NO-mediated cytoprotection: Instant adaptation to oxidative stress in bacteria

Ivan Gusarov and Evgeny Nudler*

Department of Biochemistry, New York University Medical Center, New York, NY 10016

Edited by Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved July 29, 2005 (received for review May 25, 2005)

Numerous sophisticated systems have been described that protect bacteria from increased levels of reactive oxygen species. Although indispensable during prolonged oxidative stress, these response systems depend on newly synthesized proteins, and are hence both time and energy consuming. Here, we describe an “express” cytoprotective system in Bacillus subtilis which depends on nitric oxide (NO). We show that NO immediately protects bacterial cells from reactive oxygen species by two independent mechanisms. NO transiently suppresses the enzymatic reduction of free cysteine that fuels the damaging Fenton reaction. In addition, NO directly reactivates catalase, a major antioxidant enzyme that has been inhibited in vivo by endogenous cysteine. Our data also reveal a critical role for bacterial NO-synthase in adaptation to oxidative stress associated with fast metabolic changes, and suggest a possible role for NO in defending pathogens against immune oxidative attack.

Fenton reaction | nitric oxide | thiols

NO has many of the properties of a prototypical signaling molecule. It is small, freely diffusible, short-lived, and highly reactive in biological systems. NO is synthesized by NO synthases (NOS) in a wide variety of cells and is involved in numerous physiological and pathological processes in mammals (1–4). In contrast, bacterial-derived NO has been known only as an intermediate in the process of anaerobic respiration. However, some recent evidence suggests that NO and/or its equivalents [S-nitrosothiols (SNO)] may also be involved in signaling in bacteria. Several bacterial proteins have been shown to change their properties upon interaction with NO. For example, the transcription factors OxyR, SoxR, NorR, and Fur (5–7) in Escherichia coli and ResDE in Bacillus subtilis (8) activate corresponding regulons upon reaction with NO. Furthermore, several Gram-positive bacteria, including B. subtilis, possess an enzyme orthologous to eukaryotic NOS (9–13). The ability of B. subtilis NOS to synthesize NO from arginine has been confirmed in vitro (9, 10), although its physiological role remains obscure.

NO bioactivity depends on its target (2, 3, 14). In mammals, NO/SNO influence ranges from cytoprotection to cytotoxicity (5, 15–17). NO has been shown to protect various types of eukaryotic cells from H2O2 and organic peroxide-mediated toxicity (18–24), although the molecular mechanism of NO mediated cytoprotection has not been elucidated.

In bacteria, H2O2 toxicity is attributable primarily to DNA damage (25–27). Upon interaction with free cellular iron, H2O2 forms hydroxyl radicals (OH−) (reaction 1) that react at diffusion-limited rates with DNA bases and sugar moieties causing modifications and strand breaks (25–27).

\[
Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^+
\]  
[1 (Fenton reaction)]

\[
Fe^{3+} + \text{reducatant}_{reduced} \rightarrow Fe^{2+} + \text{reducatant}_{oxidized}
\]  
[2]

Significantly, free reduced iron, which is required for the Fenton reaction, is scarce in vivo and would be depleted almost instantaneously upon H2O2 challenge (25). Thus, to persistently drive the Fenton reaction, ferric iron must be continuously rereduced to the ferrous state by cellular reductants (reaction 2). It has been shown that rereduction of ferric iron by cellular reducing equivalents (RE) such as FADH2 and cysteine sustain the Fenton reaction, ultimately leading to cellular death (25, 28). Here, we demonstrate that B. subtilis utilizes endogenous and exogenous NO for rapid protection from oxidative damage. NO suppresses the Fenton reaction by transiently inhibiting cysteine reduction. Independently, NO specifically activates catalase to detoxify excess H2O2. We explain how these two components of NO-mediated cytoprotection function in bacteria, and propose that this dual mechanism may be universal.

The ability of B. subtilis to synthesize NO from arginine has been confirmed in vitro (9, 10), although its physiological role remains obscure.

Novel Methods. B. subtilis IS75 and Staphylococcus aureus wt strain RN6734 (a gift from R. Novick, New York University Medical

This paper was submitted directly (Track II) to the PNAS office. Abbreviations: Cm, chloramphenicol; mBB, monobromobimane; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulphophenyl)2H-tetrazolium inner salt; NOS, NO synthase; RE, reducing equivalents; TepAu, chloro(triethylphosphine)gold(I); Trx, thioredoxin; TrxRed, Trx reductase.

*To whom correspondence should be addressed. E-mail: evgeny.nudler@med.nyu.edu. © 2005 by The National Academy of Sciences of the USA
Center, New York) overnight cultures grown in liquid Luria–Bertani (LB) media were diluted 1:100 in fresh LB and grown at 37°C with aeration until OD600 ~ 0.5, unless indicated otherwise. To determine H2O2 resistance, B. subtilis cells were exposed to 1 or 10 mM H2O2 for 30 min. S. aureus was challenged with 370 mM H2O2. The number of viable cells was determined by colony formation on LB agar. Colony-forming units (CFU) were counted the following day, and the percentage of survival was calculated. To prepare bacterial cell extract, B. subtilis cells were harvested, dissolved in lysis buffer (20 mM Tris·HCl, pH 7.9/150 mM NaCl) containing 125 µg/ml lysozyme (Sigma), incubated for 5 min at 37°C, sonicated, and clarified by centrifugation. Protein concentration was determined by using the Bio-Rad protein assay kit. Nitrite was measured in clarified cell culture supernatants by using the fluorimetric nitrite assay kit (Cayman Chemical, Ann Arbor, MI).

**Catalase Activity Assay.** Degradation of H2O2 was monitored in real time by spectroscopy, detected as a decrease in absorbance at 240 nm (31). Total H2O2 degrading activity was measured as the decrease of H2O2 concentration per mg of total protein per sec. OD240 was converted to the concentration of H2O2 according to the calibration curve (10 mM H2O2 = 0.36 OD240).

**Quantification of Reduced Thiols in Vitro in Vivo.** Cys and other thiols react with mBB to form a fluorescent dye (32). To determine Trx/TrxRed activity in the reconstituted system, the amount of reduced Cys was measured by reaction with mBB. The reaction mixture contained TrxRed (0.05 units per 100 µl) and Trx (2 µM) from E. coli and 1 mM cystine dissolved in 0.15 M NaCl/0.2 M Hepes (pH 7.6). Reaction was initiated by addition of 0.5 mM NADPH at 25°C. Twenty-microliter aliquots were withdrawn and mixed with 80 µl of 0.4 mM mBB in 0.15 M NaCl/0.2 M Hepes (pH 7.6). Reactions were incubated for 10 min in the dark, and the fluorescence was measured by using a PerkinElmer (LC55) fluorometer (λem = 479 nm, λex = 390 nm). Addition of NO after thiol reaction with mBB did not affect the fluorescent yield. Fluorescence was converted to the concentration of thiols according to the Cys standard curve. TrxRed activity in B. subtilis extracts was measured spectrophotometrically by reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) in the presence of NADPH (33). The concentration of reduced thiols in vivo was measured by using mBB (32). B. subtilis cells (~40 ml) were grown to the mid-log phase (OD ~ 0.6–0.8), collected by centrifugation, and resuspended in 1 ml of fresh LB with 180 µg/ml Cm. Cells were treated with lysozyme (100 µg/ml) for 2 min at 37°C to remove cell walls. At 1 min time points, 20-µl aliquots were withdrawn and mixed with 80 µl of stop solution (1 mM mBB/6 M guanidine-HCl/0.2 M Tris·HCl, pH 7.9). Reactions were vigorously shaken to facilitate cell lysis and kept in the dark for 10 min before measuring fluorescence.

**Measurement of DNA Damage by Quantitative PCR.** Total genomic DNA was isolated from 10 ml of culture and quantified by using a PicoGreen dsDNA quantitation reagent (Molecular Probes) and lambda DNA as a standard. Approximately 10-kb fragments near zwf regions were used for quantitative PCR. Primer sequences were as follows: 5′-GGATGCGCTGTCTCGGTAACAACACGG (forward primer) and 5′-GACCCAGCGTTGATTAGCTGTTACACCC (reverse primer). PCR was performed by using Phusion DNA polymerase (Finnzymes). The 50-µl PCR mixture contained 0.05–0.5 µg of genomic DNA as a template, 1.5 µM primers, 200 µM dNTPs (Fermentas), 5× Phusion GC PCR buffer, and 0.5 µl of DNA polymerase. DNA was subjected to 30 cycles of PCR (98°C for 30 sec, 58°C for 30 sec, and 72°C for 9 min). PCR products were separated by 1% agarose gel electrophoresis, stained with ethidium bromide, scanned, and quantified with an Alphalmager (Imgen Technologies).

**Determination of the Rate of RE Rereduction.** IS75 cells were grown to mid-log phase in LB, and aliquots were treated with 1 mM diamide, 50 µM chloro(triethylphosphine)gold(I) (TepAu), 30 µM NO, or water. MTS (0.1 mg/ml) was added 5 sec after NO or H2O2 and 30 sec after diamide and TepAu. Tubes were incubated at 37°C, 1-ml aliquots were withdrawn every 2 min and separated from cells, and an absorbance was measured at 490 nM (34, 35). All reactions were preformed without phenazine methosulfate, because under this condition MTS is reduced by Cys 10 times more efficiently than by NADPH.

**Results and Discussion**

**NO Rapidly Protects B. subtilis from Oxidative Stress.** To examine the possibility that cytoprotection is induced by NO, we first investigated the effect of a harmless, single dose of NO (30 µM) on the survival of B. subtilis exposed to oxidative stress. As shown in Fig. 1A, within 5 sec of NO administration, resistance to H2O2 increased ~100-fold (lane 4). The addition of NO simultaneously with or after H2O2 had no protective effect (lanes 5 and 6) apparently because of NO scavenging by radicals generated by H2O2 (26, 36). Also, no cytoprotection was observed if oxidized NO or nitrite were added instead of NO (lanes 7 and 8). Notably, pretreatment with the same low concentration of H2O2 (30 µM) could not protect cells from a
lethal dose (10 mM) of H$_2$O$_2$ (lane 9), indicating that the protective effect of NO was highly specific. The above-mentioned controls (lanes 5 and 6) and large excess (>300 times) of H$_2$O$_2$ over NO rule out the possibility that the protective effect of NO was due to direct reaction with H$_2$O$_2$ or its derivatives. A similar level of protection from H$_2$O$_2$ was achieved with the NO-donors SNAP and MAHMA NONOATE (unpublished observation). Moreover, NO also protected B. subtilis against organic peroxides such as t-butyl hydroperoxide and cumene hydroperoxide (see Fig. 9, which is published as supporting information on the PNAS web site).

NO has been shown to activate various genes in E. coli and B. subtilis to protect cells from oxidative and nitrosative stress (7, 8, 37, 38). However, in our experiments the full protective effect of NO was established within 5 sec of NO administration, eliminating the necessity of gene activation for cytoprotection. We consistently found that inhibition of protein synthesis by Cm did not compromise NO-mediated cytoprotection (Fig. 1B). Notably, the effect of a bolus of NO was transient, with a maximum attained within 5 sec of application (Fig. 1B). This finding is consistent with the short life time of NO in physiological solutions (3) and argues for a rapid reversibility of the process. Taken together, these data suggest that NO directly and reversibly activates some latent, readily available oxidative stress defense system(s) in B. subtilis.

**Catalase Activation by NO: The First Component of NO-Mediated Cytoprotection.** NO could rapidly protect cells from H$_2$O$_2$ by boosting the activity of a preexisting H$_2$O$_2$ scavenging enzyme(s). B. subtilis vegetative catalase KatA is the major antioxidant enzyme. It is an iron–heme protein, and thus a natural target for NO. To test whether NO activates KatA, we measured the rate of H$_2$O$_2$ decomposition in crude cell extracts (31). Challenging a B. subtilis extract with our standard NO dose boosted H$_2$O$_2$ degradation by 75% (Fig. 2A) but failed to do so in an extract from ΔkatA cells (Fig. 2A), demonstrating that NO indeed potentiates the activity of KatA but not that of other enzymes of related function. Our in vitro data indicate that free Cys partially inhibits KatA (see Supporting Text and Fig. 10, which are published as supporting information on the PNAS web site). NO relieves this inhibition by disrupting the KatA–Cys complex apparently via an S-nitrosylation mechanism.

**Inhibition of the Fenton Reaction: A Second Component of NO-Mediated Cytoprotection.** The above results suggested that KatA deletion would compromise NO-mediated protection from reactive oxygen species in vivo. Indeed, we found that NO failed to elicit any significant protection of ΔkatA cells from H$_2$O$_2$ after 15 min of H$_2$O$_2$ application (Fig. 2B). However, NO-treated ΔkatA cells still retained most of their resistance to H$_2$O$_2$ during the first 10 min of treatment (Fig. 2B), indicating that another mechanism unrelated to KatA activation was responsible for initial, transient cytoprotection by NO.

In E. coli, DNA damage from hydroxyl radicals generated by the Fenton reaction is a primary mechanism of H$_2$O$_2$ cytotoxicity (25, 26). If the same mechanism operates in B. subtilis, an early cytoprotective effect of NO must be in suppressing the Fenton reaction and DNA damage. To test this hypothesis, we examined the effect of a cell permeable iron chelator (dipyridyl) on H$_2$O$_2$ toxicity and NO cytoprotection. Dipyridyl protected cells from a lethal dose of H$_2$O$_2$ (Fig. 2C), whereas NO failed to further protect dipyridyl-treated cells. This result shows that H$_2$O$_2$ toxicity in B. subtilis (like in E. coli) is attributed to DNA damage induced by Fenton chemistry.

To further support this conclusion, chromosomal DNA damage was measured by quantitative PCR (28). We found that the yield of full-length PCR fragments (10 kb) decreased considerably after challenging cells with 10 mM H$_2$O$_2$ with DNA fragments of a smaller size becoming apparent (Fig. 2D, lane 4). These smaller fragments reflect multiple DNA lesions that prematurely interrupted the PCR reaction. The iron chelator and inhibitor of the Fenton reaction dipyridyl (39) eliminated all DNA damage (Fig. 2D, lane 1). NO also restored the yield of the full-length fragment and eliminated smaller bands (Fig. 2D, lane 5), implying that NO protects cellular DNA from the destructive Fenton reaction.

As mentioned in the Introduction, ferric iron must be repeatedly reoxidized to maintain the Fenton reaction. In E. coli, Cys reduces cellular iron making the Fenton reaction processive (28, 36). To verify whether Cys or other free thiols are capable of supporting the Fenton reaction in B. subtilis, we used diame, a specific thiol oxidizing reagent (40). Diamide eliminated lesions in chromosomal DNA (Fig. 2D, lane 2) and protected cells from H$_2$O$_2$ toxicity (Fig. 2C). NO failed to further protect diame-treated cells from H$_2$O$_2$ (Fig. 2C), indicating that NO protection occurs via the inhibition of Fenton chemistry.

Taken together, these results explain the biphasic NO protection pattern shown in Fig. 2B and imply that NO inhibition of the Fenton reaction along with NO activation of catalase constitute the mechanism of NO cytoprotection.

**Mechanism of NO-Mediated Inhibition of the Fenton Reaction.** Because the amount of NO used in our experiments was insufficient to eliminate OH$^-$ directly (NO:H$_2$O$_2$ = 1:300), we assumed that NO inhibited the Fenton reaction by either scavenging cellular iron or preventing its reoxidation. First, we examined the effect of NO on the Fenton reaction in vitro. Hydrogen peroxide alone did not

---

**Figure 2.** Activation of catalase and inhibition of the Fenton reaction, two components of NO-mediated cytoprotection. (A) Stimulating effect of NO on H$_2$O$_2$ degrading activity in crude extracts of wt and ΔkatA cells. Total H$_2$O$_2$ degrading activity was measured as described in Experimental Procedures (31). Where indicated, extracts were incubated with 45 μM NO for 5 sec. 100% catalase activity = 30 mM H$_2$O$_2$ min$^{-1}$mg$^{-1}$. Values shown are the means ± SE from six experiments. (B) Transient protection of ΔkatA cells from oxidative stress by NO. The graph shows the time course of H$_2$O$_2$-mediated toxicity. Ten millimolar H$_2$O$_2$ was added at t = 0. Where indicated, 30 μM NO was added 5 sec before H$_2$O$_2$. Values shown are the means ± SD from three experiments. (C) Protection of wt cells from oxidative stress by the iron chelator dipyridyl and thiol oxidizer diamide. After 5 min of incubation with Cm (50 μg/ml), aerobically grown wt cells (OD$_{600}$ = 0.5) were treated with dipyridyl (1 mM for 10 min) or diamide (200 μM for 3 min) and/or NO (30 μM for 5 sec), followed by the addition of 10 mM H$_2$O$_2$ for 5 min. Values shown are the means ± SE from four experiments. (D) Chromosomal DNA damage from the Fenton reaction. A representative agarose gel shows a 10-kb PCR fragment amplified from B. subtilis chromosome. Chromosomal DNA was isolated from cells treated with diamide, dipyridyl, or NO and H$_2$O$_2$ as described in Figs. 1A and 2C. M, 1-kb DNA ladder. The relative intensity of the full size DNA band is indicated at the bottom. Values shown are the means ± SD from three experiments.
produce any strand breaks in pBR322 DNA (Fig. 3A, lane 6). Addition of Fe³⁺ caused only a slight increase in strand breaks (Fig. 3A, lane 2), whereas addition of Cys [a major free thiol in B. subtilis (41)] along with Fe³⁺ dramatically accelerated DNA damage (Fig. 3A, lane 3). Addition of excess NO (NO:Fe³⁺ = 3:2) did not inhibit DNA damage (Fig. 3A, lane 4). These results show that NO cannot interfere with Fenton chemistry by scavenging cellular iron. Indeed, the NO–iron complex is highly unstable in biological solutions $[K_{diss} \approx 24–660 \text{sec}^{-1} (42)]$ and dissociates rapidly in the presence of H₂O₂ in vitro $[t_{1/2} = 15 \text{sec} (data \ not \ shown)]$.

An alternative mechanism by which NO could suppress the Fenton reaction in vivo is to inhibit enzymes that generate RE [RE reduce cellular iron, thus driving the Fenton reaction (see Introduction)] (25). Indeed, NO readily binds iron–heme and iron–sulfur centers of various proteins $[K_{diss} \approx 10^{-6} \text{ to } 10^{-12} \text{ M} (42)]$ and can also inhibit enzymes via S-nitrosylation (43).

We first examined the possibility that NO inhibits aconitase, a Fe-S enzyme that metabolizes citrate. Citrate is a natural iron chelator. We therefore reasoned that its accumulation due to aconitase inhibition should interfere with Fenton chemistry. However, cells bearing a chromosomal deletion of the aconitase gene $\text{citB}$ were protected by NO to the same extent as $\text{wt}$ cells (data not shown), thus excluding aconitase from the mechanism of NO cytoprotection.

We then examined whether NO suppresses major catabolic pathways (glycolysis, the pentose phosphate pathway and TCA cycle) and as a result depletes cells of RE [Cys, FADH₂, and NAD(P)H]. To address this issue, we took advantage of the MTS reagent, which forms a colored dye upon reduction by cellular RE (34, 35). The rate of MTS dye accumulation is directly correlated with the rate of RE rereduction in vivo. As shown in Fig. 3B, exponentially growing $B. subtilis$ cells accumulate RE at a steady rate as represented by the linearity of the dye formation curve; note that the slope of the line reflects the cumulative activity of reducing enzymes. Remarkably, after bolus NO application, the total cellular concentration of RE was diminished. More importantly, the rate of RE rereduction also decreased >2-fold, as reflected by the lower slope of the line ($k_1/k_2 = 2.2$) (Fig. 3B). The slower rate of RE rereduction would result in a slower rate of cellular iron reduction; hence, the inefficient Fenton reaction. Significantly, the $\approx 10$-min delay in RE accumulation correlated well with the period ($\approx 10$ min) of NO-mediated protection from H₂O₂ in $\text{KatA}^+$ cells (Fig. 2B).

MTS is reduced by Cys 10 times more efficiently than by NADPH (see Experimental Procedures and Fig. 11, which is published as supporting information on the PNAS web site), suggesting that dye accumulation in Fig. 3B is mostly attributed to MTS reaction with Cys. To confirm this hypothesis, we monitored the dye accumulation in the presence of thiol-depleting reagents diamide or TepAu (Fig. 3C). TepAu is a specific inhibitor of TrxRed (44). Trx/TrxRed is the only and essential system in $B. subtilis$ dedicated to thiol reduction (45). Inhibition of this system would quickly deplete cells of reduced thiols. Indeed, treating cells with either diamide or TepAu leads to a significant decrease in the rate and amount of MTS dye formation (Fig. 3C), i.e., thiol reduction. These results indicate that NO inhibits the rate of thiols reduction and thus inhibits Fenton chemistry (Fig. 3C).

The proposed mechanism of NO cytoprotection implies that the extent of H₂O₂ toxicity is directly proportional to the rate of thiols reduction, i.e., to the activity of glycolysis, the pentose phosphate pathway, and Trx/TrxRed. Consistently, we noticed that $B. subtilis$ cells deprived of a carbon source acquired resistance to H₂O₂ (Fig. 3D). A similar observation has been made with $E. coli$ (25, 46). Moreover, the addition of glucose sensitized $B. subtilis$ cells to H₂O₂, whereas NO reversed this effect (Fig. 3D).

We next proceeded to determine which RE [FADH₂, NAD(P)H, or Cys] serves as a major driving force of the Fenton reaction in $B. subtilis$. Cys is the major reducing agent that drives the Fenton reaction as well (25), whereas free FADH₂ is scarce in the cell. The concentration of free intracellular Cys was not affected by NO (data not shown), we proposed that NO inhibited cystine reduction. Because Trx/TrxRed is a specific inhibitor of NADPH (not shown), we oxidized $\text{KatA}^+$ cells without NADPH and observed that NO inhibited cystine reduction efficiently (Fig. 3C). Forty-five and 100 μM NO decreased the reaction rate 4- and 40-fold, respectively (Fig. 4A). NO also inhibited Trx/TrxRed in a cell extract (data not shown). These results demonstrate that Trx/TrxRed is a target for NO inhibition. Consistently, a specific inhibitor of TrxRed (TepAu) protected cells against Fenton-mediated toxicity (unpublished observations).

To confirm that NO inhibits thiol reduction in vivo, we oxidized cellular thiols with diamide and then monitored the rate of their reduction. It took $\approx 6$ min for $B. subtilis$ to rereduce its thiols after diamide challenge (Fig. 4B, open squares). Two hundred and 500 μM NO delayed thiol reduction by 3 (Fig. 4B, open circles) and 6 (Fig. 4B, open triangles) min, respectively. We used higher concentrations of NO and diamide because cells were concentrated
acquire resistance to H$_2$O$_2$. Such cells are restricted to a "single-going, oxidized iron and cystine must be continuously rereduced. back to Cys at the expense of NADPH. To keep the Fenton reaction capable of generating NO from L-arginine detoxifies excess H$_2$O$_2$ (Fig. 5).

Experimental Procedures

Many organisms use glutathione to maintain their internal reducing environment. It has been shown that, unlike Cys, glutathione does not support the Fenton reaction (28). Because $B$. subtilis uses Cys instead of glutathione, it is reasonable to speculate that it has developed a dedicated system to control the rate of Cys reduction so as to keep the Fenton reaction at bay.

As noted in the Introduction, $B$. subtilis possesses NOS that is capable of generating NO from l-arginine in vitro (9). One can therefore speculate that endogenous NO renders cells more resistant to oxidants via both catalase activation and diminishing the rate of Cys reduction. To examine a possible cytoprotective role of endogenous NO (produced by bacterial NOS), we compared the ability of wild-type (wt) and nos (yfM) deletion strains to withstand oxidative stress. Because wt and $\Delta$nos had the same sensitivity to peroxide under steady growth conditions, we hypothesized that changes in thiol availability may activate NOS. To simulate this situation, we grew cells to late exponential phase and then diluted them with fresh LB medium. The intracellular level of Cys (mostly Cys) increased 2-fold in $\Delta$nos mutant upon dilution as compared with 1.5-fold in wt (Table 1, which is published as supporting information on the PNAS web site). Such nutrient shift sensitized both wt and nos cells to H$_2$O$_2$. However, $\Delta$nos cells were ≈25 times more sensitive to oxidative stress than wt (Fig. 6A). Such a dramatic difference between the two strains became obvious within the first 2 min after dilution, arguing against any contribution from de novo protein synthesis. This situation is reminiscent of the instant antioxidant effect of exogenous NO (Fig. 1).

To prove that exogenous Cys was responsible for increased H$_2$O$_2$ sensitivity, we reproduced the above experiment using saline (with or without Cys) instead of LB for dilution. Cys sensitized $\Delta$nos cells to H$_2$O$_2$ >20 times more than wt (Fig. 6B). Notably, dilution per se could not render cells sensitive to H$_2$O$_2$ (Fig. 6B). Moreover, addition of glucose or various amino acids also did not sensitize cells to H$_2$O$_2$ (data not shown). These results strongly suggest that a rapidly increased concentration of Cys triggers (directly or indirectly) NOS-mediated NO production to protect cells against oxidative stress.

To test this hypothesis, we measured the concentration of nitrite in the medium before and after dilution. Nitrite is a direct product of NO oxidation serving as a standard quantitative marker of NO. Fig. 6C shows that the level of nitrite was higher in the wt culture than $\Delta$nos, reflecting the basal level of NOS activity. More importantly, upon dilution, the concentration of nitrite went down in the $\Delta$nos culture but increased in wt. These results demonstrate that NOS is indeed activated upon dilution thus protecting cells from oxidative stress.

To determine whether endogenous NO activates catalase, we measured KatA activity upon dilution. As noted above, NO activates catalase that has been inhibited by thiols (Fig. 10). Cellular level of reduced Cys was increased upon dilution (Table 1), and catalase activity was inhibited by 20% in $\Delta$nos cells (Fig. 6D). In contrast, dilution of wt cells did not affect catalase activity. These results indicate that NOS-derived NO reactivates catalase that has been inhibited by endogenous Cys (Fig. 6D).

These findings directly implicate NOS in the delicate regulation of cellular redox homeostasis and adaptation of $B$. subtilis to oxidative stress during rapid metabolic changes. This function of NOS may be crucial under conditions of competitive growth in natural environments. Note that dilution exaggerates an otherwise subtle difference in growth rates between wt and $\Delta$nos strains (Fig. 12, which is published as supporting information on the PNAS web site).

As mentioned above, the dilution experiment simulates the natural situation when the demand for biosynthesis temporarily stops, leading to transient accumulation of reduced Cys, a major thiol in $B$. subtilis (41). The excess of Cys would not only drive the Fenton reaction (28) but also suppress catalase activity and thus elevate the level of H$_2$O$_2$ (Fig. 5). In this regard, the protective
Fig. 6. NOS-mediated protection from oxidative stress in B. subtilis. (A) Effect of nos deletion on H2O2 sensitivity. wt and Δnos cells were grown aerobically in LB to late log phase (OD600 ~ 0.8–0.9) at 30°C. An aliquot from each culture was diluted with an equal amount of fresh prewarmed LB for 2 min (diluted). Both diluted and undiluted aliquots were treated with 1 mM or 10 mM H2O2 for 30 min. The percentage of surviving cells was determined by colony formation. Values shown are the means and SD (error bars) from four independent experiments. (B) Exogenous Cys sensitizes B. subtilis to oxidative stress and induces NOS-mediated protection. Cells were grown as in A and diluted with an equal volume of saline or saline plus Cys (100 μM). Arg (100 μM) was added to all samples. After 2 min of incubation, cells were treated with 10 mM H2O2 for 30 min. Values shown are the means ± SD from three experiments. (C) Effect of fresh medium dilution on nitrite levels in wt and Δnos cells cultures. Conditions were as in A. Samples for nitrite measurements were taken 5 min after dilution. LB, nitrite level in LB. Values shown are the means ± SD from three experiments. (D) Effect of dilution on KatA activity. Conditions were as in A. Cells were collected 1 min after dilution and lysed immediately, and catalase activity was measured as described in Experimental Procedures. Values shown are the means ± SE from three experiments.

The effect of NO is twofold. It immediately and transiently suppresses Cys reduction, thus suppressing the Fenton reaction. At the same time, NO activates a preexisting antioxidant enzyme, catalase, which quickly detoxifies the excess H2O2.

NO-Mediated Cytoprotection May Be General for NOS-Containing Bacteria and Contribute to Pathogenesis. NOS has been found only in Gram-positive bacteria, suggesting that Gram-negative bacteria do not use NO for adaptation to oxidative stress. Indeed, treating E. coli with NO does not provide any immediate protection from H2O2 toxicity (data not shown). In light of the mechanism presented above, there are at least three explanations for such a difference between bacterial species. First, in contrast to B. subtilis KatA, major catalase from E. coli is inhibited by NO (48). Second, Δys does not inhibit catalase from E. coli to the same extent as B. subtilis KatA (49). Third, Cys is a major low-molecular-weight thiol in many Gram-positive including Bacilli and S. aureus but not in E. coli (28, 41). The most abundant small thiol in E. coli, glutathione (GSH), has not been found in B. subtilis (41). Unlike Cys, GSH does not support the Fenton reaction and acts as an antioxidant rather than a prooxidant in vivo (28).

The “express” mechanism of NO-mediated cytoprotection reported here could play an important role in the adaptation of pathogens to oxidative stress imposed by the immune system. Macrophages and other phagocytes produce O2− and NO in large quantities to combat infecting bacteria (4, 50–52). The ability of pathogens to survive the immunological “respiratory burst” critically depends on the state of their oxidative stress defense system. Macrophages start to produce high levels of NO after they encounter bacteria (53). Whereas superoxide anion stays inside the phagocyte vacuole, exceptionally pervasive NO escapes from immune cells. Thus, NO should be able to reach the bacterial interior even outside the macrophage, serving as a forewarning of the impending full-scale onslaught of the host. This event would postpone the damaging Fenton reaction and activate preexisting catalase to neutralize excess H2O2, giving bacteria critical time for de novo synthesis of their major stress response components. In support of this hypothesis, we provide evidence that the notorious Gram-positive pathogen S. aureus employs the same NO-mediated cytoprotection system as B. subtilis (see Fig. 13, which is published as supporting information on the PNAS web site).

We thank Ruslan Rafikov and Nick Cowan for comments. This work was supported by National Institutes of Health Grant AI060762.