ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2007, p. 3364–3366 Vol. 51, No. 9
0066-4804/07/$08.00+0 doi:10.1128/AAC.00347-07

Innate Protection of *Mycobacterium smegmatis* against the Antimicrobial Activity of Nitric Oxide Is Provided by Mycothiol[▽]

Christopher C. Miller,[1,2] Mamta Rawat,[3] Todd Johnson,[3] and Yossef Av-Gay[1]*

Department of Medicine, Division of Infectious Diseases, University of British Columbia, Vancouver, BC, Canada[1]; Pulmonox Medical Inc., Box 1020, Tofield, AB T0B 4J0, Canada[2]; and Department of Biology, California State University—Fresno, Fresno, California, 93740[3]

Received 14 March 2007/Returned for modification 16 May 2007/Accepted 9 July 2007

Nitric oxide (NO) is an efficient antimicrobial agent. A role for mycothiol in protecting mycobacteria from nitrosative damage was revealed by showing that a *Mycobacterium smegmatis* mutant is sensitive to NO. A direct correlation between NO and mycothiol levels confirmed that mycothiol is important for protecting mycobacteria from NO attack.

Mycobacteria encounter free radicals, such as reactive oxygen and nitrogen intermediates (ROIs and RNIs), as a result of normal aerobic respiration and during infection as part of host defenses. An immediate response to free radicals is provided by the thiol analog of glutathione, mycothiol (MSH). MSH also maintains a stable intracellular redox environment (3, 18) and detoxifies toxic substances (11, 15, 18). Xenobiotic detoxification is achieved by three different thiol-dependent means: (i) donation of electrons by thiols to peroxidases that catalyze the conversion of peroxides to alcohols or water, (ii) reduction of substrates in a reaction coupled with thiol oxidation (18), and (iii) conjugation of thiols to electrophiles, such as antibiotics, and production of less toxic mercapturic acids (11, 15). More importantly, an MSNO (*S*-nitrosomycothiol)-dependent nitrosomycothiol reductase has been characterized (19).

We have shown that exogenous gaseous nitric oxide (gNO) is able to eradicate a variety of bacterial species, including clinical isolates (4, 5, 10). In this study, we examined the abilities of mycobacteria to resist killing during gNO treatment and the role of MSH in protection against RNIs.

Exogenous nitric oxide exerts an antimicrobial effect on *Mycobacterium smegmatis*. The antimicrobial action of gNO on *M. smegmatis* mc^{2}155 was assessed by exposing approximately 10^5 CFU/ml to 200 ppm gNO, using our previously described apparatus and methodology (5), and determining bacterial viability. Over time, continuous exposure decreased the concentration of viable bacteria (Fig. 1A). The sharp decrease in bacterial viability followed a 7-hour latency period, and by 10 h, no viable bacteria could be recovered from the suspension. In contrast, in the control sample, the number of viable bacteria remained relatively stable, with limited decrease in viability over a period of 11 h.

Mycobacteria are more resistant to gNO than other bacteria. *M. smegmatis* had the longest latency period (defined as the time required for a 10-fold decrease in CFU) of any of the bacteria tested (Table 1) and the highest 100% lethal dose (LD_{100}) at 200 ppm h^{-1} gNO. As a result, *M. smegmatis* required the highest dose of gNO (2,000 ppm-h) to achieve complete killing in suspension.

The *M. smegmatis mshA* mutant is more sensitive to gNO. Mycobacteria are known to possess detoxification systems that offer protection from the toxic effects of ROIs and RNIs (1, 8, 9, 11, 13, 14, 17). However, the fact that there is a delay in gNO effect that could eventually be overcome with continuous exposure to gNO suggests that a cellular component that could be depleted is involved. To determine if MSH plays a direct role in protecting mycobacteria from gNO, an *M. smegmatis mshA*::Tn*5* (A1) (12) mutant unable to produce MSH was exposed to gNO. The latency period for the mutant was reduced to 4 h, compared to 7 h for the wild-type parent (Fig. 1B).

To confirm that the shortened latency period observed for *M. smegmatis mshA*::Tn*5* was due only to the missing MSH biosynthetic activity encoded by *mshA*, the *Mycobacterium tuberculosis mshA* gene was supplied in *trans* on the plasmid

TABLE 1. Killing of bacterial isolates exposed continuously to 200 ppm gNO

Bacterium	Latency period (h)[a]	LD_{100} (h)[b]	Dose (ppm-h)[c]
S. aureus	3	4	800
Pseudomonas aeruginosa	1	3	600
Staphylococcus aureus MRSA	3	5	1,000
Serratia sp.	4	6	1,200
Klebsiella sp. no. 1	3	6	1,200
Klebsiella sp. no. 2	2	5	1,000
Klebsiella sp. no. 3	3	6	1,200
Enterobacter sp.	5	6	1,200
Acinetobacter sp.	5	6	1,200
Candida albicans	2	4	800
M. smegmatis	7	10	2,000
Escherichia coli	3	5	1,000
Group B *Streptococcus*	1	2	400

[a] Latency period was defined as the exposure time required for viability to decrease 10-fold.
[b] LD_{100} was defined as the exposure time at which viable bacteria could no longer be recovered.
[c] Dose was defined as the product of the LD_{100} and the gNO concentration to which the bacteria were exposed.

* Corresponding author. Mailing address: Department of Medicine, Division of Infectious Diseases, University of British Columbia, 2733 Heather St., Vancouver, BC, Canada V5Z 3J5. Phone: (604) 875 4588. Fax: (604) 875 4013. E-mail: yossi@interchange.ubc.ca.
▽ Published ahead of print on 16 July 2007.

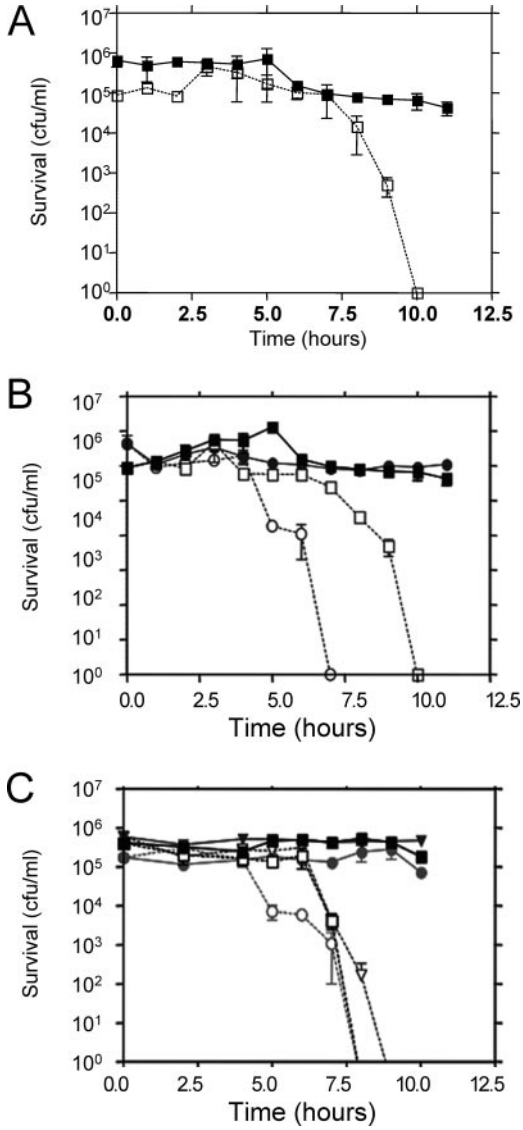

FIG. 1. Antimicrobial activity of gNO on *M. smegmatis* mc^{2}155 in 0.9% saline solution. Mycobacteria were exposed to 200 ppm gNO (mc^{2}155, open squares; *mshA*::Tn*5*, open circles; *mshA*::Tn*5* [pAL0486], open triangles) or medical air (mc^{2}155, filled squares; *mshA*::Tn*5*, filled circles; *mshA*::Tn*5* [pAL0486], filled triangles). (A) Killing pattern of *M. smegmatis*. (B) Increased sensitivity to gNO-mediated killing for MSH-deficient *M. smegmatis mshA*::Tn*5* compared to that for its wild-type parent. (C) Complementing *M. smegmatis mshA*::Tn*5* with cloned *M. tuberculosis mshA* expressed on a plasmid restored the parental level of resistance to gNO-mediated killing. In all cases, to avoid the effect of gNO on growth media compounds, bacteria were suspended in 0.9% saline. Each data point represents the mean ± standard deviation for at least three independent measurements.

pAL0486 and the complemented strain was tested. The latency period for *mshA*::Tn*5* exposed to gNO was again shorter than that for the wild-type parent strain (4 h versus 6 h), but in the complemented strain, the latency period increased to 6 h, similar to that for the wild-type parent (Fig. 1C). The basal MSH levels in the mutant, the complemented strain, and wild-type *M. smegmatis* agreed with the levels previously measured by Newton et al. (12).

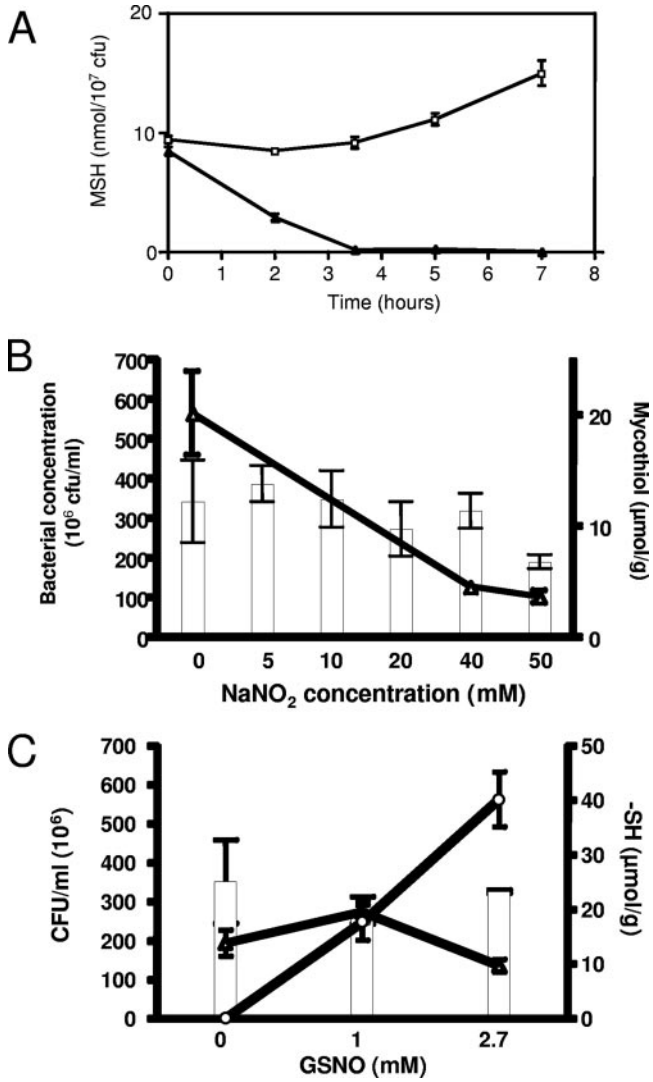

FIG. 2. MSH pool in *M. smegmatis* is decreased by exposure to NO. (A) MSH levels in *M. smegmatis* mc^{2}155 in 0.9% saline solution decreased with continuous exposure to gNO. Organisms were exposed to 400 ppm gNO (triangles) or medical air (squares) for 7 h. The amount of MSH decreased steadily to almost zero in less than 3.5 h on gNO exposure. (B) MSH levels in *M. smegmatis* mc^{2}155 decreased with exposure to the NO donor sodium nitrite, while bacterial viability remained relatively unaffected. *M. smegmatis* mc^{2}155 was grown to log phase in Middlebrook 7H9 media supplemented with 0.05% Tween and 1% glucose (pH 5.5), diluted to 0.5 optical density units at 600 nm in the same medium, and treated with various concentrations of sodium nitrite for 2 hours. Numbers of CFU (bars) and MSH levels (triangles) are shown. (C) MSH levels in *M. smegmatis* mc^{2}155 decreased with exposure to the NO donor GSNO, while viability remained unaffected. *M. smegmatis* mc^{2}155 was grown to log phase in Middlebrook 7H9 media supplemented with 0.05% Tween and 1% glucose, diluted to 0.5 optical density units at 600 nm with the same medium, and treated with various amounts of GSNO for 2 h. CFU/ml (bars), GSH levels (circles), and MSH levels (triangles) are shown. Each data point represents the mean ± standard error for four independent measurements.

MSH levels decrease on exposure to nitric oxide. To determine if MSH levels were affected by gNO, *M. smegmatis* mc^{2}155 was exposed to either 400 ppm gNO or medical-grade air for 7 h. As shown in Fig. 2A, MSH levels decreased steadily

to zero by 3.5 h upon exposure to gNO, and MSH levels in the bacterial suspension exposed to medical air slowly increased over 7 h. This result indicates that MSH is being depleted by gNO and further supports a role for MSH in detoxifying the RNIs generated by exposure to gNO. Similar observations have been made for eukaryotic systems where exposure to NO led to respiration inhibition in the mitochondria (2) due to the depletion of mitochondrial GSH (16), and a prolonged exposure to NO was required to overcome these cellular GSH levels.

To determine the NO effect on actively growing *M. smegmatis*, we also used NO donors in growth media. MSH levels were measured along with viable cell counts after $NaNO_2$ and GSNO (*S*-nitrosoglutathione) treatments in growth media. Similar to what was found for gNO treatment, MSH levels were depleted before a decrease in bacterial viability was seen (Fig. 2B). A decrease in MSH levels that was dependent on the concentration of GSNO was observed, and this decrease in MSH directly correlated with the amount of GSH resulting from NO release (Fig. 2C).

While nitrosylation of MSH by NO has not yet been demonstrated, Kharitonov et al. described the kinetics of GSNO formation from GSH and NO in the presence of O_2 in vitro (7). Under conditions of high NO concentration, S nitrosylation of GSH is favored over protein-bound cysteine residues (6). Our results support a GSH-like role for MSH as the major protectant against NO toxicity in mycobacteria.

Together, these data lead us to suggest the following as the most likely scenario for mycobacterial resistance to continuous gNO exposure. MSH interacts with NO to mitigate this molecule's toxic effects. MSH levels decrease upon exposure to NO, but mycobacteria continue to be viable during early gNO exposure, suggesting that cellular MSH is still available for protection. On prolonged exposure, NO binds to all available MSH and mycobacterial viability decreases sharply as the cellular detoxification system no longer has the capacity to bind NO or NO-generated ROIs and RNIs. This depletion of MSH likely coincides with the end of the latency period. These data, combined with those from our previous studies, provide evidence for MSH as the major detoxification system in mycobacteria for ROIs, RNIs, and antibiotics.

This work was funded by Pulmonox Medical Inc. Financial support was provided to Y.A. by the TB Veterans Charitable Foundation and to M.R. by the NIH-MBRS-SCORE 2S06 GM061223-05A1 award. M.R. and Y.A. were also supported by the British Columbia Lung Association.

We thank Barb Conway for assistance with manuscript preparation and Bevin McMullin for technical support.

REFERENCES

1. **Bryk, R., C. D. Lima, H. Erdjument-Bromage, P. Tempst, and C. Nathan.** 2002. Metabolic enzymes of mycobacteria linked to antioxidant defense by a thioredoxin-like protein. Science **295:**1073–1077.
2. **Clementi, E., G. C. Brown, M. Feelisch, and S. Moncada.** 1998. Persistent inhibition of cell respiration by nitric oxide: crucial role of S-nitrosylation of mitochondrial complex I and protective action of glutathione. Proc. Natl. Acad. Sci. USA **95:**7631–7636.
3. **Dosanjh, N. S., M. Rawat, J. H. Chung, and Y. Av-Gay.** 2005. Thiol specific oxidative stress response in Mycobacteria. FEMS Microbiol. Lett. **249:**87–94.
4. **Ghaffari, A., C. C. Miller, B. McMullin, and A. Ghahary.** 2006. Potential application of gaseous nitric oxide as a topical antimicrobial agent. Nitric Oxide **14:**21–29.
5. **Ghaffari, A., D. H. Neil, A. Ardakani, J. Road, A. Ghahary, and C. C. Miller.** 2005. A direct nitric oxide gas delivery system for bacterial and mammalian cell cultures. Nitric Oxide **12:**129–140.
6. **Herold, S., and G. Rock.** 2005. Mechanistic studies of S-nitrosothiol formation by NO*/O2 and by NO*/methemoglobin. Arch. Biochem. Biophys. **436:**386–396.
7. **Kharitonov, V. G., A. R. Sundquist, and V. S. Sharma.** 1995. Kinetics of nitrosation of thiols by nitric oxide in the presence of oxygen. J. Biol. Chem. **270:**28158–28164.
8. **Lama, A., S. Pawaria, and K. L. Dikshit.** 2006. Oxygen binding and NO scavenging properties of truncated hemoglobin, HbN, of Mycobacterium smegmatis. FEBS Lett. **580:**4031–4041.
9. **Master, S. S., B. Springer, P. Sander, E. C. Boettger, V. Deretic, and G. S. Timmins.** 2002. Oxidative stress response genes in Mycobacterium tuberculosis: role of ahpC in resistance to peroxynitrite and stage-specific survival in macrophages. Microbiology **148:**3139–3144.
10. **McMullin, B. B., D. R. Chittock, D. L. Roscoe, H. Garcha, L. Wang, and C. C. Miller.** 2005. The antimicrobial effect of nitric oxide on the bacteria that cause nosocomial pneumonia in mechanically ventilated patients in the intensive care unit. Respir. Care **50:**1451–1456.
11. **Newton, G. L., Y. Av-Gay, and R. C. Fahey.** 2000. A novel mycothiol-dependent detoxification pathway in mycobacteria involving mycothiol S-conjugate amidase. Biochemistry **39:**10739–10746.
12. **Newton, G. L., T. Koledin, B. Gorovitz, M. Rawat, R. C. Fahey, and Y. Av-Gay.** 2003. The glycosyltransferase gene encoding the enzyme catalyzing the first step of mycothiol biosynthesis (*mshA*). J. Bacteriol. **185:**3476–3479.
13. **Ng, V. H., J. S. Cox, A. O. Sousa, J. D. MacMicking, and J. D. McKinney.** 2004. Role of KatG catalase-peroxidase in mycobacterial pathogenesis: countering the phagocyte oxidative burst. Mol. Microbiol. **52:**1291–1302.
14. **Pathania, R., N. K. Navani, A. M. Gardner, P. R. Gardner, and K. L. Dikshit.** 2002. Nitric oxide scavenging and detoxification by the Mycobacterium tuberculosis haemoglobin, HbN in Escherichia coli. Mol. Microbiol. **45:**1303–1314.
15. **Rawat, M., M. Uppal, G. Newton, M. Steffek, R. C. Fahey, and Y. Av-Gay.** 2004. Targeted mutagenesis of the *Mycobacterium smegmatis mca* gene, encoding a mycothiol-dependent detoxification protein. J. Bacteriol. **186:** 6050–6058.
16. **Riobo, N. A., E. Clementi, M. Melani, A. Boveris, E. Cadenas, S. Moncada, and J. J. Poderoso.** 2001. Nitric oxide inhibits mitochondrial NADH:ubiquinone reductase activity through peroxynitrite formation. Biochem. J. **359:** 139–145.
17. **Ruan, J., G. St. John, S. Ehrt, L. Riley, and C. Nathan.** 1999. *noxR3*, a novel gene from *Mycobacterium tuberculosis*, protects *Salmonella typhimurium* from nitrosative and oxidative stress. Infect. Immun. **67:**3276–3283.
18. **Ung, K. S., and Y. Av-Gay.** 2006. Mycothiol-dependent mycobacterial response to oxidative stress. FEBS Lett. **580:**2712–2716.
19. **Vogt, R. N., D. J. Steenkamp, R. Zheng, and J. S. Blanchard.** 2003. The metabolism of nitrosothiols in the Mycobacteria: identification and characterization of S-nitrosomycothiol reductase. Biochem. J. **374:**657–666.