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Signaling mechanisms of NO-mediated host defense are yet to be elucidated. In this study, we report a unique signal pathway for cytoprotection during Salmonella infection that involves heme oxygenase 1 (HO-1) induced by a nitrated cyclic nucleotide, 8-nitroguanosine 3',5'-cyclic monophosphate (8-nitro-cGMP). Wild-type C57BL/6 mice and C57BL/6 mice lacking inducible NO synthase (iNOS) were infected with Salmonella enterica serovar Typhimurium LT2. HO-1 was markedly up-regulated during the infection, the level being significantly higher in wild-type mice than in iNOS-deficient mice. HO-1 up-regulation was associated with 8-nitro-cGMP formation detected immunohistochemically in Salmonella-infected mouse liver and peritoneal macrophages. Our results suggest that HO-1 induction by 8-nitro-cGMP formation contributes, via its potent cytoprotective function, to host defense during murine salmonellosis.

effects (38, 39), and inhibition of T cell proliferation (40). In fact, HO-1 protects against cell and tissue damage induced by LPS both in vitro and in vivo (24, 28). We have also demonstrated that, in several solid tumor models, expression of HO-1 in tumor cells that was up-regulated by NO could contribute to tumor growth, possibly because of potent cytoprotective activity (26, 27, 41, 42). Although host cell apoptosis induced by various pathogens and microbial toxins is critical in the pathogenesis of many infectious diseases, no study has yet confirmed the antiapoptotic and host defense roles of HO-1 during bacterial infections in vivo.

The host defense effects of NO have been reported for many microbiological infections including leishmaniasis (5), tuberculosis (43, 44), salmonellosis (2, 44–49), and listeriosis (50). Murine salmonellosis has been studied extensively so as to investigate host defense mechanisms and immune responses occurring during typhoid fever: the infection induces excessive production of NO as a host defense response (2, 44–49). In earlier studies, we found much greater bacterial growth and apoptotic changes in iNOS-deficient (iNOS−/−) mice than in wild-type mice (iNOS+/+) during Salmonella infection (2, 45, 46, 49). However, the mechanism of NO-mediated cytoprotection that occurred during Salmonella infection remained unclear.

In the present experiments, we sought to clarify NO-dependent cytoprotective and antimicrobial host defense, with a particular focus on the signaling mechanism of HO-1 induction as a cytoprotective response for Salmonella enterica serovar Typhimurium (S. typhimurium) infection in vivo and in vitro. We also investigated formation of 8-nitro-cGMP in Salmonella-infected mouse liver cells and macrophages in culture and its possible signaling role in the cytoprotective host defense mediated by HO-1 induction.

### Materials and Methods

#### Animals

Littermates of wild-type C57BL/6 mice and C57BL/6-NOS2−/− (iNOS−/−) mice were used throughout the study, conducted according to the guidelines in the Laboratory Protocol of Animal Handling, Kumamoto University Graduate School of Medical Sciences.

#### Bacterial infection in mice

The bacteria S. typhimurium LT2 were used throughout the study. Bacteria were grown in still culture of brain-heart infusion broth (Difco Laboratories) at 37°C under 5% CO2. Peritoneal macrophages were harvested in DMEM containing 10% heat-inactivated FCS and rabbit polyclonal anti-cytochrome 

#### Treatment of Salmonella-infected mice with HO-1 inhibitor

To inhibit HO-1 activity, mice were treated with polyethylene glycol-conjugated zinc-protoporphyrin (PEG-ZnP) at 100 mg/kg. PEG-ZnP, a water-soluble derivative of HO-1 inhibitor ZnP-P, was synthesized according to our method reported previously (51). Mice were given 0.1 ml of 1 mM PEG-ZnP at 12 h before infection and 1 h after gentamicin treatment. At 6, 12, and 24 h after infection, mice were washed twice with PBS and lysed with 0.1% Triton X-100. Serially diluted cell lysates were subjected to the colony formation assay for quantification of intracellular bacteria. To obtain bacterial count in siRNA-transfected cells, cells were collected in DMEM after washing twice with PBS and counted the number of cells followed by centrifugation and lysis with 0.1% Triton X-100 for colony assay. Bacterial count was adjusted with the number of cells because HO-1 siRNA-transfected cells tended to washout from the plate compared with control siRNA.

#### Immunohistochemistry and immunocytochemistry

Livers and tissues were fixed and cryostat tissue sections were prepared as described earlier (20, 45). Immunostaining was achieved by using biotin-conjugated monoclonal mouse Abs for 8-nitro-cGMP (clones 1G6 and H77) and by using rabbit polyclonal anti-HO-1 IgG (Stressgen Biotechnologies), as reported in a recent publication (17, 20). For immunofluorescence staining for 8-nitro-cGMP, Abs were labeled with Alexa Fluor 555 (Molecular Probes-Invitrogen). Images were obtained by means of an Olympus DP70 digital camera and software.

#### Apoptosis assay

Apoptosis in mouse liver tissues infected with S. typhimurium was analyzed by use of the TUNEL assay with an in situ apoptosis detection kit (TACS; Trevigen), as described previously (45, 46). For in vitro TUNEL analysis, RAW 264 cells and peritoneal macrophages were cultured at densities of 2 × 105 and 5 × 106 cells/well of 4-well chamber slides, respectively. At 12 h after infection, cells were washed twice with PBS and fixed with 3.7% buffered formaldehyde for 15 min at room temperature.

#### Western blot analysis

Western blotting was performed according to our standard procedure (20), unless otherwise specified. The tissue homogenate and cell lysate protein samples were separated via SDS-PAGE (12%), followed by electroblotting to polyvinylidene fluoride membranes. Membranes were subjected to immunoblotting with rabbit polyclonal anti-HO-1 Ab (Stressgen Biotechnologies). Caspase 3 and cytochrome c were detected by means of rabbit polyclonal anti-cleaved caspase 3 (ASP175; Cell Signaling Technology) and rabbit polyclonal anti-cytochrome c (H-104; Santa Cruz Biotechnology), respectively.

#### Measurement of CO in blood

We estimated CO production of RBC by measuring hemoglobin-associated CO in blood from Salmonella-infected mice. Specifically, 350 μl of freshly collected mouse blood was diluted with 3.65 ml of PBS in a 10-ml test tube

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sealed with an airtight rubber cap. To remove residual oxygen gas from the solution in the tube, nitrogen gas was forced into the solution for 2 min via a needle inserted through the rubber cap. As the source of NO, 40 μl of 2.5 mM 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-methyl-1-propanamine (NOC7; Dojindo Laboratories) was added to the solution, and the sample was incubated for 2 h at room temperature under anaerobic conditions. Because NO has greater affinity for heme iron than does CO, exogenously added NO effectively replaces hemoglobin-bound CO, and CO purged from heme accumulates in the gaseous phase in the tube. After NO treatment, each aliquot in the tube was injected into a gas chromatography-based biogas analyzer (Taiyo Instruments) to measure the CO content. In fact, using CO-saturated hemoglobin, we confirmed that almost 100% of CO bound with hemoglobin was recovered in the gaseous phase.

Quantification of HO-1 mRNA expression

Expression of HO-1 mRNA was quantified by real-time RT-PCR analysis. Total RNA was isolated by use of TRIzol reagent (Invitrogen). cDNA was synthesized using 2.5 μg of RNA in a reaction mixture of 20 μl containing 1.0 μl of 10 U/μl RNase inhibitor, 1.0 μl of 3 μg/μl random primers, and 1.0 μl of 200 U/μl Moloney murine leukemia virus reverse transcriptase. Real-time RT-PCR was performed by using an Applied Biosystems Prism 7700 Real-Time PCR System with TaqMan Universal PCR master mix and Assays-on-Demand gene expression probes (Applied Biosystems). The amount of mRNA was quantified by means of the standard curve method. A standard sample was serially diluted and used for constructing this standard curve. Simultaneous quantification of 18S rRNA, with TaqMan RO-dent 18S rRNA Control Reagents VIC (Applied Biosystems) was used as an endogenous control to normalize the differences in reverse transcription efficiencies and amount of template in the reaction mixture.

Measurement of ROS

The intracellular ROS level was assessed with 2′,7′-dichlorodihydrofluorescein diacetate, diacetoxymethylester (H2-DCFDA; Molecular Probes) as described earlier (42). After macrophages were incubated with H2-DCFDA, 2′,7′-dichlorodihydrofluorescein fluorescence intensity was measured via a flow cytometer (BD Biosciences).

Statistical analysis

All data are presented as means ± SEM or SD. Statistical significance between two groups was determined by means of a two-tailed unpaired Student’s t test. A value of p < 0.05 was considered statistically significant.

Results

HO-1 expression induced by NO during Salmonella infection in vivo

We first examined whether HO-1 is up-regulated in murine salmonellosis by infecting wild-type mice with S. typhimurium LT2. At days 1–5 after infection, mouse livers were collected for analysis of HO-1 expression. In vivo HO-1 activity, as evidenced by CO produced in the blood, increased in a time-dependent fashion (Fig. 1A), in parallel with growth of Salmonella organisms in the liver (bacterial yield (log10 CFU/g liver); 4.1 ± 0.7 (day 1), 5.1 ± 0.3 (day 3), 6.1 ± 0.2 (day 5)). HO-1 expression in mouse liver induced by S. typhimurium infection was further confirmed via
Western blot analysis for HO-1 (Fig. 1A, inset) and immunostaining for HO-1 protein (data not shown). Induction of HO-1 activity in the liver was also confirmed by measuring the production of biliverdin spectrophotometrically as reported earlier (27) (data not shown).

We then investigated the effect of NO on HO-1 induction in livers of infected mice. Both wild-type and iNOS−/− mice were infected with S. typhimurium LT2 at a dose of either 1 × 10^4 CFU/mouse (low dose) or 4 × 10^5 CFU/mouse (high dose). On day 5 after infection, the livers from iNOS−/− mice had significantly higher bacterial growth than did livers from wild-type mice, regardless of the S. typhimurium dose (Fig. 1B). In contrast, HO-1 activity, as evidenced by CO production (Fig. 1C), HO-1 protein (Fig. 1D), and HO-1 mRNA (Fig. 1E) were higher in wild-type mice than in iNOS−/− mice. Even under the conditions where similar bacterial yields were detected (wild-type mice at high dose vs iNOS−/− mice at low dose in Fig. 1B), a significantly higher HO-1 activity (Fig. 1C) and expression (Fig. 1, D and E) were determined for wild-type mice than for iNOS−/− mice. These findings suggest that NO derived from iNOS plays an important role in HO-1 induction during Salmonella infection.

Cells expressing HO-1 were identified by double immunostaining with Abs for HO-1 and macrophages or neutrophils. Most macrophages were positive for HO-1, whereas some but not all neutrophils were HO-1 positive (data not shown). Microscopic observation revealed that hepatocytes did not have strong staining for HO-1, although a weak immunoreaction was observed in Salmonella-infected liver compared with control liver.

**HO-1 expression induced by NO in Salmonella-infected macrophages in culture**

NO-dependent HO-1 induction in macrophages was investigated in more detail by means of an in vitro study using peritoneal macrophages from both wild-type and iNOS−/− mice. These peritoneal macrophages were infected with S. typhimurium at a MOI of 10, and 24 h after infection HO-1 expression was determined by Western blotting and real-time RT-PCR. Consistent with in vivo results, macrophages from wild-type mice had significantly higher HO-1 protein and mRNA levels than macrophages from iNOS−/− mice during Salmonella infection (Fig. 2, A and B). Treatment of S. typhimurium-infected wild-type macrophages with a NO inhibitor, L-NMMA or L-NIL, resulted in a significant reduction in HO-1 expression (Fig. 2C).

NO-mediated induction of HO-1 in Salmonella-infected macrophages was also examined by using exogenous NO donors. Uninfected iNOS−/− peritoneal macrophages were incubated with different concentrations of the NO donors SNAP, S-nitroso-glutathione (GSNO), and NOC7. HO-1 induction was assessed via Western blot and immunocytochemistry at 12 h after treatment with these NO donors. HO-1 was markedly induced by SNAP (Fig. 2D) and by other NO donors such as GSNO and NOC7 (data not shown) in a dose-dependent manner. Similarly, when iNOS−/− peritoneal macrophages infected with Salmonella were treated with 200 μM NOC7, GSNO, or SNAP, the HO-1 level was higher in cells treated with the NO donors than in untreated cells (data not shown).

Upon bacterial infection, it is known that phagocytic oxidase (Phox; NADPH oxidase, Nox) is activated to generate ROS (47, 48, 52, 53). We measured ROS generation during Salmonella infection by using oxidant-sensitive probe (H2-DCFDA). This analysis showed that similar levels of ROS were generated in wild-type and iNOS−/− macrophages early in the infection (i.e., at 6 h; data not shown), although the amount of ROS tended to be higher in iNOS−/− macrophages than in wild-type macrophages at a later time (at 24 h; data not shown). In addition, similar to the in vivo results (Fig. 1B), the number of bacteria grown intracellularly was significantly higher for iNOS−/− macrophages than for wild-type cells (data not shown).

These results strongly support the interpretation mentioned earlier of NO-dependent HO-1 induction during Salmonella infection.

**Formation of 8-nitro-cGMP and its signaling functions during marine salmonellosis**

We recently reported formation of 8-nitro-cGMP in immunostimulated mouse macrophages in culture (20). We also determined that
8-nitro-cGMP reacts with the sulfhydryl group of proteins and forms protein-cysteine-cGMP adducts by a process that we called S-guanylation (20). S-Guanylation of proteins by 8-nitro-cGMP is a new concept in cell biology and may have a significant role in unique posttranslational modification of proteins. More important, in this earlier work we reported that transcription of phase 2 antioxidant enzymes including HO-1 involved 8-nitro-cGMP-dependent S-guanylation of Kelch-like ECH-associated protein 1 (Keap1), a cysteine-rich redox-sensitive regulatory protein involved in sequestration of transcription factor NF-E2-related factor 2 (Nrf2).

To study mechanisms how NO can mediate HO-1 expression during Salmonella infection, we focused on 8-nitro-cGMP formation. For immunohistochemical detection of 8-nitro-cGMP, we produced two different clones of mAbs, 1G6 and 1H7. Clone 1G6 detected 8-nitro-cGMP and 8-nitroguanosine, whereas 1H7 was specific for 8-nitro-cGMP. Wild-type and iNOS−/− mice were infected with S. typhimurium at a dose of 4 × 10⁸ CFU/mouse. Liver sections from uninfected wild-type mice (control; day 0) and Salmonella-infected wild-type and iNOS−/− mice were fixed and processed for immunostaining with 1G6 Ab. Peritoneal macrophages from wild-type and iNOS−/− mice were seeded at a density of 5 × 10⁵ cells/well in 4-well chamber slides and were infected with S. typhimurium at 10 MOI. At 24 h after infection, cells were fixed and stained with either 1G6 or 1H7 Ab. Uninfected macrophages from wild-type mice served as control.

FIGURE 3. HO-1 induction and formation of 8-nitro-cGMP in S. typhimurium-infected mouse liver and peritoneal macrophages. A, Wild-type and iNOS−/− mice were infected with S. typhimurium at a dose of 4 × 10⁸ CFU/mouse. Liver sections from uninfected wild-type mice (control; day 0) and Salmonella-infected wild-type and iNOS−/− mice at day 5 were fixed and processed for immunostaining with 1G6 Ab. B, Peritoneal macrophages from wild-type and iNOS−/− mice were infected with S. typhimurium at 10 MOI. At 24 h after infection, cells were fixed and stained with 1G6 Ab. Uninfected macrophages from wild-type mice served as control.

8-Nitro-cGMP as a second messenger in NO-mediated HO-1 induction

We then investigated the possible signaling function of 8-nitro-cGMP in HO-1 induction during Salmonella infection. The HO-1 induction potential of 8-nitro-cGMP was examined by adding chemically synthesized 8-nitro-cGMP to uninfected iNOS−/− macrophage cultures. Marked, dose-dependent induction of HO-1 was seen in iNOS−/− macrophages and was highest between 12 and 18 h (Fig. 4, A and B). We then treated Salmonella-infected iNOS−/− macrophages with 8-nitro-cGMP and measured HO-1 induction at 24 h after infection via Western blot analysis. Fig. 4C shows that Salmonella-infected iNOS−/− macrophages had reduced expression of HO-1 compared with that of wild-type-infected macrophages, but the decreased HO-1 expression was almost restored by 30–100 μM 8-nitro-cGMP treatment. This observation clearly demonstrated that 8-nitro-cGMP, which forms in wild-type macrophages during Salmonella infection, has a signaling function as a second messenger of NO in HO-1 induction.

Impaired host defense against Salmonella caused by HO-1 antidotes

To address whether HO-1 induced by NO possesses host defense functions during S. typhimurium infection, we tested the effect of HO-1 inhibition on bacterial growth in wild-type mice by treating infected mice with PEG-ZnPP at a dose that was equivalent to 2.5 mg of ZnPP IX/kg of body weight. We used three bacterial doses (1 × 10⁷, 2 × 10⁷, and 4 × 10⁷ CFU/mouse). PEG-ZnPP treatment started after infection of bacteria and continued until day 3 after infection. At day 5 after infection, we determined blood CO levels for PEG-ZnPP-treated groups compared with controls, at all three bacterial doses (Fig. 5A), the difference being significant (p < 0.05) for the two higher bacterial
doses. The effect of inhibited HO-1 activity was associated with significantly enhanced bacterial yield (greater number of bacteria) in PEG-ZnPP-treated mice compared with controls at all bacterial doses (Fig. 5B; \( p < 0.05 \)).

A similar effect of PEG-ZnPP treatment on bacterial growth was observed in vitro. Fig. 6, A and B, show that at each time point after infection, 6, 12, and 24 h, the number of bacteria in PEG-ZnPP-treated RAW 264 cells and peritoneal macrophages was significantly higher than that in controls (no PEG-ZnPP treatment; \( p < 0.01 \)).

We also evaluated intracellular bacterial growth in PEG-ZnPP-treated and -untreated peritoneal macrophages in culture via microscopic methods. *S. typhimurium* organisms were immunostained with FITC-conjugated anti-*Salmonella* Ab at 24 h after infection. PEG-ZnPP-treated macrophages showed greater fluorescence intensity compared with untreated cells (Fig. 6C). Thus,

**FIGURE 5.** Effects of HO inhibition by PEG-ZnPP on growth of bacteria in mouse liver. A, Wild-type mice were infected with *S. typhimurium* at different doses and were treated or not treated with PEG-ZnPP. CO production in blood was measured at day 5 after infection. Data are means ± SEM (\( n = 5 \)) of measurements from three independent experiments. The inset shows the PEG-ZnPP structure. *, \( p < 0.05 \) vs PEG-ZnPP-treated groups (unpaired Student’s \( t \) test). B, Bacterial growth in livers from PEG-ZnPP-treated and control mice was measured via the colony formation assay at day 5 after infection. Data are means ± SEM (\( n = 5–7 \)). *, \( p < 0.05 \) vs controls (unpaired Student’s \( t \) test).

**FIGURE 6.** Effects of HO-1 inhibition by PEG-ZnPP on growth of bacteria in cultured macrophages. RAW 264 cells (A) and mouse peritoneal macrophages (B) were seeded at densities of \( 2 \times 10^5 \) cells/well and \( 5 \times 10^5 \) cells/well, respectively, in 24-well plates and were infected with *S. typhimurium* at 10 MOI. Cells were treated or not with 20 \( \mu \)M PEG-ZnPP to inhibit HO-1. Bacterial growth at 6, 12, and 24 h after infection was measured by means of the colony formation assay. Data are means ± SD of triplicate wells. ***, \( p < 0.01 \) vs controls (unpaired Student’s \( t \) test). C, Immunostaining for *Salmonella* in PEG-ZnPP-treated and -untreated peritoneal macrophages. Peritoneal macrophages were infected with *S. typhimurium* at 10 MOI and were treated with 20 \( \mu \)M PEG-ZnPP at 12 h before infection and 1 h after gentamicin treatment. Intracellular growth of *Salmonella* at 24 h was visualized by immunostaining with anti-*Salmonella*-FITC Ab.
inhibition of HO-1 activity enhanced susceptibility to *Salmonella* infection, which suggests the potential involvement of HO-1 in defense of macrophages against *Salmonella*.

Furthermore, we examined, via TUNEL analysis, apoptotic cell death occurring in liver tissues from PEG-ZnPP-treated and -untreated mice. Liver samples were collected at day 5 after infection with *S. typhimurium* at a dose of $2 \times 10^4$ CFU/mouse. Cell death was detected in PEG-ZnPP-treated mouse livers than in untreated mouse livers ($p < 0.05$). Most TUNEL-positive cells were confined to the area of microabscesses, which were composed of infiltrated macrophages, neutrophils, and degenerated hepatocytes. This finding indicates that inhibition of HO-1 activity by PEG-ZnPP treatment during *S. typhimurium* infection may accelerate tissue injury and cell death, which in turn would facilitate bacterial growth and histopathological changes in infected livers.

We evaluated the effects of PEG-ZnPP treatment on apoptosis in vitro by in situ TUNEL analysis with *Salmonella*-infected (10 MOI) RAW 264 cells and macrophages at 12 h after infection. Both RAW 264 cells (Fig. 7, C and D) and peritoneal macrophages (Fig. 7, E and F) had significantly higher levels of apoptosis after PEG-ZnPP treatment compared with untreated controls. As a control compound for PEG-ZnPP, we used PEG-PP, which lacks the zinc ion and thus HO inhibitory activity. PEG-PP had no significant effect on apoptosis induction and intracellular growth of *Salmonella* in RAW 264 cells (data not shown).

Results obtained by pharmacological inhibition of HO-1 were also confirmed by an alternative approach involving the use of siRNA for HO-1. Peritoneal macrophages from wild-type mice were treated with either HO-1 siRNA or control siRNA for 48 h, after which cells were infected with *Salmonella* at 10 MOI. Bacterial growth was measured at 6 and 18 h after infection, and apoptosis was measured by TUNEL analysis at 15 and 24 h after infection. Transfection of macrophages with HO-1 siRNA caused a significantly reduced expression of HO-1 protein as detected by Western blotting (Fig. 8 A). HO-1 siRNA-treated cells had a significantly higher expression of HO-1 as compared with control siRNA-treated cells. These results therefore support the findings obtained with PEG-ZnPP and suggest that HO-1 is protective in *S. typhimurium* infection.

All of these findings together suggest that HO-1 plays a critical role in host defense during murine salmonellosis.

**Cytoprotective effect of 8-nitro-cGMP**

Because 8-nitro-cGMP is involved in HO-1 induction and HO-1 has a cytoprotective function during *Salmonella* infection, we investigated whether 8-nitro-cGMP has a direct cytoprotective effect. iNOS$^{-/-}$ macrophages were treated with 30 mM 8-nitro-cGMP at 6 h after *Salmonella* infection. As shown in Fig. 9 A, higher apoptosis was observed in iNOS$^{-/-}$ macrophages compared with wild-type macrophages, but the difference was nullified when iNOS$^{-/-}$ cells were treated with 8-nitro-cGMP. No apparent

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*FIGURE 7.* Induction of apoptosis after PEG-ZnPP treatment of *Salmonella*-infected mouse livers and cultured macrophages. A, Wild-type mice were infected with *S. typhimurium* at a dose of $2 \times 10^4$ CFU/mouse and treated or not with PEG-ZnPP. The TUNEL assay of liver tissues collected at day 5 after infection provided a measure of apoptosis. Morphometric analysis of TUNEL-positive cells was performed with five fields in two different sections from each mouse (three mice per group). Data are means ± SD of measurements from two different experiments. *, $p < 0.05$ vs no PEG-ZnPP (control; unpaired Student’s t test). B, Representative TUNEL results for data shown in A for PEG-ZnPP-treated and -untreated mouse livers. Dark blue identifies TUNEL-positive cells. RAW 264 cells (C) seeded at a density of $2 \times 10^5$ cells/well and thioglycolate-elicited peritoneal macrophages from wild-type mice (E) seeded at a density of $5 \times 10^5$ cells/well were infected with *S. typhimurium* at 10 MOI. Cells were treated or not with 20 μM PEG-ZnPP. Apoptosis was determined via the TUNEL assay at 12 h after infection. Data are means ± SD of 25 different microscopic fields from two independent experiments. **, $p < 0.01$ vs no PEG-ZnPP (control; unpaired Student’s t test). D and F, Representative TUNEL results for data shown in C and E for PEG-ZnPP-treated and -untreated cells.
cytoprotection was observed for 8-bromoguanosine 3',5'-cyclic monophosphate (8-bromo-cGMP), a widely used cGMP analog, which indicates that the cytoprotective effect of 8-nitro-cGMP occurred via a pathway independent of classical cGMP signal transduction. The apoptotic index was also measured by means of caspase 3 activation at 24 h after infection and was detected by Western blot analysis of active caspase 3 in Salmonella-infected macrophages. Consistent with TUNEL analysis results, higher caspase 3 activation was found in iNOS−/− macrophages compared with wild-type cells, and this activation was suppressed by 8-nitro-cGMP treatment (Fig. 9B). Release of cytochrome c was used to investigate the upstream pathway of the caspase cascade. iNOS−/− macrophages had showed greater mitochondrial release of cytochrome c in cytosol compared with wild-type macrophages (Fig. 9B). This higher cytochrome c release was partially blocked by 8-nitro-cGMP treatment (Fig. 9B). HO-1 may have a direct or indirect role in this 8-nitro-cGMP-mediated inhibition of caspase 3 activation, because greater activation of caspase 3 occurred after inhibition of HO-1 by PEG-ZnPP (data not shown).

These findings further confirmed the hypothesis that 8-nitro-cGMP has a signaling function in NO-mediated host defense via induction of HO-1.

Discussion
Although NO-dependent host defense and cytoprotection have been documented in a series of studies of S. typhimurium infection in mice (2, 45–49), biochemical and molecular mechanisms of NO-mediated host defense, particularly cytoprotection, have not been clearly addressed. Our study here clarified that NO-dependent...
HO-1 induction is mediated, at least in part, by 8-nitro-cGMP and contribute to the host defense potential for Salmonella infection.

NO has diverse physiological and pathological functions during infection and inflammation. Its two opposite biological effects, i.e., cytotoxicity and cytoprotection, depend on the chemical reactivities of NO and RNS. For example, NO reacts with \( \text{O}_2^- \) and produces RNS such as \( \text{ONOO}^- \) and \( \text{NO}_2^- \). These species interact with biological molecules, including thiols, lipids, proteins, and nucleic acids via nitration reactions and thereby form nitrated derivatives (54, 55).

Our previous studies indicated that nitration of proteins and tyrosine occurs in a manner dependent on NO produced from iNOS during murine salmonellosis (2, 45, 49). More important, we recently observed that excessive production of NO mediates guanine nitration in influenza virus-infected mouse lung (10). Nitration of guanine and/or guanosine in an environment in which NO is produced was also recently verified in other inflammatory lung diseases in humans (17). We reported in our recent study that, among several derivatives of 8-nitroguanine, 8-nitro-cGMP was the major product in biological systems and possessed certain unique features such as electrophilic, redox active, and cell signaling potentials (20). Moreover, 8-nitro-cGMP reacted readily with cysteine sulfhydryl groups of proteins to form cysteine-cGMP adducts, this reaction being called S-guanylation (20). In that study, we demonstrated S-guanylation of Keap1, a cysteine-rich protein involving sequestration of transcription factor Nrf2 in cytosol of macrophages stimulated with LPS plus IFN-\( \gamma \) or with Salmonella infection. The reaction of 8-nitro-cGMP with Keap1 presumably dissociates Nrf2 from the Keap1-Nrf2 complex, which leads to its nuclear translocation and the subsequent transcriptional activation of antioxidant factors including HO-1.

We therefore hypothesized that 8-nitro-cGMP-mediated S-guanylation of Keap1 may produce a functional modification of the Keap1 protein. We also postulated that S-guanylation of Keap1 may induce transcription of HO-1 through an Nrf2 pathway as a major mechanism of NO-mediated HO-1 induction. It was thus of great importance to see whether 8-nitro-cGMP indeed formed during Salmonella infection and participated in HO-1 induction. In this study, we clearly demonstrated that 8-nitro-cGMP was formed in the pathophysiological condition of salmonellosis and that 8-nitro-cGMP possessed a strong potential for HO-1 induction. This study provides critical information in research exploring the formation and functional role of a newly identified nitrated cyclic nucleotide, 8-nitro-cGMP, in a pathophysiological context.

Under inflammatory conditions, electrophilic substances formed endogenously and potentially involved in the Keap1/Nrf2 pathway, include 15-deoxy-\( \Delta^{12,14} \)-PGJ\(_2\) (15d-PGJ\(_2\)), a product of cyclooxygenase, and 4-hydroxy-2-nonenal (4-HNE). Keap1 cysteine modification by 15d-PGJ\(_2\) was suggested to occur by using biotinylated derivatives of 15d-PGJ\(_2\) added exogenously to cells (56). However, it is still unclear whether endogenous 15d-PGJ\(_2\) can indeed modify Keap1. It was recently reported that intracellular concentrations of 15d-PGJ\(_2\) formed endogenously is as low as 1 nM (57). Furthermore, cysteine adducts of 15d-PGJ\(_2\) are so unstable to readily dissociate spontaneously under physiological conditions (58). These factors may hamper detection of Keap1-PGJ\(_2\) adducts. Further study is needed to clarify the relative contribution of the iNOS-dependent 8-nitro-cGMP pathway and cyclooxygenase-dependent 15d-PGJ\(_2\) pathway for activation of Keap1/Nrf2 signaling by developing a sensitive method to detect unstable Keap1-PGJ\(_2\) adducts. 4-HNE is formed during the process of lipid peroxidation, and hence, is associated with oxidative stress (59). We observed that oxidant formation in macrophages during Salmonella infection was higher in iNOS-deficient cells than in wild-type cells (data not shown). This suggests that oxidative stress-associated production of 4-HNE may not be a major pathway to induce Keap1/Nrf2-dependent HO-1 induction in our murine salmonellosis model.

Earlier studies documented HO-1 induction by NO in various cultured cells (22–24), including macrophages treated with LPS (25), and in in vivo solid tumor models (26, 27), rat models of sepsis produced by LPS treatment (60, 61), and a mouse ischemia-reperfusion model (62). All of these data are thus consistent with our current investigations of murine salmonellosis. In contrast, however, Barreiro et al. (63) did not find NO-dependent HO-1 induction in diaphragm muscle of rats treated with LPS. They suggested that oxidative stress induced by LPS in muscle may be the major contributor to HO-1 induction. These discrepant results may be due to the different tissues and animal models used. In any event, macrophage-specific induction of HO-1 up-regulated via NO production seems to have some significance in host defense.

We used two approaches to investigate the exact function of HO-1 induced by NO and 8-nitro-cGMP in our Salmonella infection models. One was enzyme inhibition by ZnPP IX, which is commonly used to examine the effect of HO-1 expression in experimental models. However, a potential problem with using ZnPP IX in such studies is its insolubility in water. To overcome this problem, we developed a water-soluble ZnPP IX derivative by conjugating it with PEG (Ref. 51; the inset in Fig. 5A shows its structure). The resultant PEG-ZnPP is highly water soluble, can be administered in vivo by the i.v. route, and is as effective as ZnPP IX in inhibiting HO-1 (41, 42, 51). Also, we found PEG-ZnPP to be more effective than ZnPP in targeted inhibition of HO-1 in solid tumors because it demonstrated an increased retention time in tissues, which was a result of its being a macromolecule (41, 42). In addition, increased PEG-ZnPP accumulated in liver and spleen without affecting the physiological functions of these organs (41, 42). In fact, in our present study, PEG-ZnPP treatment effectively inhibited HO-1 activity, in that PEG-ZnPP suppressed the blood CO level in \( S. \) typhimurium-infected mice (Fig. 5A). The other approach that we used was HO-1 gene knockdown by siRNA. As Fig. 8 illustrates, the trends of increased apoptosis and greater bacterial growth after inhibition of HO-1 by siRNA support the finding of a host defense function of HO-1 as shown by studies using PEG-ZnPP just mentioned.

Catalytic products of HO-1 reaction have been known to possess cytoprotective and antiapoptotic activities. Bilirubin is formed as a result of intracellular reduction of biliverdin by biliverdin reductase (32, 33). Bilirubin thus formed is known as a potent antioxidant (32, 33). ROS and RNS produced by phagocytes after stimulation with particular pathogens play a role in elimination of phagocytosed bacteria, but also are cytotoxic to the phagocytes themselves, particularly at high concentrations. Bilirubin may protect phagocytes from ROS/RNS-induced oxidative cellular damage. In addition, another HO product, CO possesses cytoprotective and antiapoptotic activities. Accordingly, up-regulation of HO-1 protects macrophages from apoptosis induced by Salmonella infection, thereby facilitating the killing of Salmonella organisms. In fact, our preliminary study performed in a separate experiment indicates that HO-1 induced by 8-nitro-cGMP formed endogenously in the cells could exert potent antioxidant and cytoprotective functions in cells in culture (our unpublished observation). On the other hand, Nobre et al. (64) recently demonstrated that exogenous CO can inhibit growth of pathogenic bacteria \( S. \) enterica and \( S. \) aureus (64). Thus, cytoprotection by HO-1 may be mediated by suppression of bacterial growth as an
alternative possibility. The contribution of HO-1-derived CO on the antimicrobial effect should be clarified in the future study.

We observed in this study that HO-1 was induced in iNOS−/− mice/macrophages during Salmonella infection, although the level of HO-1 was much lower in iNOS−/− cells than in wild-type cells. It has been reported that HO-1 can be induced in response to various stimuli, including ROS (29) and bacterial component LPS (24, 25). We found that Salmonella infection induced ROS generation in both wild-type and iNOS−/− macrophages; the level was slightly higher in iNOS−/− macrophages (data not shown). Therefore, it can be assumed that ROS and LPS may contribute to the induction of HO-1 in iNOS−/− cells during Salmonella infection.

One of the reasons for lethality in murine salmonellosis is septic shock (2, 45). The pathogenesis of sepsis is characterized by pathological events caused by uncontrolled production of proinflammatory cytokines (such as TNF-α and IL-1β) and macrophage inflammatory proteins, which would lead to leukocyte recruitment, capillary leaking, and tissue damage, and ultimately lethality (39, 65). Recent studies have suggested that HO-1 inhibits production of proinflammatory cytokines (66). We therefore measured the TNF-α level in S. typhimurium-infected mouse serum and culture supernatant of peritoneal macrophages with or without PEG-ZnPP treatment. We found a markedly higher TNF-α level in PEG-ZnPP-treated mouse serum than in untreated mouse serum after S. typhimurium infection (data not shown). We obtained a similar result in an in vitro study of cultured infected peritoneal macrophages (data not shown). HO-1 may thus have an anti-inflammatory activity during S. typhimurium infection by inhibiting proinflammatory cytokines such as TNF-α.

In summary, this study described a unique mechanism of NO-mediated host defense in which NO causes formation of a novel signaling molecule, 8-nitro-cGMP, which possesses several important biological functions including induction of HO-1. This study also demonstrated that HO-1 induced by 8-nitro-cGMP plays an important role in innate immunity by protecting cells from apoptosis and possibly by promoting phagocytic bacterial killing by macrophages. The up-regulation of HO-1 induced by NO and involvement of HO-1 in host defense may aid understanding of the molecular mechanisms of NO-mediated host defense (67). It would be extremely interesting to explore other target proteins for S-glycation by 8-nitro-cGMP during NO-mediated host defense. Additional studies of host defense activities mediated by 8-nitro-cGMP and HO-1 in other microbial infections are thus warranted.

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Disclosures
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References