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Nitric Oxide Generated from Isoniazid Activation by KatG: Source of Nitric Oxide and Activity against *Mycobacterium tuberculosis*

Graham S. Timmins,[1]* Sharon Master,[2] Frank Rusnak,[3]† and Vojo Deretic[2]

College of Pharmacy, Toxicology Program,[1] and Department of Molecular Genetics and Microbiology,[2] University of New Mexico Health Sciences Center, Albuquerque, New Mexico 87131, and Department of Biochemistry and Molecular Biology and Section of Hematology Research, Mayo Clinic and Foundation, Rochester, Minnesota 55905[3]

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Isonicotinic acid hydrazide (INH) is a frontline antituberculosis agent. Once taken up by *Mycobacterium tuberculosis*, INH requires activation by the catalase-peroxidase KatG, converting INH from its prodrug form into a range of bactericidal reactive species. Here we used ^{15}N-labeled INH together with electron paramagnetic resonance spin trapping techniques to demonstrate that nitric oxide ($NO^{\cdot}$) is generated from oxidation at the hydrazide nitrogens during the activation of INH by *M. tuberculosis* KatG. We also observed that a specific scavenger of $NO^{\cdot}$ provided protection against the antimycobacterial activity of INH in bacterial culture. No significant increases in mycobacterial protein nitration were detected, suggesting that $NO^{\cdot}$ and not peroxynitrite, a nitrating metabolite of $NO^{\cdot}$, is involved in antimycobacterial action. In conclusion, INH-derived $NO^{\cdot}$ has biological activity, which directly contributes to the antimycobacterial action of INH.

Mycobacterium tuberculosis infections cause 2 million deaths every year and latently persist in over 1 billion individuals worldwide (33). Isoniazid (isonicotinic acid hydrazide [INH]) is a frontline antituberculosis agent that is prescribed daily in millions of doses worldwide. While its mechanism(s) of action and cellular targets continue to be uncovered (16, 20), its full range of effects on mycobacterial cells still remains to be resolved (14, 27). The tubercle bacillus is exceptionally sensitive to INH (7, 36), a prodrug, which is peroxidatively activated by the *M. tuberculosis* catalase-peroxidase KatG to produce damaging species within the bacteria.

In keeping with the critical role of INH activation via KatG, the main site for mutations associated with resistance to INH is the *katG* gene (37). Several INH-derived intermediates generated during INH activation, such as isonicotinic acyl NADH (20) and mycobacterial targets including enzymes from the mycobacterial type II fatty acid synthase system (1, 16), have been identified. Other hypotheses regarding INH activation have focused upon INH-derived free radicals as important antimycobacterial intermediates (12, 24, 31). Despite this progress, the exact mechanism(s) of INH action that underlies its exceptional and specific potency against *M. tuberculosis* remains to be fully delineated, as multiple targets and pathways have been considered (7, 14, 17, 26).

$NO^{\cdot}$ and other reactive nitrogen species have previously been demonstrated to have appreciable activity against *M. tuberculosis* (5, 15, 35). The immune response-derived $NO^{\cdot}$ is considered to contribute to defenses against mycobacterial infection (22). In this study, we were prompted by reports indicating tyrosine nitration during oxidation of INH (29), $NO^{\cdot}$ formation from hydroxyurea in vivo (11), and $NO^{\cdot}$ formation during horseradish peroxidase-catalyzed oxidation of hydroxyurea in vitro (10) to examine whether $NO^{\cdot}$ is generated during INH activation by KatG. Based on partial molecular similarities between INH and hydroxyurea, we hypothesized that pathways might exist for $NO^{\cdot}$ production from INH during its oxidation catalyzed by KatG.

Here we describe the previously unappreciated generation of nitric oxide during activation of INH from its prodrug form into reactive intermediates by the *M. tuberculosis* catalase-peroxidase KatG. We also present in vivo analysis indicating that $NO^{\cdot}$ production during INH activation may potentially contribute to the antimycobacterial action of INH.

MATERIALS AND METHODS

In vitro spin trapping of INH-derived NO. A sensitive and $NO^{\cdot}$-specific electron paramagnetic resonance (EPR) spin trapping technique was used (13). A 10 mM Fe^{II} (*N*-methyl-D-glucamine dithiocarbamate)$_2$ complex was incubated with 0.471 mg of purified *M. tuberculosis* H37Rv KatG (30) ml^{-1} with 35 mM INH and 10 mM H_2O_2 in 10 mM phosphate buffer, pH 7, at 37°C for 5 min. [$^{15}N_2$, $^{15}N_3$]INH was synthesized by using the method of Todorovic et al. (28), and purity was confirmed by thin-layer chromatography and ^{15}N nuclear magnetic resonance. *N*-methyl-D-glucamine dithiocarbamate was synthesized by the method of Shinobu et al. (23). EPR spectrometry of these incubations was performed by using a Bruker Elexsys series spectrometer operating at X-band frequencies, at 25°C with samples held in 20-μl capillaries. Recombinant KatG was expressed in *Escherichia coli* and purified by chromatography on DEAE Sepharose CL-6B, Sephacryl S300-HR, and MonoQ HR5/5 columns as previously described (32).

Treatment of bacterial cultures with INH and $NO^{\cdot}$ scavenger. Exponentially growing cultures in aerobic roller bottles containing *M. tuberculosis* var. *bovis* BCG were exposed to 2 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO; a specific $NO^{\cdot}$ scavenger) (Alexis Inc., Carlsbad, Calif.) and/or 3.7 μM INH for 7 days at 37°C. CPTIO alone had a minor effect, reducing viability by from 100% ± 6.3% to 68% ± 15.5% (number of cultures, 3; $P < 0.05$). CPTIO showed no interactions with INH as tested by EPR: the reaction of INH with the nitroxide functional group of CPTIO would cause a loss of EPR signal, and a reaction with the nitrone functional group of CPTIO would cause an alteration in the hyperfine coupling pattern. Bacterial cultures were serially diluted and plated on 7H11 plates for CFU determination.

Nitrotyrosine levels upon exposure to INH. Exponentially growing cultures of *M. tuberculosis* var. *bovis* BCG were treated overnight with 73 μM INH with or

* Corresponding author. Mailing address: College of Pharmacy, University of New Mexico Health Sciences Center, Albuquerque, NM 87131. Phone: (505) 272-4103. Fax: (505) 272-6749. E-mail: gtimmins@salud.unm.edu.
† Deceased.

without 1 mM plumbagin (Sigma, St. Louis, Mo.) at 37°C. Cell extracts were obtained by bead beating with 0.1-mm zirconia beads (two 30-s cycles) in a Mini-Beadbeater (Biospec Products Inc., Bartsville, Okla.). Cell extracts were assayed for protein by the Pierce BCA kit (Pierce, Rockford, Ill.) and for nitrotyrosine by using the Hycult Biotechnology Hbt nitrotyrosine enzyme-linked immunosorbent assay (ELISA) kit (Uden, The Netherlands).

RESULTS AND DISCUSSION

NO· production from KatG activation of INH. We tested whether NO· was produced during INH activation in an in vitro system consisting of purified components. One advantage of using purified and defined components is the elimination of complications associated with complex mixtures that may contain NADH and other chemical species known to complex with INH in the presence of NAD-binding proteins (20). NO· production was detected during KatG-mediated oxidation of INH in a reaction mixture comprised of purified and previously characterized *M. tuberculosis* H37Rv KatG (31), INH, and H_2O_2 (32) (Fig. 1a, spectrum i). This mixture, consisting of purified components, was identical to the previously published INH activation system (31), with the single modification of substituting the previously used catalase-insensitive *t*-butyl hydroperoxide (31) with H_2O_2 at concentrations bypassing the intrinsic catalase activity of KatG. The identification of NO· was based on the ^{14}N hyperfine coupling (1.25 mT) and *g* value (2.04) and by comparison with an authentic NO· standard (not shown).

Conclusive confirmation that NO· detected during oxidation with KatG and H_2O_2 is derived from INH was obtained by synthesis of INH containing the hydrazide group that was the doubly ^{15}N-labeled [$^{15}N_2$, $^{15}N_3$]INH. Using ^{15}N-labeled INH in the KatG activation system resulted in a characteristic transformation of the ^{14}NO· triplet spectrum (Fig. 1a) to a ^{15}NO· doublet EPR spectrum (Fig. 1a, spectrum ii), with hyperfine coupling of 1.78 mT, resulting from the different nuclear spins and gyromagnetic ratios of ^{14}N and ^{15}N. The generation of NO· in this in vitro system was consistently reproducible and showed absolute requirements for all components of the system: enzyme (KatG), substrate (INH), and oxidant (H_2O_2) (Fig. 1b). No signals were observed in the absence of the spin trap Fe(*N*-methyl-D-glucamine dithiocarbamate)$_2$.

Biological activity of NO· generated during INH-derived NO· activation. We next tested whether exposure of *M. tuberculosis* to NO· generated during the KatG oxidation of INH is potentially toxic to the bacteria. As a control, cultures of *M. tuberculosis* var. *bovis* BCG were treated with an NO· donor, 2,2-(hydroxynitrosohydrazono)-bis-ethanamine (DETA NONOate). Treatment with 1 mM DETA NONOate resulted in a 70% decrease in bacterial survival (Fig. 2a), in accord with the known potent action of NO· against *M. tuberculosis* (5, 15, 35). Next, *M. tuberculosis* was treated with INH in the presence or absence of the NO· scavenger CPTIO. CPTIO was chosen as it is a widely used and specific scavenger of NO· (19). Although hemoprotein NO· scavengers such as myoglobin and hemoglobin are effective, they were not used to avoid complications due to the addition of iron to the medium and also because they are capable of oxidatively activating INH themselves to form radicals (25). The addition of CPTIO to INH-treated cultures inhibited the killing of aerobically growing *M. tuberculosis* var. *bovis* BCG (Fig. 2b). CPTIO increased bacterial survival, relative to INH treatment in the absence of CPTIO, by 30-fold from 0.53 to 17.5% (Fig. 2b). A direct reaction between INH and CPTIO was excluded (assayed by EPR of the nitroxide CPTIO), confirming that the action of CPTIO was mediated by the scavenging of INH-derived NO· and not by a fortuitous sequestering or reaction with INH.

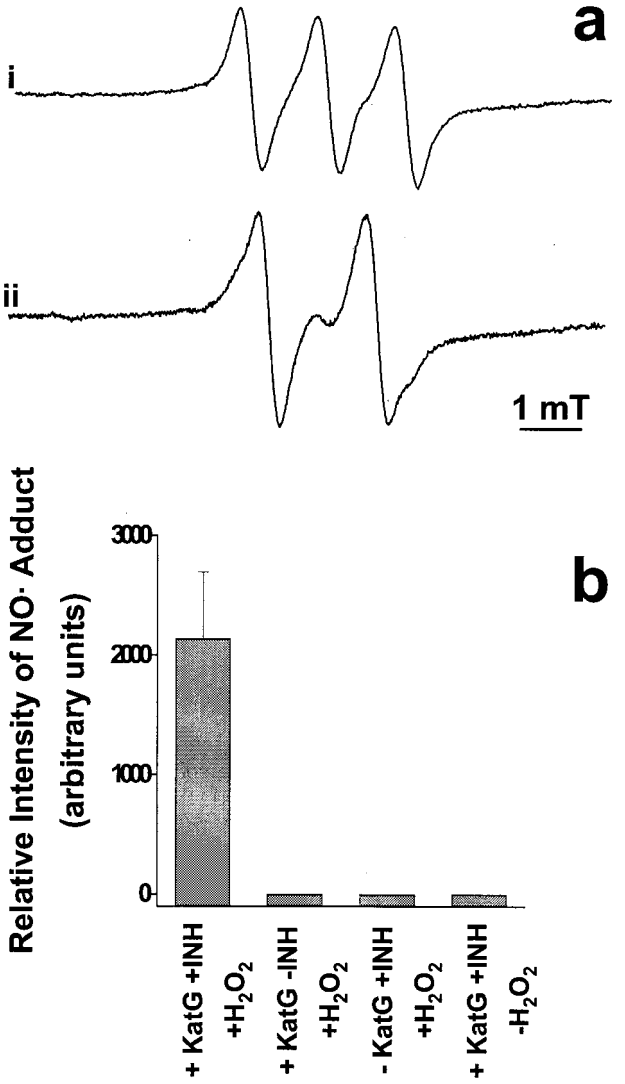

FIG. 1. Nitric oxide is generated from oxidation hydrazide nitrogen atoms during INH activation by KatG. (a) EPR spectra of NO· derived from KatG activation of INH. NO· was spin trapped with 10 mM Fe^{II} (*N*-methyl-D-glucamine dithiocarbamate)$_2$ complex after incubation of 0.471 mg of KatG ml^{-1} with 35 mM INH and 10 mM H_2O_2 in 10 mM phosphate buffer, pH 7, at 37°C for 5 min. Spectrum i is all [^{14}N]INH; spectrum ii is hydrazide-labeled [$^{15}N_2$, $^{15}N_3$]INH. The EPR spectrometer settings were as follows: microwave power, 10 mW; modulation, 0.2 mT at 100 kHz; *x*-axis resolution, 1,024 points; conversion time, 82 ms; time constant, 164 ms; sweep, 8 mT; number of scans, 45. (b) Dependence of NO· production from INH on KatG and H_2O_2. Conditions are the same as in panel a except that the concentration of INH is 10 mM. Data are the results of four experiments.

Analysis of biological indications for peroxynitrite formation. NO· is often thought to exert antimicrobial activity through its reaction with superoxide ($O_2^{\cdot -}$) to form peroxynitrite, a reactive species capable of oxidizing and nitrating

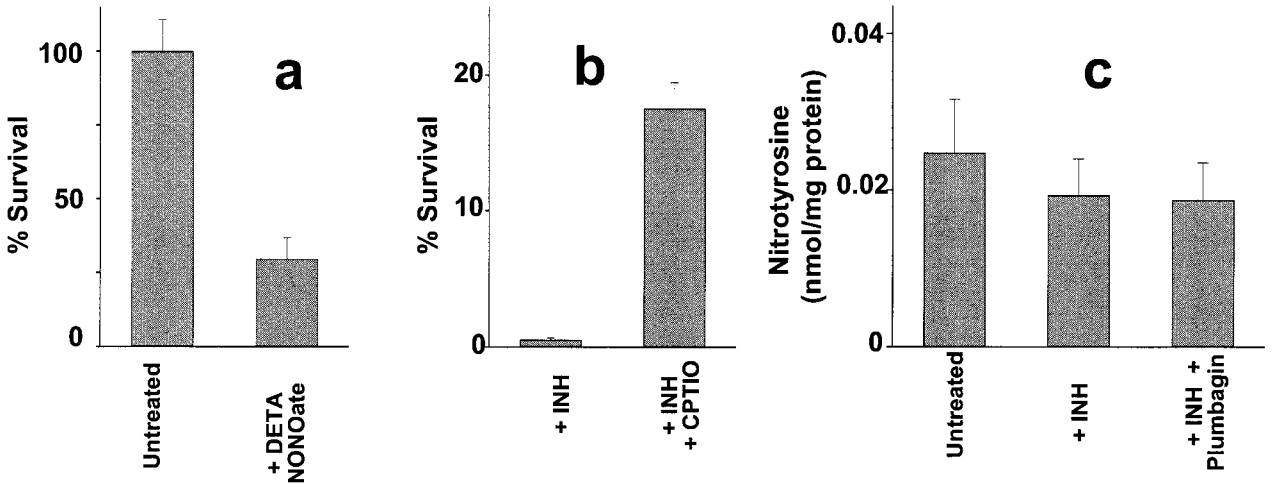

FIG. 2. Antimycobacterial action of INH-derived nitric oxide. (a) Survival data for *M. tuberculosis* var. *bovis* BCG upon exposure to 1 mM DETA-NONOate. Exponentially growing cultures in aerobic roller bottle culture of *M. tuberculosis* var. *bovis* BCG were exposed to 1 mM DETA-NONOate for 7 days at 37°C. Data are normalized to the respective day 0 values and represent triplicate experiments ($P = 0.026$). (b) The $NO^{\cdot}$ scavenger CPTIO protects against the antimycobacterial action of INH. Exponentially growing cultures in aerobic roller bottle cultures of *M. tuberculosis* var. *bovis* BCG were exposed to 2 mM CPTIO and/or 3.7 μM INH for 7 days at 37°C. CPTIO alone had no effect on viability (data not shown). Cultures were serially diluted and plated on 7H11 plates for CFU determination. Values are normalized to CFU values of untreated controls at day 0 and represent triplicate experiments ($P = 0.0009$). The actual survival rate in culture with CPTIO was 17.5% compared to day 0 controls. (c) Nitrotyrosine levels upon exposure to INH. Exponentially growing cultures of *M. tuberculosis* var. *bovis* BCG (as in panel a) were treated overnight with 73 μM INH with or without 1 mM plumbagin at 37°C. Cell extracts (obtained by bead beating with 0.1-mm ZrSi beads) were assayed for nitrotyrosine by using the Hycult Biotechnology Hbt nitrotyrosine ELISA. Data represent the means of three replicate cultures. No levels of nitrotyrosine were significantly different from others ($P < 0.05$).

biomolecules (21). We examined the potential evidence for peroxynitrite-mediated killing by determining the levels of nitrotyrosine, a widely used marker for peroxynitrite formation and damage (2), in the proteins extracted from INH-treated *M. tuberculosis* var. *bovis* BCG. However, ELISAs of nitrotyrosine in protein extracts from INH-treated *M. tuberculosis* (Fig. 2c) showed no increase above background levels. There was no increase in nitrotyrosine even in the presence of plumbagin, a known redox-cycling agent stimulating $O_2^{\cdot -}$ production (4), which is known to potentiate INH action (3) and is thus expected to increase peroxynitrite formation (which is formed by reaction of $O_2^{\cdot -}$ and $NO^{\cdot}$). Western blot analysis (data not shown) also showed no increases in nitrotyrosine levels above the background in *M. tuberculosis* var. *bovis* BCG protein extracts upon INH treatment.

Conclusions. The studies presented here with ^{15}N-labeled INH unequivocally demonstrate that $NO^{\cdot}$ is generated from the INH hydrazide group during activation by *M. tuberculosis* KatG. A potential pathway of $NO^{\cdot}$ formation is shown in Fig. 3, based upon the known importance of the hydrogen at the hydrazide N_2 (i.e., a lack of substitution by alkyl or other groups) (8) and the requirement for oxygen for maximal INH activity (34). However, the reaction pathway after the N-O bond formation from the reaction of the nitrogen-centered radical with oxygen (9) is as yet unclear. Our bacterial survival assays suggest that the $NO^{\cdot}$ generated in vivo during oxidation of INH may have appreciable activity. Although the $NO^{\cdot}$ scavenger CPTIO caused substantial protection of mycobacteria against INH action, it is evident from the experimental data that it did not fully protect *M. tuberculosis* var. *bovis* BCG

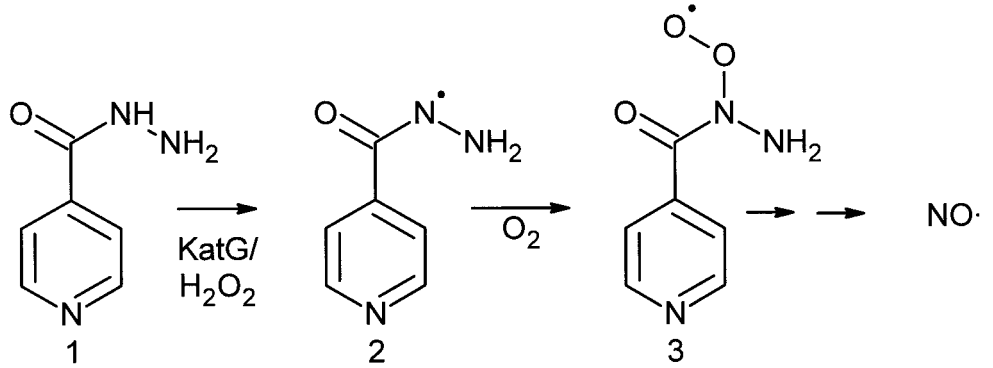

FIG. 3. Postulated pathway of $NO^{\cdot}$ production. Oxidation of INH (1) at N_2 (18, 25) to form the hydrazyl radical (2) is followed by oxygen addition to the hydrazyl radical (causing N-O bond formation) (3), followed by as yet undelineated fragmentation or elimination.

against INH. This is consistent with the presence of other known antimycobacterial products of INH, such as isonicotinic acyl-NADH (20). Alternatively, the incomplete protection by CPTIO can be explained as resulting from any of the following: (i) incomplete scavenging of $NO^{\cdot}$, (ii) antimycobacterial activity of one of the products of $NO^{\cdot}$ scavenging by CPTIO, (iii) the activity of additional INH-derived radical species (31), and/or (iv) the slight effects of CPTIO on growth itself. It should be acknowledged that it is unlikely that $NO^{\cdot}$ is the only product of INH contributing to its overall antituberculosis action, as other studies have shown that INH-NAD adducts are another class of major toxic products responsible for the antimycobacterial action of INH (1, 20). Nevertheless, the unambiguous generation of $NO^{\cdot}$ from INH and its detectable effects on mycobacteria suggest the possibility of enhancing this property of INH as a potential strategy for generating new antituberculosis drugs. Such ideas already have preliminary validation, as it has recently been shown that the addition of $NO^{\cdot}$-releasing groups to ciprofloxacin greatly increases its activity against *M. tuberculosis* (6).

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