

Antimicrobial Actions of the NADPH Phagocyte Oxidase and Inducible Nitric Oxide Synthase in Experimental Salmonellosis. I. Effects on Microbial Killing by Activated Peritoneal Macrophages In Vitro

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Abstract

The contribution of the NADPH phagocyte oxidase (phox) and inducible nitric oxide (NO) synthase (iNOS) to the antimicrobial activity of macrophages for *Salmonella typhimurium* was studied by using peritoneal phagocytes from C57BL/6, congenic gp91phox^{-/-}, iNOS^{-/-}, and doubly immunodeficient phox^{-/-}iNOS^{-/-} mice. The respiratory burst and NO radical (NO·) made distinct contributions to the anti-*Salmonella* activity of macrophages. NADPH oxidase-dependent killing is confined to the first few hours after phagocytosis, whereas iNOS contributes to both early and late phases of antibacterial activity. NO-derived species initially synergize with oxyradicals to kill *S. typhimurium*, and subsequently exert prolonged oxidase-independent bacteriostatic effects. Biochemical analyses show that early killing of *Salmonella* by macrophages coincides with an oxidative chemistry characterized by superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and peroxynitrite (ONOO⁻) production. However, immunofluorescence microscopy and killing assays using the scavenger uric acid suggest that peroxynitrite is not responsible for macrophage killing of wild-type *S. typhimurium*. Rapid oxidative bacterial killing is followed by a sustained period of nitrosative chemistry that limits bacterial growth. Interferon γ appears to augment antibacterial activity predominantly by enhancing NO· production, although a small iNOS-independent effect was also observed. These findings demonstrate that macrophages kill *Salmonella* in a dynamic process that changes over time and requires the generation of both reactive oxidative and nitrosative species.

Key words: phagocyte • *Salmonella* • innate immunity • nitrosative • oxidative

Introduction

Salmonella pathogenesis commences in the ileal mucosa with invasion of M cells or ingestion by CD18-expressing phagocytes (1, 2). For the duration of the infection, *Salmonella* can be found principally within mononuclear phagocytes (3), which can serve as a vehicle of extraintestinal dissemination (1) and as a protected site for intracellular bacterial replication (3). The capacity to survive within macrophages is an absolute requirement for *Salmonella* virulence in vivo (4). Macrophages contribute to resistance to

Salmonella by forming granulomas and limiting bacterial growth (5), but the effector mechanisms by which mononuclear phagocytes combat this intracellular pathogen are incompletely understood.

Radicals generated by the NADPH oxidase and inducible nitric oxide (NO)¹ synthase (iNOS) are cytotoxic for a variety of microorganisms as phylogenetically diverse as viruses, bacteria, protozoa, and fungi. The NADPH oxidase expressed by myeloid cells catalyzes the univalent reduction of molecular oxygen to O₂⁻. This radical has only limited

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¹Abbreviations used in this paper: iNOS, inducible nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; RNS, reactive nitrogen species.

membrane diffusibility and modest antibacterial activity, but also serves as a precursor to more toxic reactive oxygen species (ROS [6]). The critical role of the phagocyte oxidase (phox) is reflected by the enhanced susceptibility of patients with chronic granulomatous disease to a wide range of microbial pathogens, including *Salmonella* species (6–8). Immunodeficient gp91phox^{-/-} mice, an animal model for X-linked chronic granulomatous disease (CGD), are accordingly very susceptible to experimental *Salmonella typhimurium* infections (9, 10). The reduced killing capacity of cell lines impaired in their ability to sustain a respiratory burst further attests to the importance of the NADPH oxidase in the anti-*Salmonella* activity of macrophages (11).

NOS isoforms generate NO· in a complex reaction that consumes NADPH, oxygen, and L-arginine (12). NO· by itself poses only weak antimicrobial activity against *Salmonella* (13), but congeners resulting from NO· autooxidation such as NO₂·, N₂O₃, and S-nitrosothiols (14) enhance its cytotoxic potential. In addition, concerted actions of the NADPH oxidase and iNOS can synergize to form highly potent antimicrobial species. For example, NO· reacts at a rate of 6.7×10^9 M/s with O₂⁻ to form ONOO⁻ (15, 16), an oxidant capable of damaging lipids, proteins, and DNA; ONOO⁻ has been associated with enhanced killing of *S. typhimurium*, *Escherichia coli*, and *Candida albicans* (13, 17, 18). NO· can also act in concert with H₂O₂ to kill *E. coli* in vitro by a mechanism that appears to be at least partially iron dependent (19).

The contribution of NO· is of particular significance in resistance to intracellular pathogens. Development of a Th1 immune response dominated by IFN-γ, IL-2, and IL-12 synthesis in combination with NO·-mediated effector functions has been correlated with resistance to *Leishmania* and mycobacterial infections (20, 21). Similarly, IFN-γ, IL-12, and TNF-α production is associated with resistance to *S. typhimurium* (22, 23). However, the contribution of NO· in innate immunity to *Salmonella* is still a matter of active debate. Evidence obtained from several laboratories using several nitrogen oxide donors such as S-nitrosoglutathione, acidified NO₂⁻, and ONOO⁻ has unequivocally established that *S. typhimurium* is susceptible to reactive nitrogen species (RNS) in vitro (13, 24–26). Furthermore, *S. typhimurium* deficient in DNA repair systems (e.g., *umuC* and *recBC*), small thiol molecules (e.g., homocysteine and glutathione), or detoxifying enzymes (e.g., flavohemoprotein or copper zinc superoxide dismutase) is hypersusceptible to NO· congeners in vitro, and shows reduced macrophage resistance and virulence in vivo (13, 25, 27–29). Yet, despite this compelling evidence favoring distinct NO· actions against *Salmonella*, some studies have failed to demonstrate that NO· plays a role in macrophage inhibition or killing of wild-type *S. typhimurium* (9, 24, 30, 31).

In this work, we employed macrophages from C57BL/6 mice and their congenic *iNOS*^{-/-}, gp91phox^{-/-}, and doubly immunodeficient *iNOS*^{-/-}gp91phox^{-/-} derivatives to elucidate the contributions of the NADPH phagocyte oxidase and iNOS to antibacterial actions of macrophages for wild-type *S. typhimurium*.

Materials and Methods

Bacterial Strains. Wild-type *S. typhimurium* strains American Type Culture Collection 14028s (1) and M525P (10, 32) were used for this study. For immunofluorescence microscopy, *rpmM::gfp* was moved by P22-mediated transduction from strain SMO22 (1) into *S. typhimurium* 14028s to yield *S. typhimurium* AF991 (*gfp*⁺).

Mice. Wild-type C57BL/6 mice were purchased from The Jackson Laboratory. Congenic *iNOS*^{-/-} (33), gp91phox^{-/-} (34), and doubly immunodeficient *iNOS*^{-/-}gp91phox^{-/-} (9) mice were bred in our animal facility according to Institutional Animal Care and Use Committee guidelines. The *iNOS*^{-/-} mice were the progeny (N₃–N₅) of mice backcrossed onto a C57BL/6 background for 10 generations (gift of C. Nathan, Cornell University, New York, NY). The gp91phox^{-/-} and *iNOS*^{-/-}gp91phox^{-/-} mice were progeny (N₄–N₆) of mice described previously (9, 34). gp91phox^{-/-} and *iNOS*^{-/-}gp91phox^{-/-} mice were maintained on drinking water containing 15 mg/ml itraconazole, 0.2 mg/ml trimethoprim, and 40 mg/ml sulfamethoxazole up to 4 d before experimentation to prevent spontaneous infections.

Macrophages. Peritoneal macrophages from C57BL/6 and congenic *iNOS*^{-/-} (33), gp91phox^{-/-} (34) and doubly immunodeficient *iNOS*^{-/-}gp91phox^{-/-} (9) mice were harvested 4 d after intraperitoneal inoculation of 1 mg/ml sodium periodate as described (25). The peritoneal exudate cells were resuspended in RPMI 1640 supplemented with 10% heat-inactivated FCS (Gemini Bioproducts), 1 mM sodium pyruvate, 10 mM Hepes, and 2 mM L-glutamine (all reagents from Sigma-Aldrich). The macrophages were selected by adherence in a 96-well plate and cultured for 48 h at 37°C in a 5%–CO₂ incubator. Unless otherwise indicated, adherent macrophages were treated in vitro overnight with 20 U/ml of IFN-γ (Life Technologies) from a 10⁵ U/ml stock containing 0.8 ng/ml LPS.

Macrophage Killing Assays. Periodate-elicited macrophages were challenged with *S. typhimurium* opsonized with 10% normal mouse serum at a 10:1 multiplicity of infection, allowed to internalize the bacteria for 15 min, and washed with prewarmed medium containing 6 μg/ml gentamicin (25). At several time points after infection, the macrophages were lysed with 0.5% sodium deoxycholate, and surviving bacteria were enumerated on Luria-Bertani agar plates. The results are expressed as percentage survival.

Chemiluminescence. Macrophage chemiluminescence was estimated by the reduction of 25 μM lucigenin (bis-*N*-methylacridinium) and the oxidation of 100 μM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma-Aldrich) with a Lumistar chemiluminometer (BMG Lab Technologies) in Microlite flat-bottomed microtiter plates (Dinex Technologies, Inc.). The macrophages were challenged with wild-type *S. typhimurium* American Type Culture Collection 14028s as described in the macrophage killing assays above. Extracellular bacteria were removed by washing and the addition of 6 μg/ml gentamicin. At specified time intervals, medium was harvested and replaced by fresh medium for performance of the assays. Lucigenin and luminol were used as indicators of O₂⁻ and ONOO⁻ production (35, 36), respectively. 1 mM uric acid (37, 38) inhibited ~80% of luminol-dependent chemiluminescence generated by IFN-γ-activated periodate-elicited macrophages 1 h after bacterial challenge, whereas 1,000 U/ml catalase (gift of Dr. S. Libby, North Carolina State University, Raleigh, NC) inhibited only 20%. These observations, along with the dependence of chemiluminescence on both the NADPH oxidase and iNOS, indicate that the majority of this luminol chemiluminescence is mediated by ONOO⁻, whereas a

minor proportion reflects H₂O₂. Periodate-elicited macrophages from wild-type, *iNOS*^{-/-}, and *phox*^{-/-} mice were compared.

Superoxide Anion Determination. O₂^{·-} was quantified by the superoxide dismutase-inhibitable reduction of ferricytochrome c (39). At different time points after infection with *Salmonella*, medium was removed from cultured IFN-γ-treated 48 h-aged periodate-elicited macrophages and replaced with fresh medium containing 60 μM ferricytochrome c in phenol red-free Earle's balanced salt solution. After 1 h incubation in 5% CO₂ at 37°C, the OD of the supernatants was determined spectrophotometrically at 550 nm. The concentration of O₂^{·-} was calculated by using an ε₅₅₀ of 2.1 × 10³ M⁻¹ cm⁻¹. All reagents were purchased from Sigma-Aldrich.

Hydrogen Peroxide Determination. H₂O₂ was measured by the horseradish peroxidase-dependent oxidation of phenol red (39). The macrophages were challenged with *Salmonella* as described above, and extracellular bacteria were removed by washing and the addition of gentamicin before incubation in Earle's balanced salt solution containing 0.56 mM phenol red and 20 U/ml horseradish peroxidase. At indicated time intervals, medium was harvested and replaced with fresh medium. After 1 h incubation at 37°C in a 5%-CO₂ atmosphere, the absorbance of the supernatants was read at 600 nm after mixing with 10 μl of 1 N NaOH per well. H₂O₂ was quantitated by comparison with a standard curve prepared with known concentrations of H₂O₂. All reagents were purchased from Sigma-Aldrich.

NO_x Determination. NO synthesis by periodate-elicited macrophages challenged with *Salmonella* as described above was estimated by measuring the accumulation of nitrite (NO₂⁻) and nitrate (NO₃⁻), stable metabolites of the reaction of NO with oxygen, using the Griess reaction. The NO₂⁻ present in supernatants of *Salmonella*-infected macrophages was measured spectrophotometrically at 550 nm after mixing with an equal volume of Griess reagent (0.5% sulfanilamide and 0.05% *N*-1-naphthylethylenediamide hydrochloride in 2.5% acetic acid). The NO₂⁻ concentration was determined from a standard curve prepared with NaNO₂. The NO₃⁻ accumulated in the supernatants was estimated by the Griess reaction after enzymatic reduction of NO₃⁻ to NO₂⁻ (40, 41) in pH 7 sodium phosphate buffer containing 1.6 U/ml nitrate reductase, 16 U/ml glucose dehydrogenase, 10 μM NADPH, and 10 mM glucose-6-phosphate. NO₃⁻ concentration was calculated as the difference between NO₂⁻ accumulated in the presence and absence of nitrate reductase. At designated time points, the NO_x concentrations were determined from *S. typhimurium*-challenged macrophages cultured for 1 h in serum-free IMDM (Sigma-Aldrich) supplemented with 7.5% minimal essential amino acid solution (Life Technologies), 1% minimal nonessential amino acid solution, 1.1 mM sodium pyruvate (Life Technologies), 1 mg/ml streptomycin sulfate (Sigma-Aldrich), 0.5 mg/ml gentamicin (Sigma-Aldrich), 0.6 mg/ml penicillin G (Life Technologies), 0.75% wt/vol dextrose (Sigma-Aldrich), 0.85% wt/vol NaHCO₃ (Sigma-Aldrich), and 1% nutridoma-SP (Boehringer). Low background levels of nitrate (~1 μM) detected in parallel wells were subtracted to exclude a contribution by residual levels of nitrate present in the culture medium.

Immunocytochemistry. Macrophages plated onto sterile coverslips were incubated for 48 h at 37°C in a 5%-CO₂ atmosphere. The macrophages were stimulated with IFN-γ during the last 20 h and challenged with *S. typhimurium* strain 14028s as described above. After 90 min of infection, the coverslips were washed with PBS, and the cells were fixed with 2% paraformaldehyde in PBS for 20 min. After extensive washing with 0.1% Tween in

PBS, the macrophages were incubated with a 3% normal goat serum solution in Tris balanced solution, pH 7.3. The cells were stained with 5 μg/ml of a rabbit antinitrotyrosine polyclonal antibody (42) or a rabbit antidinitrophenol (Zymed Laboratories) polyclonal control antibody for 1 h, followed by a rhodamine-conjugated goat anti-rabbit polyclonal antibody (Jackson ImmunoResearch Laboratories) for 1 h. After washing, the coverslips were mounted with Vectashield® (Vector Laboratories) and examined with an Olympus IX70 inverted microscope, a Photometrics PXL camera with Kodak KAF1400 chip (6.7 × 6.7 μm physical pixels giving 67 nm per image pixel with a ×100 oil immersion objective), and a Silicon Graphic O₂ computer with DeltaVision deconvolution software (Applied Precision).

Results

NADPH Oxidase- and *iNOS*-derived Chemical Species Contribute to the Antimicrobial Activity of Macrophages for *Salmonella*. Macrophages from *iNOS*^{-/-} or *gp91phox*^{-/-} mice exerted less antimicrobial activity to *S. typhimurium* strain 14028s than macrophages from congenic wild-type control animals (Fig. 1). Macrophages lacking the NADPH oxidase were less effective in their ability to contain *Salmonella* than congenic cells lacking *iNOS*, suggesting that O₂^{·-} or its derivatives play a greater role than NO· congeners in *Salmonella* killing (Fig. 1). The addition of IFN-γ enhanced bactericidal activity of wild-type macrophages (Fig. 1), and this was correlated with enhanced NO· production (Fig. 2). IFN-γ did not increase O₂^{·-} production by wild-type macrophages as measured by reduction of cytochrome c (Fig. 2), and only very modestly increased the antimicrobial activity of *iNOS*^{-/-} macrophages (Fig. 1). Similar results were obtained for wild-type *S. typhimurium* American Type Culture Collection strain 14028s and wild-type strain M525P (data not shown).

Temporal Differences in *iNOS*- and NADPH Oxidase-mediated Macrophage Cytotoxicity. To study the relative con-

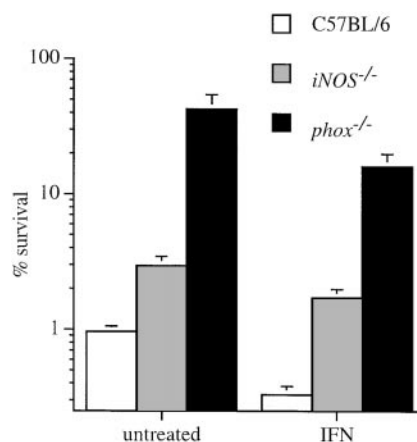


Figure 1. Both ROS and RNS contribute to the antimicrobial activity of macrophages for *Salmonella*. Intracellular bacterial counts of untreated and IFN-γ-activated periodate-elicited macrophages from C57BL/6, *iNOS*^{-/-}, and *gp91phox*^{-/-} mice were compared. Viable intracellular bacteria were quantified by plating 20 h after the macrophages were challenged with *S. typhimurium* 14028s. The data are the mean ± SEM of 12–18 independent observations obtained on at least four separate days.

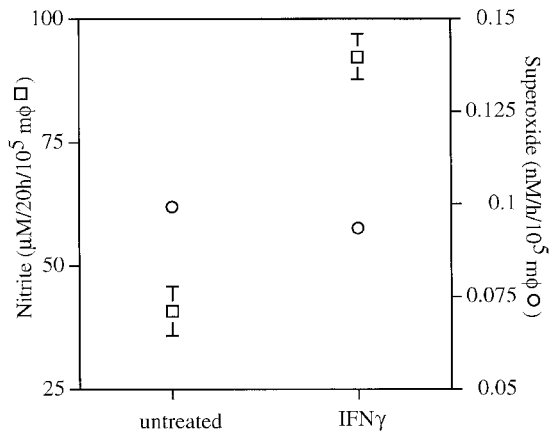


Figure 2. Nitrite and superoxide production by untreated and IFN- γ -activated macrophages. NO₂⁻ (□) accumulated by macrophages (mφ) from C57BL/6 mice in response to *S. typhimurium* 14028s was determined by the Griess reaction at 20 h after challenge, and O₂⁻ (○) production was measured by the reduction of cytochrome c over a 1-h interval 20 h after challenge. The data are the mean \pm SEM of six independent observations obtained on at least two separate days.

tribution of iNOS and the NADPH oxidase to phagocyte-mediated killing of *S. typhimurium* in more detail, the cytotoxicity of IFN- γ -activated macrophages from wild-type, *iNOS*^{-/-}, and *gp91phox*^{-/-} mice was studied over a period of 14 h (Fig. 3). Macrophages from wild-type C57BL/6 mice exhibited pronounced bactericidal activity towards *Salmonella* during the first 6 h after challenge. In fact, these macrophages eliminated >99% of the original inoculum during the first 6 h of infection. At later time points, macrophages exhibited cytostatic behavior, confining the bacterial burden to a steady level. Although the NADPH oxidase contributed more than iNOS to overall macrophage antimicrobial activity, substantial temporal differences were observed in the contribution of these systems. Macrophages deficient in the NADPH oxidase did not reduce the original inoculum, but were still able to maintain the bacterial burden at a steady level over time (Fig. 3 A). In contrast, macrophages deficient in iNOS considerably reduced the initial inoculum but were unable to control bacterial replication at later time points (Fig. 3 B). The contribution of iNOS to macrophage antibacterial activity was detectable as early as 2 h after the initial challenge ($P < 0.05$), but became more substantial over time.

In accord with these observations, macrophages deficient in both the NADPH oxidase and iNOS did not reduce the inoculum at early time points nor achieve subsequent control of bacterial replication (Fig. 3 C). A comparison of macrophages from *phox*^{-/-} and *iNOS*^{-/-}*gp91phox*^{-/-} mice revealed an NO \cdot -dependent antimicrobial activity at later time points that is independent of the NADPH oxidase.

Production of ROS and RNS in Response to Salmonella. Production of ROS and RNS by IFN- γ -activated macrophages from wild-type, *iNOS*^{-/-}, and *gp91phox*^{-/-} mice was determined over a 10-h period by chemiluminescence using lucigenin and luminol, and by spectro-

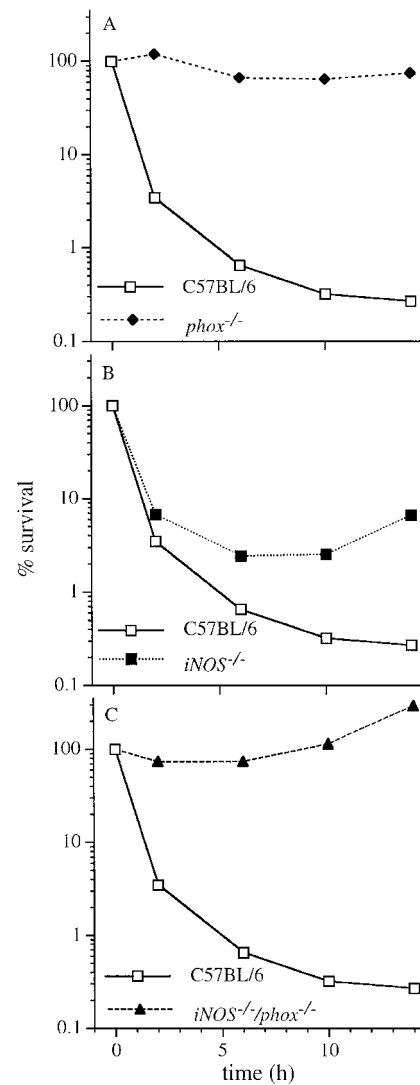


Figure 3. The contribution of iNOS and the NADPH phagocyte oxidase to the antimicrobial activity of macrophages for *S. typhimurium* varies over time. The killing activity of IFN- γ -activated, periodate-elicited macrophages from (A–C) C57BL/6, (A) *phox*^{-/-}, (B) *iNOS*^{-/-}, and (C) *iNOS*^{-/-}*gp91phox*^{-/-} mice was recorded over a 1-h period after challenge with wild-type *S. typhimurium* strain 14028s. The data are the mean \pm SEM of 3–11 independent observations obtained on at least two separate days.

tometry using the Griess reagent (Fig. 4). Production of ROS by macrophages from wild-type mice was initiated immediately after phagocytosis, decreasing to undetectable levels by 6 h thereafter (Fig. 4 A). Lucigenin-dependent chemiluminescence was three times lower than that mediated by luminol (Fig. 4, A and B), suggesting that a substantial proportion of the O₂⁻ formed by the NADPH oxidase reacts with NO \cdot to form ONOO⁻. As anticipated, macrophages from *phox*^{-/-} mice exhibited neither lucigenin- nor luminol-dependent chemiluminescence. Macrophages from *iNOS*^{-/-} mice showed a considerable diminution in

luminol-dependent chemiluminescence. However, these macrophages generated a prolonged lucigenin-dependent chemiluminescence that persisted for the duration of the experiment.

The rate of NO_x production by macrophages from wild-type mice infected with *S. typhimurium* increased over time (Fig. 4 C). In agreement with previous observations (9), macrophages from *gp91phox*^{-/-} mice consistently produced more NO_x than wild-type controls, likely reflecting

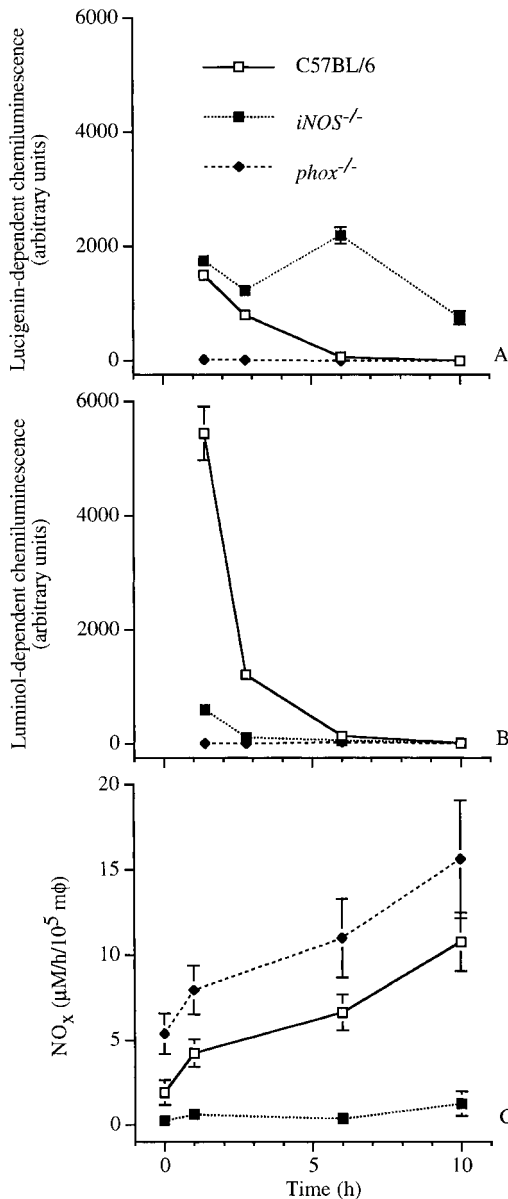


Figure 4. The lucigenin- and luminol-dependent chemiluminescence and NO_x production of *Salmonella*-infected macrophages vary over time. The capacity of IFN- γ -activated macrophages (m ϕ) to produce ROS and RNS was measured at selected 1-h intervals over a 10-h period from independent wells. The respiratory burst and NO_x production were measured as (A) lucigenin- and (B) luminol-dependent chemiluminescence, and (C) by the Griess reaction, respectively. The data are the mean \pm SEM of 3–11 independent observations obtained on at least four separate days.

a lack of $\text{NO}\cdot$ scavenging by $\text{O}_2\cdot^-$. Macrophages from *iNOS*^{-/-} mice produced very low residual quantities of NO_x , possibly reflecting constitutive NOS activity.

The relative abundance of ROS and RNS produced by macrophages during the first hour after challenge with *S. typhimurium* strain 14028s was characterized further (Fig. 5). Of the ROS measured, H_2O_2 was the most abundant, accounting for $\sim 0.15 \pm 0.01$ nmol/h/ 10^5 macrophages, closely followed by $\text{O}_2\cdot^-$ (0.10 ± 0.01 nmol/h/ 10^5 macrophages; Fig. 5, A and B). Macrophages from *iNOS*^{-/-} mice produced more $\text{O}_2\cdot^-$ and H_2O_2 in response to *Salmonella* infection than those from wild-type control animals ($P < 0.05$). Macrophages from *iNOS*^{-/-} mice appeared to produce very low levels of NO_x that almost exclusively consisted of NO_3^- (Fig. 5, C and E), suggesting that a significant proportion of the $\text{NO}\cdot$ attributed to constitutive NOS might react with $\text{O}_2\cdot^-$ to form ONOO^- (Fig. 4 B). Macrophages from *gp91phox*^{-/-} mice did not produce detectable quantities of $\text{O}_2\cdot^-$, H_2O_2 , or chemiluminescence.

Even at the earliest time point, ~ 0.4 nmol/h/ 10^5 macrophages of NO_x was produced (Fig. 5 C). The majority of the $\text{NO}\cdot$ was oxidized to NO_3^- (~ 0.3 nmol/h/ 10^5 macrophages), although a third was metabolized to NO_2^- (Fig. 5, D and E). After 10 h of infection, the macrophages increased their production of both NO_3^- and NO_2^- . NO_3^- production greatly exceeded that of NO_2^- in wild-type, *iNOS*^{-/-}, and *gp91phox*^{-/-} macrophages. $\text{NO}\cdot$ synthesized by constitutive NOS appear to have a negligible contribution to macrophage antimicrobial activity, as the NOS inhibitor N^G-monomethyl L-arginine did not reduce the inhibition of *Salmonella* by macrophages from *iNOS*^{-/-} mice (data not shown).

Nitrotyrosine Formation in *Salmonella*-infected Macrophages. To determine whether ONOO^- produced by macrophages targets intracellular *Salmonella*, formation of nitrotyrosine, a product that can be formed from the reaction of ONOO^- with tyrosine residues, was investigated by immunofluorescence microscopy (Fig. 6). In agreement with the biochemical data (Fig. 4), nitrotyrosine was present in wild-type macrophages infected with wild-type *S. typhimurium*, but absent from NADPH oxidase-deficient control macrophages (Fig. 6, A and C). The presence of nitrotyrosine was markedly reduced, but not totally absent, in macrophages from *iNOS*^{-/-} mice (Fig. 6 B). Nitrotyrosine labeling failed to colocalize with green fluorescent protein (GFP)-tagged *Salmonella* in any instance (Fig. 6, A and B), suggesting that ONOO^- is formed but may not contribute to bacterial killing. In further support of this notion, the scavenger uric acid did not diminish, but rather enhanced by twofold the bactericidal activity of wild-type macrophages (Fig. 7), suggesting that formation of ONOO^- is actually detrimental to macrophage anti-*Salmonella* activity.

Discussion

Macrophages can kill or limit the replication of intracellular bacteria by producing antimicrobial peptides, lyso-

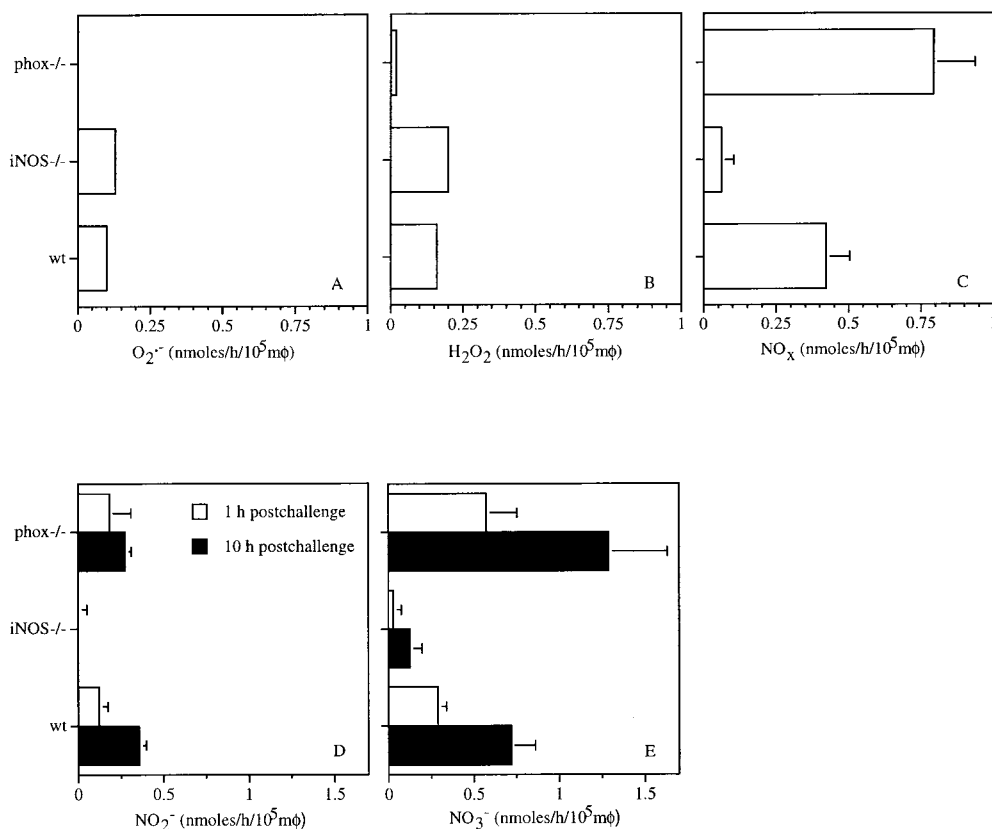


Figure 5. Macrophage production of ROS and RNS in response to *Salmonella* challenge is largely dependent on the NADPH oxidase and iNOS. (A) O₂^{·-}, (B) H₂O₂, and (C) NO_x production were measured by reduction of cytochrome c, horseradish peroxidase-dependent oxidation of phenol red, and the Griess reaction, respectively. A–C represent metabolite production during the first hour after challenge. D and E represent NO₂⁻ and NO₃⁻ accumulated over a 1-h interval at 1 and 10 h after challenge. The data are the mean ± SEM of 3–10 independent observations obtained on at least two separate days. mφ, macrophages.

mal enzymes, ROS, and RNS. The importance of ROS for macrophage killing of *S. typhimurium* has been demonstrated (9, 11, 30); however, the participation of RNS has been less clear (9, 24, 30, 31). In this work, we demonstrate that although the NADPH oxidase is more essential than iNOS for *Salmonella* killing by peritoneal macrophages, iNOS nevertheless contributes to macrophage antibacterial activity against *Salmonella* in distinct and important ways. The NADPH oxidase is required for rapid initial *Salmonella* killing by macrophages (Fig. 3 A), and iNOS provides a subsequent sustained bacteriostatic effect (Fig. 3 B). The failure of previous investigators to demonstrate macrophage NO₂⁻-dependent anti-*Salmonella* activity may be attributable to a reliance on phagocyte killing assays of relatively brief duration (9, 24, 30). Effector functions of macrophage-derived nitrogen oxides may explain why iNOS is required for resistance to *Salmonella* infection (10, 43), despite the apparent immunosuppressive actions of NO[·] on T cells (43, 44).

NO[·]-dependent anti-*Salmonella* activity was demonstrable in both untreated and IFN- γ -treated macrophages, suggesting that bacterial products such as LPS and DNA (45) can trigger sufficient NO[·] synthesis to exert antimicrobial activity, at least in partially activated peritoneal cells elicited by sodium periodate. Nevertheless, the addition of IFN- γ enhances macrophage anti-*Salmonella* activity predominantly by increasing NO[·] production (Figs. 1 and 2).

Although ROS and RNS can exert synergistic antimicrobial actions (14, 17–19, 25), they principally act in sequential fashion in assays of macrophages infected with *Sal-*

monella. An early phase of rapid oxidative killing occurs during the peak respiratory burst, followed by a nitrosative bacteriostatic phase (Figs. 3 and 4). The functional separation of these two phases of antimicrobial activity is illustrated by the capacity of macrophages deficient in the NADPH oxidase to maintain their bacterial load at a steady level despite an inability to reduce the original inoculum. In contrast, congenic iNOS-deficient macrophages retained early bacterial killing but were unable to maintain control of subsequent bacterial replication over time. These temporal differences in the antimicrobial behavior of macrophages were paralleled by the early detection of oxidative products followed by a later rise in nitrogen oxides (Fig. 4). The respiratory burst of infected macrophages peaked shortly after phagocytosis, decreasing rapidly thereafter. The brief duration of the respiratory burst may result in part from direct inhibition of NADPH oxidase assembly by NO[·] congeners (46–49), as macrophages from iNOS^{-/-} mice sustained a more prolonged respiratory burst. Additionally, the increasing abundance of nitrogen oxides over time may be quenching the oxidative chemistry of O₂^{·-} and ONOO⁻ (50, 51).

The sequential, functionally distinct, and essential roles of ROS and RNS in *Salmonella* killing or inhibition by macrophages contrast with earlier studies of *Leishmania*, in which ROS did not appear to play a role (52). Studies of *Listeria* killing by macrophages have yielded somewhat conflicting results, with various investigators reporting that antilisterial activity is RNS independent (9, 53), ROS de-

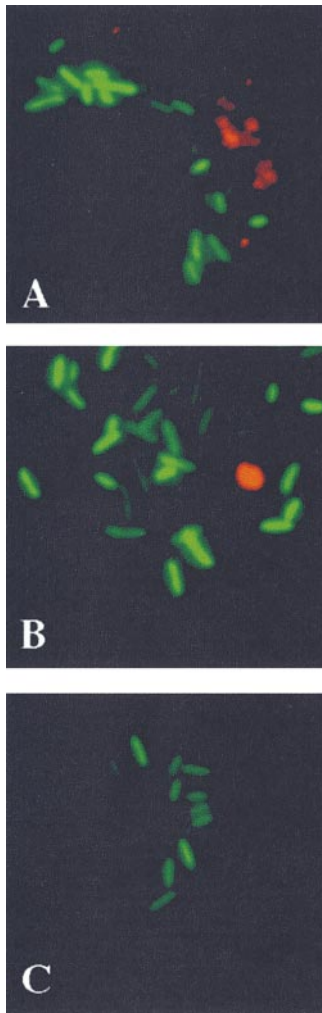


Figure 6. Nitrotyrosine staining fails to colocalize with bacteria in *Salmonella*-containing macrophages. The presence of nitrotyrosine (red) was examined by immunofluorescence microscopy of IFN- γ -activated macrophages from (A) C57BL/6, (B) *iNOS*^{-/-}, and (C) *gp91phox*^{-/-} mice that were challenged in vitro with GFP-expressing *S. typhimurium* (green). The pictures are representative of data obtained on two separate days.

pendent (9), or either RNS or ROS dependent depending on the timing of activation (54).

S. typhimurium killing by macrophages appears to be completely dependent upon the NADPH oxidase. H₂O₂ is likely to make a major contribution to bacterial killing, as it diffuses rapidly through membranes and reacts with transition metals to form highly toxic hydroxyl radicals. O₂^{•-} has also been implicated by observations that *sodC* mutant bacteria deficient in periplasmic Cu,Zn-superoxide dismutase are hypersusceptible to macrophage killing and exhibit reduced virulence (25, 55, 56).

NO[•] makes a relatively minor contribution to early macrophage oxidative killing (Fig. 3). ONOO⁻, a reactive molecule capable of mediating 1- and 2-electron oxidations (16) with potent in vitro microbicidal activity for *E. coli*, *S. typhimurium*, and *C. albicans* (17, 18, 25), might account for this activity. However, intracellular bacteria fail to colocalize with nitrotyrosine (Fig. 6), a molecular signature that can be associated with ONOO⁻ synthesis (15, 42), suggesting that ONOO⁻ may not be responsible for early bactericidal effects. The scavenger uric acid actually potentiates *Salmonella* killing (Fig. 7), further indicating that ONOO⁻ production by *Salmonella*-containing macrophages may be responsible

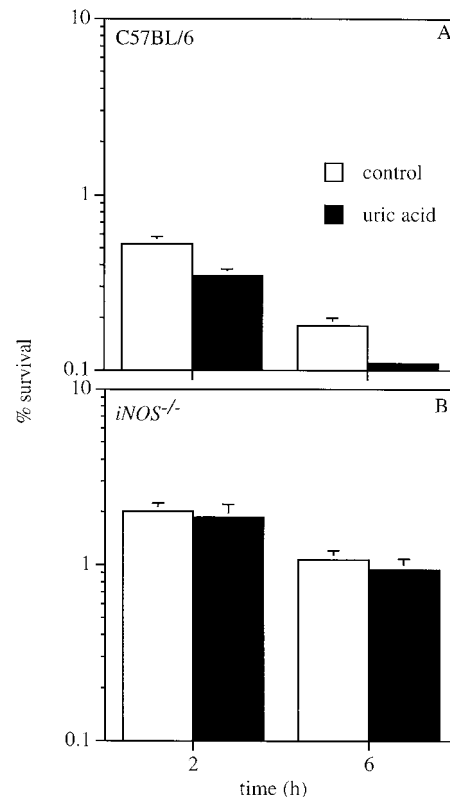


Figure 7. Uric acid improves killing of *Salmonella* by macrophages. 1 mmol uric acid enhances killing of *Salmonella* by (A) IFN- γ -activated macrophages, but this enhancement is iNOS dependent (B). The data are the mean \pm SEM of six independent observations obtained on two separate days.

for host cell autotoxicity rather than antimicrobial activity. Synergistic actions of metal ions, NO[•] redox congeners, and H₂O₂, or the production of singlet oxygen from the reaction of NO[•] and H₂O₂, are alternative mechanisms by which NO[•]-derived species may potentiate NADPH oxidase-dependent macrophage killing (19, 57, 58).

At this time, the identity of the nitrogen oxides responsible for the sustained NO[•]-dependent inhibition of bacterial growth are not known, but possibilities include NO[•] itself, N₂O₃, and *S*-nitrosothiols, which have been shown to exert bacteriostatic activity for *S. typhimurium* in vitro (13, 14). The predominant accumulation of NO₃⁻ rather than NO₂⁻ at late time points in the macrophage assay when ONOO⁻ is no longer detectable suggests that dioxygenase activity (59) might be involved in NO[•] oxidation.

Of the three NOS isoforms that can catalyze the enzymatic production of NO[•], iNOS is most closely associated with antimicrobial activity. The contribution of cNOS to the antibacterial activity of macrophages in this study was negligible. The small quantity of NO[•] attributed to cNOS was oxidized to NO₃⁻ (~0.12 nmol/h/10⁵ macrophages). A small amount of nitrotyrosine staining and NO_x production detectable in iNOS-deficient macrophages infected with *Salmonella* suggests that NO[•] derived from cNOS can react with O₂^{•-}, as has been demonstrated in murine *Myco-*

plasma pulmonis infection (60). These results might indicate that murine mononuclear phagocytes are able to express both inducible and constitutive NOS, as has been suggested by others (61, 62), although functional cNOS has not yet been definitively demonstrated in primary murine peritoneal macrophages.

In conclusion, both the NADPH phagocyte oxidase and iNOS contribute to the ability of macrophages to inhibit or kill *S. typhimurium*. Analysis of murine peritoneal macrophages reveals a temporally coordinated action of ROS and RNS. Rapid bacterial killing coinciding with production of $O_2^{\cdot-}$ by the NADPH phagocyte oxidase is followed by a prolonged period of inhibition of bacterial growth associated with the production of iNOS-derived nitrosative species. The sequential roles of oxidative and nitrosative phagocyte antimicrobial mediators in vitro are mirrored by the temporal relationship of early NADPH oxidase-dependent and late iNOS-dependent antimicrobial effector mechanisms observed during murine *Salmonella* infection in vivo (10).

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