Isoniazid (INH) activation in vitro is associated with reduction of the mycobacterial ferric KatG catalase-peroxidase by hydrazine and reaction with O$_2$ to form an oxyferrous enzyme complex. Since this complex could also form directly via reaction of ferric KatG with superoxide, intracellular activation might be responsive to superoxide concentration. When Mycobacterium smegmatis carrying the M. bovis katG gene was treated with nontoxic levels of plumbagin, a generator of superoxide, the bacteriostatic activity of INH increased unless a plasmid-borne superoxide dismutase gene was also present. Thus, endogenous superoxide probably contributes to intracellular activation of INH.
and AcpM-KasA in *M. tuberculosis*. INH (INH*) blocks mycolic acid synthesis by inactivating InhA in a comparable line because it is unlikely to be significant in vivo (see text); in vivo, there may be inhibition by superoxide dismutase (SOD). This pathway is shown with a dashed line because it is unlikely to be significant in vivo (see text); in vivo, there may be a comparable katG-independent, peroxide-dependent activation (18). Activated INH (INH') blocks mycolic acid synthesis by inactivating InhA in *M. smegmatis* and AcpM-KasA in *M. tuberculosis*. Reactive oxygen species (ROS) arise during activation of INH (19, 20) or from the presence of superoxide. Wavy lines indicate inhibition of a pathway; lines with a perpendicular bar indicate inhibition of enzymes.

FIG. 1. Potential involvement of superoxide in INH action. INH is shown as a prodrug activated by KatG protein (catalase-peroxidase) or Mn³⁺ action. At the left of the figure is a schema showing the resting form of KatG (Fe³⁺ KatG) being converted to an active form by two pathways, one of which (a) requires superoxide (O₂⁻). On the right is a schema showing activation of INH by an Mn³⁺-dependent pathway that also involves superoxide (b), as indicated by inhibition by superoxide dismutase (SOD). This pathway is shown with a dashed line because it is unlikely to be significant in vivo (see text); in vivo, there may be a comparable katG-independent, peroxide-dependent activation (18). Activated INH (INH') blocks mycolic acid synthesis by inactivating InhA in *M. smegmatis* and AcpM-KasA in *M. tuberculosis*. Reactive oxygen species (ROS) arise during activation of INH (19, 20) or from the presence of superoxide. Wavy lines indicate inhibition of a pathway; lines with a perpendicular bar indicate inhibition of enzymes.

nau, Public Health Research Institute). The resulting plasmid, pJYW1333, lacks a mycobacterial origin of replication and has an att site for integration; consequently, transformants were expected to carry chromosomal insertions of katG. Southern transfer hybridization confirmed the presence of *M. bovis* BCG katG in the chromosome of *M. smegmatis* (data not shown). The resulting strain, KD1337, was 30-fold more susceptible to INH than the parental strain (BH1) when assayed for colony-forming ability on 7H10 agar containing INH.

The effect of elevation of the superoxide concentration on INH activity was observed by plating strain KD1337 (katG*) on 7H10 agar containing combinations of INH and plumbagin (technical grade; Sigma Chemical Co., St. Louis, Mo.; 20 μM). As shown in Fig. 2A, the presence of plumbagin made INH considerably more effective at blocking colony formation. Under these conditions, plumbagin by itself reduced colony formation by less than 20%. As a control, the same experiment was carried out with the parental strain (BH1), which is deficient in catalase-peroxidase. In this case, cultures were quite resistant to INH, and 20 μM plumbagin had no stimulatory effect (Fig. 2B). Similar results were obtained when the two strains were grown in 7H9 liquid medium and growth was determined by measuring changes in culture turbidity (data not shown). The inability of plumbagin to enhance INH potency in the katG mutant (strain BH1) indicates that a KatG-independent pathway for INH activation (pathway b in Fig. 1) plays at most a minor role in living cells.

We also performed the same plating experiment in cells transformed with plasmid pSOD3, which expresses superoxide dismutase (6). Strain KD1337 was transformed with pSOD3, and the effect of plumbagin on INH activity was measured as described above. As shown in Fig. 2C, the presence of plasmid pSOD3 limited the enhancement by plumbagin of INH toxicity. A vector identical to pSOD3 except for the presence of the katG gene and associated linking sequences had no effect on the ability of plumbagin to enhance INH activity (data not shown). These results argue that the effect of plumbagin is via production of superoxide anion.

As an additional control, we examined the ability of plumbagin treatment to elevate expression of KatG, since that would provide an explanation for increased INH activity unrelated to activation of the catalase-peroxidase. Catalase activity was assayed (8) in extracts prepared from KD1337 grown in 7H9 medium with or without INH (50 μg/ml) or plumbagin (30 μM). INH treatment caused a 50% increase in catalase activity, from 0.1 to 0.15 U/mg of total protein; plumbagin reduced activity by 20%, to 0.08 U/mg of total protein.

In summary, chemical considerations led to the expectation that superoxide stimulates INH activity in mycobacteria. We showed that plumbagin, a superoxide generator, enhanced INH action except when a plasmid-borne superoxide dismutase gene was present. It should be possible to improve INH therapy for mycobacterial diseases by supplementing INH treatments with INH and agents such as clofazimine. This superoxide-producing compound (22) is sometimes used for treatment of leprosy (12, 13) and shows activity against *M. tuberculosis* (17).

FIG. 2. INH activity during perturbation of the intracellular superoxide concentration. (A) Enhancement by plumbagin of INH activity against *M. smegmatis* containing a katG gene from *M. tuberculosis*. An exponentially growing culture of strain KD1337 (10⁷ cells/ml) was serially diluted and plated on 7H10 agar containing the indicated concentration of INH plus 0 μM (solid symbols) or 20 μM (open symbols) plumbagin. Numbers of colonies are expressed as fractions of the number of colonies obtained on control plates lacking INH or plumbagin. (B) katG is required to observe plumbagin enhancement of INH activity. *M. smegmatis* BH1 was plated on agar containing the indicated concentrations of INH in the presence or absence of plumbagin, as in panel A. (C) Antagonism of INH activity by expression of plasmid-borne superoxide dismutase. Strains KD1337 (parental) (circles) and KD1593 (pSOD3) (diamonds) were plated on 7H10 agar containing the indicated concentrations of INH with no addition (solid symbols) or 40 μM plumbagin (open symbols). Numbers of colonies are expressed as fractions of the number of colonies obtained on control plates lacking INH or plumbagin.

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