical role that Hmp plays in protecting intracellular SPI2 expression in various populations of macrophages, N_2O_3 was quantified as an indicator of the nitrosative potential of *Salmonella*-infected phagocytes in different states of activation. Hmp was found to prevent the formation of 300 nM N_2O_3/h/bacteria in IFNγ-primed macrophages, accounting for about a 60% reduction of the nitrosative power of activated phagocytes. Utilization of the vacuolar ATPase inhibitor bafilomycin indicates that a fourth of the ~200 nM N_2O_3/h sustained by IFNγ-primed macrophages is generated in endosomal compartments via condensation of HNO_2. In sharp contrast, control macrophages infected with wild-type *Salmonella* produce as little N_2O_3 as iNOS-deficient controls. Collectively, these findings indicate that the NO-metabolizing activity of *Salmonella* Hmp is functional in both control and IFNγ-primed macrophages. Nonetheless, a substantial amount of the NO generated by IFNγ-primed macrophages gives rise to N_2O_3, a species that not only enhances the nitrosative potential of activated phagocytes but also avoids detoxification by *Salmonella* Hmp.

Keywords

Hmp; IFNγ; macrophages; N_2O_3; nitric oxide; nitrosative stress; *Salmonella*

Introduction

NO is an integral part of host defense. Mice rendered pharmacologically or genetically deficient in their ability to sustain high NO synthesis are hypersusceptible to experimentally-induced systemic salmonellosis (Alam et al., 2002; Mastroeni et al., 2000; Shiloh et al., 1999; White et al., 2005). The contribution of NO to resistance to this enteric pathogen strongly correlates with the iNOS-dependent anti-*Salmonella* activity of macrophages (Vazquez-Torres et al.,

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2000a). Toll-like receptor-4 agonists, such as *Salmonella* lipopolysaccharide (Vazquez-Torres et al., 2004), stimulate innate transcription of iNOS through direct activation of NF-κB and indirect induction of IRF-1 upon autocrine release of IFNβ (Fujihara et al., 1994; Gao et al., 1998; Ohmori and Hamilton, 2001; Xie et al., 1994; Zhang et al., 1994). The pro-inflammatory cytokine IFNγ, possibly through further activation of IRF-1 (Kamijo et al., 1994; Martin et al., 1994), synergizes with innate responses to *Salmonella* ligands to augment iNOS transcription (Vazquez-Torres et al., 2000a; Vazquez-Torres et al., 2004). Involvement of NO in the anti-*Salmonella* activity of IFNγ-treated macrophages is well accepted (Vazquez-Torres et al., 2000a; Vazquez-Torres et al., 2004; Webb et al., 2001); however, the role that this diatomic radical plays in the anti-*Salmonella* arsenal of macrophages has proven to be a contentious topic (Chakravortty et al., 2002; Ekman et al., 1999; Saito et al., 1991; Shiloh et al., 1999; Shiloh et al., 1997; Vazquez-Torres et al., 2000a; Vazquez-Torres et al., 2004).

The ability of *Salmonella* to cause disseminated disease greatly depends on the type III secretion system encoded within the SPI2 pathogenicity island 2 (Hensel et al., 1995; Ochman et al., 1996; Shea et al., 1996). SPI2 enhances *Salmonella* intracellular fitness by remodeling the phagosome, and thus it minimizes the cytotoxicity of lysosomal hydrolytic enzymes and oxyradicals generated by NADPH oxidase enzymatic complexes (Gallois et al., 2001; Suvarnapunya and Stein, 2005; Uchiya et al., 1999; Vazquez-Torres et al., 2000b). In addition, SPI2 boosts antinitrosative defenses of *Salmonella* by preventing close interactions of phagosomes with iNOS-containing vesicles (Chakravortty et al., 2002). However, we have shown that nitrogen oxides produced by IFNγ-primed macrophages render this type III secretion system nonfunctional, ultimately leading to progression of *Salmonella* phagosomes along the degradative pathway (McCollister et al., 2005). It is seemingly paradoxical that NO can selectively mediate strong anti-*Salmonella* activity of IFNγ-primed macrophages, but exerts negligible or minimal effects in populations of phagocytes that had not been stimulated by IFNγ. We have used herein a combination of bacterial genetics and biochemical assays to study the nitrosative potential of control and IFNγ-primed macrophages in response to *Salmonella* infection.

Material and Methods

Bacterial strains

Salmonella enterica serovar Typhimurium strain ATCC 14028s was used throughout this study as wild-type and as background for the construction of mutants (Table 1). *S.* Typhimurium strain AV0615 (*ΔspiC::FRT Δhmp::km*) was constructed after P22-mediated transduction of the *Δhmp::km* allele from strain AV0468 into strain AV0201 carrying a *ΔspiC::FRT* mutant allele (McCollister et al., 2005). The *ΔspiC::lacZ* transcriptional fusion was transduced from strain AV0207 into AV0468 (McCollister et al., 2005), generating strain AV0539 (*ΔspiC::lacZ Δhmp::FRT*). Pseudolysogens were eliminated by streaking on Evans blue uranine agar plates. The green-fluorescent protein expressed under the control of the P_{tac} promoter was amplified from pRSET::*gfp* using the primer pair F-5′-AGC TGT TGA CAA TTA ATC ATC GGC TCG TAT AAT GTG TGG AAT TGT GAG CGG ATA ACA ATT TCA CAC AGG AAC AGA AAT GAG TAA AGG AGA AGA ACT TTT C-3′ (the P_{tac} promoter is underlined) and R-5′-TAA TAC GAC TCA CTA TAG GG-3′. The PCR product was ligated into pCR®-Blunt (Invitrogen, Carlsbad, CA), and the resulting plasmid was transformed into *Salmonella* to generate strain AV0101.

Macrophages

Macrophages were collected from C57BL/6 and congenic gp91*phox*$^{-/-}$, *iNOS*$^{-/-}$ and doubly immunodeficient gp91*phox*$^{-/-}$ iNOS$^{-/-}$ (MacMicking et al., 1995; Pollock et al., 1995; Shiloh et al., 1999) mice by peritoneal lavage 4 d after intraperitoneal inoculation of 1 mg/ml sodium

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periodate as described (De Groote et al., 1997). The peritoneal exudate cells were resuspended in RPMI 1640 medium (Cellgro, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum (BioWhittaker, Walkersville, MD), 15 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO) and $100U \cdot ml^{-1}/100mg \cdot ml^{-1}$ of penicillin/streptomycin (Cellgro). Peritoneal exudate cells were seeded at 2×10^5 cells/well in 96-well plates for macrophage killing assays, or 10^6 cells/well in 24-well plates (Falcon, Franklin Lakes, NJ) for RNA transcriptional studies. Peritoneal macrophages were selected by adherence following 48 h of culture at 37°C in a 5% CO_2 incubator. IFNγ (Life Technologies, St. Paul, MN) was added at a final concentration of 200 U/ml to selected groups of macrophages 20 h prior to *Salmonella* infection.

Macrophage killing assay

Macrophages were challenged at an MOI of 2 with stationary phase *Salmonella* opsonized with 10% normal mouse serum for 20 min. Extracellular bacteria were removed from the monolayers 25 min after challenge by washing with pre-warmed RPMI medium containing 6 μg/ml of gentamicin (Sigma) (De Groote et al., 1997). *Salmonella*-infected macrophages were lysed at the indicated time points after challenge, and the surviving bacteria were enumerated on Luria-Bertani agar plates. The results are expressed as % survival.

Synthesis of cDNA

Total RNA was isolated from *Salmonella*-infected macrophages as previously described (McCollister et al., 2005) using a combination of the TRIzol reagent (Invitrogen, Carlsbad, CA) and an RNAeasy kit (Qiagen, Valencia, CA). Complementary cDNA was synthesized at 42°C for 30 min using MMLV reverse transcriptase (Promega), RNasin (Promega), dNTPs, and random primers or a poly-dT oligomer (Promega). The synthesized cDNA was then used as template for quantitative real-time PCR and standard or nested PCR.

Transcriptional profiling

Real-time PCR reactions contained cDNA, Takara OmniMix™ HS (Takara Bio Inc., Otsu, Shiga, Japan), 200 nM forward and reverse primers and 240 nM fluorescent-labeled DNA probes. Oligonucleotide primers and probes for transcriptional analysis of *Salmonella spiC* and *rpoD* expression were used as previously described (McCollister et al., 2005). Real-time PCR analysis of macrophage *iNOS* and *GAPDH* expression was performed using primers and probes described in supplementary Table 2. Real-time PCR reactions consisted of a cycle of 94°C for 45 s followed by 45 cycles of 94°C for 5 s and 59°C for 30 s. The resulting fluorescence was recorded using the SmartCycler®II thermocycler (Cepheid, Sunnyvale, CA). *Salmonella spiC* and murine *iNOS* transcripts were normalized with respect to house-keeping *Salmonella* RNA polymerase sigma factor *rpoD* or eukaryotic *GAPDH*, respectively. Transcription of *rpoD* and *sdhA* and *sdhB Salmonella* genes in macrophages isolated from gp91*phox*- or gp91*phox* iNOS-deficient mice was assessed by nested PCR using primers described in Table 2. Nested PCR consisted of an initial 15 cycle amplification using the long primers. The number of amplification cycles used in the nested PCR were adjusted according to *rpoD* transcript levels determined by real-time PCR.

Expression of *spiC::lacZ* transcriptional fusions

SPI2 expression was induced *in vitro* by culturing *Salmonella* in low osmolarity N salts medium as described (Deiwick et al., 1999). Briefly, *S.* Typhimurium strains harboring a *spiC::lacZ* transcriptional fusion were grown overnight in high Mg^{++} N salts medium [5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 38 mM glycerol, 0.1% casamino acids] supplemented with 10 mM $MgCl_2$ and 100 mM Tris-HCl, pH 7.6. The bacteria were subcultured in high Mg^{++} N salts medium and grown at 37°C in a shaker incubator to OD_{600}

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of 0.5. SPI2 expression was induced by switching the bacteria to 8 μM $MgCl_2$ N salts medium, pH 6.9. The expression of *spiC::lacZ* was quantified spectrophotometrically as β-galactosidase enzymatic activity using the substrate o-nitrophenyl-β-D-galactopyranoside (Sigma) (Miller, 1972). β-galactosidase activity is expressed in Miller units according to the equation $1000 \times [(OD_{420} - 1.75 \times OD_{550})]/(T_{(min)} \times V_{(ml)} \times OD_{600})$. The NO donor spermine NONOate (Cayman Chemical, Ann Arbor, MI) was used to determine the effects of RNS on SPI2 transcription. Spermine NONOate dissolved in 10 mM Tris-HCl, pH 8.0 was quantified spectrophotometrically using $\varepsilon_{252} = 8500\ M^{-1}\ cm^{-1}$. The NO donor was added to the cultures after *Salmonella* had been grown for 1 h in 8 μM $MgCl_2$ N salts medium, a time at which the bacterial cells had reached early stationary phase.

Intracellular *hmp::lacZ* expression was determined 20 h after wild-type and iNOS-deficient macrophages were infected with strain AV0305 at an MOI of 2. Selected populations of macrophages were activated with 200 U/ml IFNγ 20 h prior to infection. β-galactosidase was monitored in an Lmax luminometer (Molecular Devices, Sunnyvalle, CA) following directions of the Galacton-Plus® kit (Applied Biosystems, Foster City, CA). Intracellular *hmp::lacZ* expression is represented as β-galactosidase/10^6 bacteria.

NO_x determination

Rates of macrophage NO synthesis were estimated over time as previously described (Vazquez-Torres et al., 2000a) by measuring the accumulation of nitrite (NO_2^-) and nitrate (NO_3^-), which are stable metabolites of the reaction of NO with oxygen. *Salmonella*-challenged macrophages were washed and resuspended in prewarmed IMDM medium (Sigma) supplemented with 0.3% wt/vol sodium bicarbonate (Sigma), 2 mM L-glutamine, 1% Nutridoma-SP (Boehringer), and 6 μg/ml gentamicin 1 h prior to the designated time points, untreated or IFNγ-treated. NO_x were allowed to accumulate for 1 h prior to collection. Enzymatic reduction of NO_3^- to NO_2^- was performed as described before (Vazquez-Torres et al., 2000a) using nitrate reductase (Sigma). NO_x were then estimated from total NO_2^- concentrations measured spectrophotometrically at 550 nm using the Griess reagent (0.5% sulfanilamide and 0.05% *N*-1-naphthylethylenediamide hydrochloride in 2.5% phosphoric acid). Standard curves were prepared with $NaNO_2$.

Analysis of nitrosative stress

Nitrosative stress can be associated with a variety of RNS, including peroxynitrite, dinitrosyl iron complexes and N_2O_3. N_2O_3 was quantified as an indicator of the nitrosative capacity of control and IFNγ-treated macrophages. N_2O_3 was indirectly visualized as the N-nitroso 2,3-naphthotriazole (NAT) derivative of 2,3-diaminonaphthalen (DAN) (Sigma) as described (Espey et al., 2000). A 100 mM stock of DAN prepared in dimethylformamide was used at a final concentration of 200 μM in MEM medium supplemented with 1% nutridoma and 6 μg/ml gentamicin. Accumulation of NAT was recorded for 1 h at the indicated times after infection. Fluorescence was measured on a Synergy HT fluorometer (BioTek, Winooski, Vermont) set at λ_{ex}=375 nm, λ_{em}=460 nm. Concentration of N_2O_3 released by the macrophages was estimated by linear regression analysis of serially diluted NAT prepared after 30 min incubation of 25 μM DAN with 1 mM spermine NONOate.

Immunofluorescence microscopy

Macrophages plated on 13 mm glass coverslips in a 24-well plate at a density of 2.5×10^5 cells per well were infected as described above. Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) 20 h post-infection, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS (Sigma), and blocked at 37°C with PBS containing 4% donkey serum. Cells were incubated with an anti-iNOS polyclonal rabbit antibody (Upstate, Charlottesville, VA) for 45 min at 37°C followed by a Cy3-conjugated

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donkey anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 2 μg/ml, Sigma) for 45 min at 37°C. After washing with PBS, coverslips were mounted onto glass slides with Prolong Antifade (Molecular Probes, Eugene, OR) and viewed on a Zeiss Axiophot fluorescence microscope.

Statistical analysis

Data are expressed as mean ± SEM. The data were analyzed using a paired Student's *t* test.

Results

Hmp protects SPI2 expression from NO produced during the innate response of macrophages

We have previously shown that the expression of SPI2 by intracellular *Salmonella* residing in macrophages is repressed in an iNOS-dependent manner upon activation of the phagocytes with IFNγ (McCollister et al., 2005). Therefore, we examined whether the NO-metabolizing capacity of Hmp (Gardner et al., 1998; Hausladen et al., 2001; Hausladen et al., 1998) preserves SPI2-dependent *Salmonella* survival in macrophages that have not been treated with IFNγ. Hmp significantly ($p<0.05$) enhanced wild-type but not *spiC*-deficient *Salmonella* survival in macrophages that have not been treated with IFNγ (Fig. 1A, left panel). The protective effects of Hmp appear to be related to its NO-consuming activity because survival of *hmpA*-deficient *Salmonella* was restored to wild-type levels in iNOS-deficient macrophages (Fig. 1A, left panel). The contribution of Hmp to *Salmonella* survival in macrophages was related to protection of SPI2 transcription as *spiC* transcripts were significantly ($p<0.05$) reduced by NO in the absence of Hmp (Fig. 1B). Accordingly, the expression of *spiC* by *hmpA*-deficient *Salmonella* returned to wild-type levels in iNOS-deficient macrophages (Fig. 1B). Remarkably, the protective role of Hmp was lost in IFNγ-activated macrophages as bacterial survival was equally reduced in the presence or absence of *hmpA* (Fig. 1A, right panel).

Quantification of *hmpA* expression by intracellular *Salmonella*

Hmp is the most important antinitrosative defense of *Salmonella* (Bang et al., 2006). We calculated the degree by which Hmp protects SPI2 expression against nitrosative stress and the extent of *hmpA* expression during innate and acquired responses of macrophages. An IC_{50} value of 563 μM was estimated for the inhibition of SPI2 transcription by the NO donor spermine NONOate as measured by β-galactosidase activity of a *spiC::lacZ* transcriptional fusion expressed by wild-type *Salmonella* (Fig. 2A). In the absence of Hmp, the spermine NONOate IC_{50} was, however, reduced to 176 μM. In comparison, we have estimated an IC_{50} value of 538 μM for the inhibition of respiration by the NO donor spermine NONOate in wild-type *Salmonella* (data not shown) indicating that the electron transport chain may be as susceptible as SPI2 to the inhibitory effects of NO. These data support a role for the NO-consuming activity of Hmp in protecting SPI2 transcription in macrophages expressing iNOS. In contrast, SPI2 expression has been shown to be inhibited in IFNγ-activated macrophages. The inability of Hmp to protect SPI2 expression against the 4-fold increased NO_x rates sustained by IFNγ-primed macrophages (Fig. 2B) is surprising since we found that 7-fold more *hmpA* was expressed by *Salmonella* in activated macrophages (Fig. 2C).

Distribution of iNOS in IFNγ-activated macrophages

The iNOS enzymatic complex can partition in vesicular as well as cytosolic and cortical cytoskeletal fractions (Vodovotz et al., 1995; Webb et al., 2001). Because IFNγ enhances killing of nonfusogenic microbes such as *Mycobacterium* and *Chlamydia* by enhancing vesicular trafficking (MacMicking et al., 2003; Nelson et al., 2005), we tested whether abrogation of SPI2-dependent *Salmonella* survival manifested in IFNγ-activated macrophages

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may be related to the redistribution of iNOS-containing vesicles. Fluorescence microscopy at 20 h post *Salmonella* infection revealed punctate iNOS staining consistent with localization of the hemoprotein within membrane-bound vesicles as suggested in left insert of Fig. 3. In agreement with previous investigations (Chakravortty et al., 2002; Webb et al., 2001), no obvious clustering of iNOS-containing vesicles was observed around *Salmonella* within macrophages. No apparent differences were seen for the cellular distribution of iNOS in control and IFNγ-primed macrophages. Nonetheless, IFNγ induced a marked increase in iNOS staining throughout *Salmonella*-infected macrophages (Fig. 3, middle panel), consistent with the synergistic effects of IFNγ and bacterial ligands on iNOS transcription (Ding et al., 1988; Kamijo et al., 1993; Lorsbach et al., 1993; Xie et al., 1993). Staining for iNOS protein was enriched in both control and IFNγ-activated macrophages in perinuclear areas occupied by *Salmonella* (Fig. 3, panel insets).

IFNγ enhances nitrosative chemistry of *Salmonella*-infected macrophages

We explored the ability of control and IFNγ-primed macrophages to generate the strong nitrosating species N_2O_3. Similar to iNOS-deficient controls, periodate-elicited macrophages synthesized very little NAT during the innate response to *Salmonella* (Fig. 4A). In contrast, IFNγ-primed macrophages generated significant amounts of NAT after 12 h of infection. DAN can be nitrosated to NAT by N_2O_3 produced in the autooxidation of NO or by the condensation of HNO_2 arising from acidified NO_2^-. Since the *Salmonella* phagosome acidifies to pH 5.0 (Rathman et al., 1996), it is possible that the N_2O_3 detected in IFNγ-primed macrophages represents a mixture of these two chemistries. To quantify the contribution of acidified NO_2^- to global N_2O_3 engendered by IFNγ-primed macrophages, the vacuolar ATPase inhibitor bafilomycin was added at the time of infection. Fig. 4B indicates that about one fourth of the N_2O_3 produced by macrophages can be accounted for by condensation of HNO_2 in the acidified lumen of the *Salmonella* phagosome. Bafilomycin did not, however, reduce the overall formation of NO_2^- (Fig. 4C), demonstrating that reduction on N_2O_3 by bafilomycin does not reflect indiscriminate toxicity of the cell.

Hmp prevents generation of N_2O_3 intracellularly

We tested the extent by which Hmp inhibits generation of N_2O_3 in the cytoplasm of *Salmonella* residing in control and IFNγ-activated macrophages (Fig. 5). Because the bacterial burden recovered from control and IFNγ-primed wild-type macrophages from C57BL/6 mice is so disparate (Fig. 1), the ability of Hmp to prevent N_2O_3 synthesis was studied in gp91*phox*$^{-/-}$macrophages that are known to inhibit SPI2 transcription in an iNOS-dependent manner, while they unable to reduce *Salmonella* viability (McCollister et al., 2005;Vazquez-Torres et al., 2000a). To increase the sensitivity of the assay, N_2O_3 generation was measured in macrophages cultured in 24 well plates. Macrophages generated 3 times more N_2O_3 when infected with *hmpA*-deficient bacteria. A functional Hmp was calculated to prevent formation of ~40 nM N_2O_3/bacteria/h in macrophages that had not been treated with IFNγ, whereas this enzyme blocked generation of ~300 nM N_2O_3 in IFNγ-treated macrophages. Despite this robust diminution in N_2O_3 synthesis, Hmp-expressing bacteria were exposed to ~200 nM/h N_2O_3, which represents about nine times the amount of N_2O_3 encountered by *Salmonella* in control macrophages not treated with IFNγ.

Hmp protects intracellular transcription of *sdhA*-encoding succinate dehydrogenase of the electron transport chain

Repression of SPI2 transcription in activated macrophages despite the abundant expression of Hmp represents an apparent paradox. To assess whether the Hmp expressed in IFNγ-primed macrophages is functionally active, we followed transcription of the respiratory *sdhA* and *sdhB* genes, which are tightly controlled by the ArcB sensor of the electron transport chain

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(Georgellis et al., 2001; Shen and Gunsalus, 1997). Expression of *sdhA* and *sdhB* was studied in gp91*phox*-deficient macrophages, because these phagocytes produce nitrosative chemistry inhibitory for SPI2 expression (McCollister et al., 2005) while preserving bacterial viability (Vazquez-Torres et al., 2000a). Hmp protected *sdhA* and *sdhB* expression in both control and IFNγ-primed macrophages (Fig. 6). Inhibition of *sdhA* and *sdhB* transcription in *ΔhmpA::FRT Salmonella* within gp91*phox*-deficient macrophages is NO-mediated, because transcription of these genes was restored in doubly immunodeficient phagocytes lacking both gp91*phox* and iNOS (Fig. 6).

Discussion

The enzymatic activity of iNOS is an integral component of the antimicrobial arsenal of macrophages; however, the biological chemistry mediating host defense associated with this hemoprotein is very little understood. We have used here a combination of bacterial genetics and biochemical assays to shed light into the nature of the RNS differentially expressed by populations of macrophages exhibiting strong iNOS-dependent antimicrobial activity against the intracellular pathogen *Salmonella*. Transcriptional analysis of *sdhA* and *sdhB* genes encoding subunits of the complex II of the electron transport chain indicates that *Salmonella* Hmp is functional in both control and IFNγ-primed macrophages. The 4-fold increases in NO synthesis in IFNγ-primed macrophages are met by a 7-fold up-regulation in transcription of *hmpA*. This probably explains the sustained expression of *sdhA* and *sdhB* in control and IFNγ-activated macrophages. Transcription of *sdhA* and *sdhB* is tightly regulated by ArcB (Georgellis et al., 2001; Shen and Gunsalus, 1997), which senses the reduced pool of quinones in the electron transport chain. Transcription of *sdhA* and *sdhB* in macrophages sustaining disparate levels of nitrosative stress is probably an indication of preserved respiratory activity by the NO-consuming activity of Hmp. These findings suggest that a functional Hmp detoxifies NO quite effectively at the various rates generated by professional phagocytes in various states of activation.

Hmp also protects SPI2 transcription against nitrosative stress generated by resting macrophages. *Salmonella* lacking Hmp experienced three times more N_2O_3 in resting macrophages than in wild-type controls, resulting in comparable reductions in SPI2 expression and intracellular survival. Because SPI2 is critical for intracellular survival of *Salmonella* in a variety of phagocytic and nonphagocytic cells and is essential for the development of systemic salmonellosis (Ochman et al., 1996; Shea et al., 1996; Vazquez-Torres et al., 2000b), protection of SPI2 transcription against NO toxicity represents a novel mechanism by which Hmp may contribute to *Salmonella* virulence (Bang et al., 2006). It should be noted, nonetheless, that SPI2-dependent survival in resting macrophages was not completely abrogated in *hmpA*-deficient *Salmonella*. In the absence of Hmp, the low yields of nitrosating species invoked by innate LPS-TLR4 host signaling are likely to be antagonized by the small thiol-containing molecules homocysteine and glutathione (De Groote et al., 1996; Hausladen et al., 1996). In addition, the vacuolar-remodeling capacity of SPI2 may also lessen NO-mediated toxicity in resting populations of macrophages (Chakravortty et al., 2002). Together, the ability of *Salmonella* to avoid, scavenge and detoxify NO lessen nitrosative stress evoked by the innate expression of iNOS.

Hmp fails, however, to protect SPI2 transcription from the toxicity associated with nitrogen oxides produced by IFNγ-activated macrophages. Repression of SPI2 in view of Hmp-protected *sdhA sdhB* transcription is even more remarkable when one considers that the spermine NONOate IC_{50} values for inhibition of SPI2 expression and respiration are quite similar *in vitro*. These data suggest that IFNγ-primed macrophages generate a variety of nitrogen oxides with distinct biological chemistries. We therefore evaluated the ability of IFNγ-primed macrophages to generate N_2O_3 in response to *hmpA*-proficient and -deficient

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Salmonella. Hmp prevented formation of 300 nM N_2O_3/h in IFNγ-primed macrophages. This activity indicates that at least 60% of the total nitrosative capacity of activated macrophages is directed towards *Salmonella*. Activated macrophages produced 200 nM N_2O_3/h that is not susceptible to detoxification by Hmp, an amount of N_2O_3 that represents nine times that produced by control phagocytes. Studies with the vacuolar inhibitor bafilomycin indicate that at least 50 nM/h are generated in the vacuoles by the condensation of HNO_2. Once N_2O_3 is formed by either the autooxidation of NO or the condensation of HNO_2, the NO-detoxifying activity of Hmp cannot protect *Salmonella* against the cytotoxicity derived from these RNS. N_2O_3 is a potent nitrosative species that could increase the anti-*Salmonella* potential of IFNγ-primed macrophages.

In summary, Hmp detoxifies NO produced by control and IFNγ-primed macrophages, thus effectively preserving the function of NO-sensitive targets in disparate intracellular conditions. However, high NO fluxes sustained by IFNγ-primed macrophages generate nitrogen oxides such as N_2O_3 for which Hmp offers no protection. Further investigations will be needed to determine the extent by which products of the autooxidation of NO abrogate SPI2-transcription, thereby mediating the anti-*Salmonella* activity of IFNγ-primed macrophages.

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Abbreviations

IFNγ
: interferon-γ

iNOS
: inducible nitric oxide synthase

N_2O_3
: dinitrogen trioxide

NO
: nitric oxide

RNS
: reactive nitrogen species

SPI2
: *Salmonella* pathogenicity island 2

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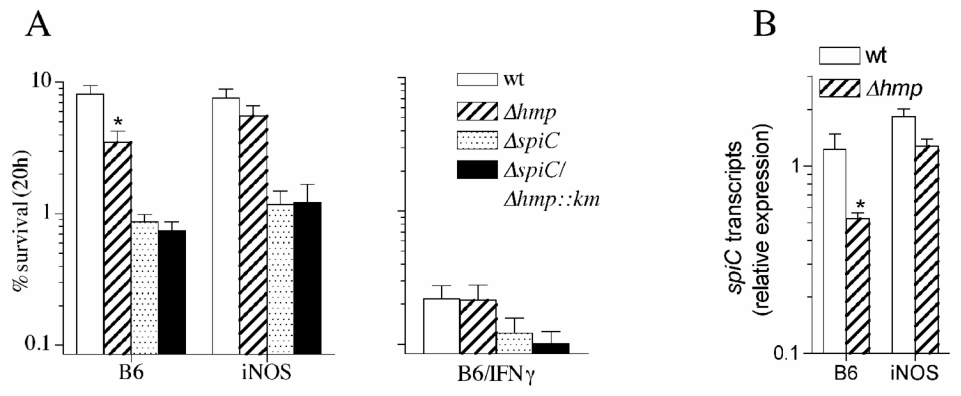

Fig. 1.

The flavohemoprotein Hmp protects SPI2-dependent *Salmonella* survival. (A) The % of wild-type (WT) *Salmonella* and its isogenic *ΔhmpA*-, *ΔspiC*-, and *ΔspiC/ΔhmpA::km*-deficient controls recovered from untreated (left panel) or IFNγ-treated (right panel) macrophages from immunocompetent (B6) or *iNOS*-deficient (iNOS) mice was determined 20 h after infection. (B) *spiC* transcripts were measured by real-time PCR of samples isolated from B6 or iNOS-deficient macrophages 20 h after challenge. *, $p<0.05$ compared to wild-type *Salmonella* controls.

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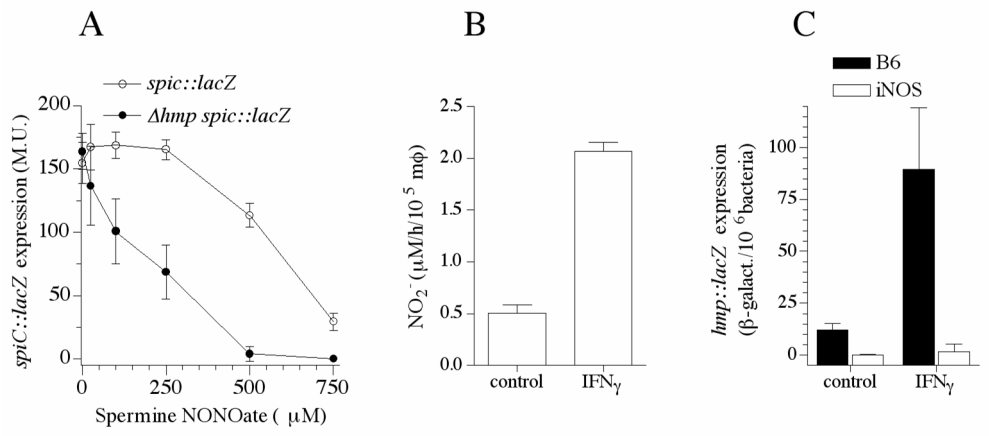

Fig. 2.

Hmp lessens NO-mediated inhibition of SPI2 transcription. (A) Expression of SPI2 was estimated as β-galactosidase activity of a *spiC::lacZ* transcriptional fusion expressed in *hmpA*-proficient or –deficient *Salmonella*. Bacterial samples were collected 2 h after culture in 8 μM $MgCl_2$ N salts medium in the presence of increasing concentrations of the NO donor spermine NONOate. (B) The NO_2^--producing capacity of control and IFNγ-primed macrophages was measured by the Griess reaction 12 h after challenge. (C) Intracellular *hmpA* expression was quantified following expression of the *hmpA::lacZ* transcriptional fusion 20 h after infection.

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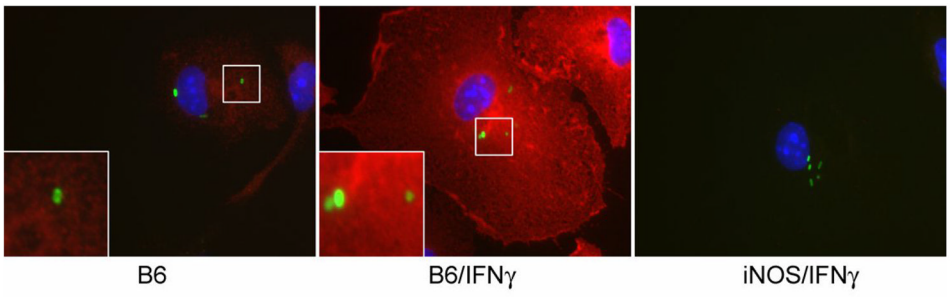

Fig. 3.

IFNγ increases the expression of iNOS but does not stimulate the intracellular redistribution of iNOS. Distribution of iNOS (red) within untreated (B6) or IFNγ-treated (B6/IFNγ) macrophages was visualized 20 h post-infection with *gfp*-expressing *Salmonella* (green). Insets show detailed intracellular distribution of iNOS and *Salmonella*. IFNγ-treated macrophages isolated from iNOS-deficient mice (iNOS/IFNγ) were used as controls. Original magnification of 1000×.

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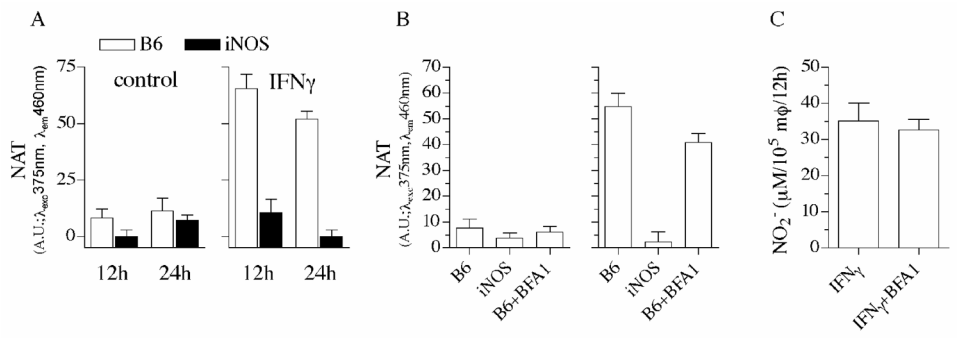

Fig. 4.

Effects of IFNγ treatment on the nitrosative capacity of *Salmonella*-infected macrophages. (A) Nitrosative stress was measured as the N-nitroso (NAT) derivative of diaminonaphthalene by wild-type and iNOS-deficient macrophages infected with *Salmonella* at an MOI of 2. Contribution of acidified NO_2^- to the nitrosative capacity of *Salmonella*-infected macrophages was estimated by studying the effects of the vacuolar ATPase inhibitor bafilomycin A1 (BFA1) on the generation of NAT (B) and NO_2^- (C).

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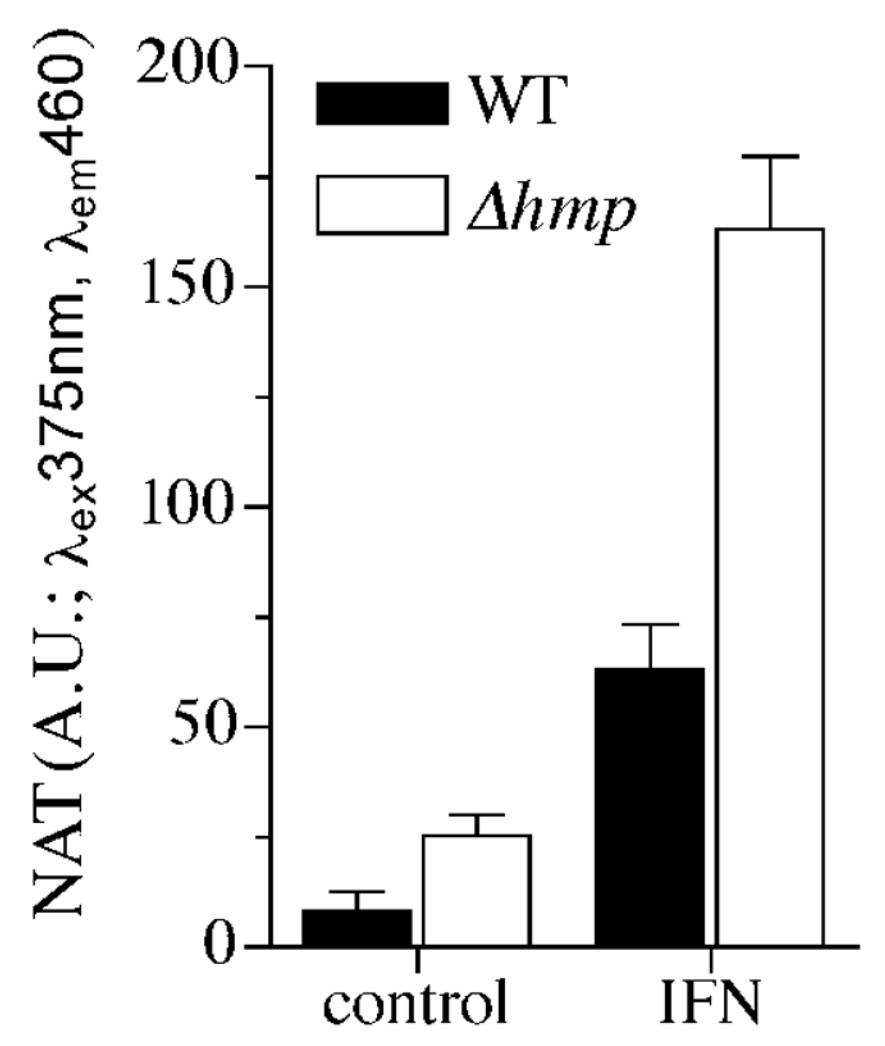

Fig. 5.

Contribution of N_2O_3 to the nitrosative capacity of IFNγ-primed macrophages. The ability of Hmp to diminish N-nitrosation by macrophages from C57BL/6 and gp91*phox*-deficient mice was studied 12 h after infection by following the generation of triazol (NAT) from DAN. Selected groups of macrophages were treated *in vitro* with 200 U/ml IFNγ 12-16 h before infection with wild-type (WT) or *ΔhmpA*-mutant *Salmonella*. To increase sensitivity of the assay, NAT formation was monitored in macrophages cultured in 24 well plates at a density of 5×10^5 cells/well and challenged with *hmpA*-proficient or -deficient *Salmonella* at an MOI of 5. After 12 h of infection, macrophages were incubated for 1 h in MEM^+ medium supplemented with 200 μM DAN and 6 μg/ml gentamycin and the amount of NAT formed measured as above.

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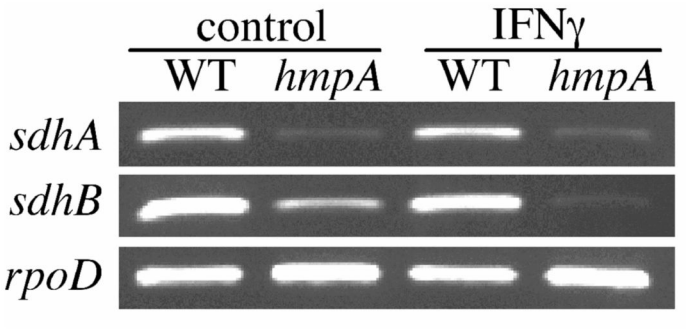

Figure 6A.

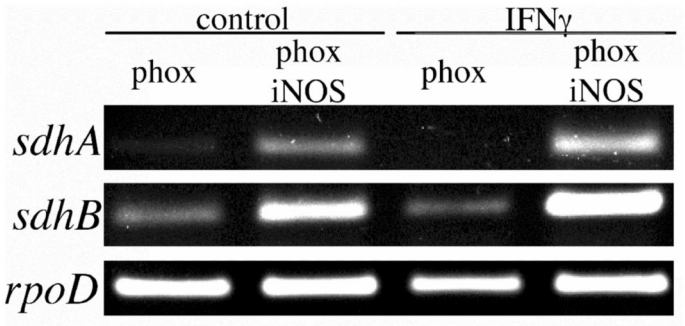

Figure 6B

Fig. 6.

HmpA protects the *Salmonella* electron transport chain during innate and acquired responses of macrophages. (A) Expression of the *sdhA* and *sdhB*-encoding subunits of succinate dehydrogenase was studied by nested PCR in WT and *Δhmp::FRT Salmonella* RNA samples isolated from gp91*phox*-deficient macrophages 12 h after *Salmonella* infection. (B) Expression of *sdhA* and *sdhB Δhmp::FRT Salmonella* in gp91*phox*- or doubly gp91*phox* iNOS-deficient macrophages. Intracellular expression of the *rpoD* housekeeping sigma factor was used as a control.

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Table 1

Bacterial strains and plasmids.

Strains	Genotype	Parent strain	Source
S. Typhimurium 14028s	Wild-type		ATCC
AV0101	pCRGFP	14028s	This study
AV0201	*ΔspiC::FRT*	14028s	(McCollister et al., 2005)
AV0207	φ(*spiC'-lac*$^+$)	AV0201	(McCollister et al., 2005)
AV0305	*Δhmp::lacZ*	AV0468	(McCollister et al., 2005)
AV0468	*Δhmp::FRT*	14028s	(McCollister et al., 2005)
AV0539	φ(*spiC'-lac*$^+$) *Δhmp::FRT*	AV0207/AV0468	This study
AV0615	*ΔspiC::FRT Δhmp::km*	AV0201/AV0468	This study
Plasmid			
PCRGFP	PCR®-Blunt::P_{tac} gfp$^+$		This study

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Table 2

Oligonucleotides used for RT-PCR, real-time RT-PCR and nested PCR.

Gene amplified	Primer/probe sequence[a]
spiC	F:5′-CTTGGATCCGGATTCATGCTGGCAGTTTT R:5′-TGGAAGCTTTCCAGGTCATTTAAGAACAAAGAA Probe: 5′-6-FAM™-CATCCTGCCAGAGGAGAAATTTTCTCA-BHQ™-1
rpoD	F:5′-GTGGCTTGCAATTCCTTGAT real time and nested primer R:5′-AGCATCTGGCGAGAAATACG real time and nested primer F:5′-GCGAACTTGCGTCTGGTTAT long primer R:5′-TTTTATCTTCCGGCATCAGC long primer Probe: 5′-6-FAM™-ATAAGTTCGAATACCGTCGCGGCTACA-BHQ™-1
hmpA	F:5′-CTTGGATCCTTAATGCTATCGCGGCCTAC R:5′-TGGAAGCTTTCAAAGCTGGTGATCAGTGC
sdhA	F:5′-GTTGTGGTGTGGGGTGTGTA long primer R: 5′-GACCCCTTAACGGTGTCGTA long primer F:5′-GATGCTGTTGTGATTGGTGC nested primer R:5′-CTTCATGGGTATTGCCGAGC nested primer
sdhB	F: 5′-AATTGCGGAGACAGGATGAT long primer R: 5′-GGGTAATACAGGCCAGACCA long primer F: 5′-TCGTTATAACCCGGATGTCG nested primer R: 5′-TCAGAACCGCACACACCTTC nested primer
iNOS	F-5′-CTTGGATCCGTGGTGACAAGCACATTTGG R-5′-CAGCAATGGGCAGACTCTGAAGAAAT Probe: 5′-6-FAMTM-CCAGCAATGGGCAGACTCTGAAGAAAT-BHQTM-1
GAPDH	F-5′-AACTTTGGCATTGTGGAAGG R-5′-GGATGCAGGGATGATGTTCT-3′ Probe: 5′-6-FAMTM-ACTGCCACCCAGAAGACTGTGGAT-BHQTM-3′

[a] Dual-labeled oligonucleotide probes contain both the fluorescent dye 6-carboxyfluorescein (6-FAM™) and Black Hole Quencher™ 1 (BHQ™-1).