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Isoniazid Induces Expression of the Antigen 85 Complex in *Mycobacterium tuberculosis*

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Exposure to isoniazid induced the expression of several secreted proteins in *Mycobacterium tuberculosis* H37Rv. Two-dimensional gel electrophoresis and immunoblot analyses indicated that two of the prominent isonicotinic acid hydrazide-inducible polypeptides were members of the antigen 85 complex, recently demonstrated to have mycolyltransferase activity. We postulate the existence of an intermediate, whose production is inhibited by isonicotinic acid hydrazide, which plays a negative feedback regulatory role in the metabolism of mycolic acids as revealed by the overexpression of the antigen 85 complex. The approach described here relies on analyses of differential gene expression following exposure to inhibitors and may become a more general tool in dissecting the effects of antimicrobial agents.

The resurgence of tuberculosis, compounded by the emergence of multidrug resistance in *Mycobacterium tuberculosis* (7), demands continuing improvement of our understanding of the mechanisms of action of antimycobacterial agents. A case in point is the sustained and renewed interest in the antimycobacterial action of isoniazid (isonicotinic acid hydrazide [INH]), a frontline antituberculosis drug. Recent advances have provided indications that several key elements participate in the mechanism of INH toxicity in *M. tuberculosis*: (i) the biosynthetic machinery for mycolic acids, e.g., InhA (5, 12), and other enzymes or intermediates may represent a target for activated INH; (ii) catalase-peroxidase (KatG) may activate the drug (15, 25, 26); and (iii) alkyl hydroperoxide reductase (Ahp) and OxyR (because of the multiple lesions in *oxyR*) may result in a reduced detoxification capacity for *M. tuberculosis* relative to those of other mycobacteria, thus resulting in the exquisite sensitivity of *M. tuberculosis* to INH (11).

Despite significant progress made in recent years, the metabolic effects of INH on *M. tuberculosis* are still not completely understood. We hypothesized that exposure of *M. tuberculosis* to INH could enhance the expression of proteins in biosynthetic pathways leading to the products whose synthesis is inhibited by INH. By metabolic labelling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of newly synthesized proteins (14), we found a weak induction of a 32-kDa polypeptide and a 55-kDa polypeptide in *M. tuberculosis* cell homogenates that were exposed to INH (Fig. 1A). The apparent M_rs of these polypeptides were reminiscent of those of the previously described fibronectin-binding proteins (1, 2). Since the 32-kDa fibronectin-binding proteins are secreted (2, 4), we next tested the culture supernatants for newly synthesized proteins (precipitated by 66% ethanol). These experiments indicated that INH concentrations of 0.1 μg/ml or higher caused a significant induction of the polypeptide band with an apparent M_r of 32 kDa (Fig. 1B). The induction of the 32-kDa polypeptides at a concentration of 0.1 μg/ml coincides with the MIC of INH for *M. tuberculosis* H37Rv (25). The induction reached its maximum at 0.1 μg of INH per ml (339% of the unstimulated control as determined by densitometry) and was slightly reduced at 10 μg of INH per ml (285% of the unstimulated control). Exposure to INH concentrations of 0.05 μg/ml failed to stimulate synthesis of the 32-kDa polypeptide compared with the levels in the untreated control (Fig. 1B). Exposure to INH also enhanced the expression of secreted 27- and 55-kDa polypeptides (Fig. 1B). Expression of the 55-kDa polypeptide was stronger at an INH concentration of 10 μg/ml, but at higher INH concentrations, protein synthesis declined (data not shown). The induction of proteins (Fig. 1) was specific and was not observed with other types of agents, e.g., the superoxide-generating redox cycling compound menadione (Fig. 1B, lane 6), consistent with previously published analyses (14, 24). Induction of these polypeptides was not detected in INH-resistant strains of *M. tuberculosis* (data not shown).

Supernatants from *M. tuberculosis* cultures exposed to INH were analyzed by two-dimensional (2-D) gel electrophoresis and autoradiography, and these analyses showed that the major upregulated 32-kDa band (Fig. 1B) could be resolved into two polypeptides by isoelectric focusing (Fig. 2A). The antimycobacterial agent ethionamide, structurally related to INH and believed to affect the same pathway in *M. tuberculosis* (5, 18, 23), induced a similar response (Fig. 2B). The high drug concentration used in the case of ethionamide may not be necessary for this effect, but this proposal was not evaluated. In addition, a 27-kDa protein that was probably identical to the 27-kDa polypeptide observed in the experiments with INH was detected (Fig. 1B). Induction of the 27- and 55-kDa polypeptides was weaker and less reproducible than that of the 32-kDa protein species.

An example of Western blot (immunoblot) analyses of culture supernatant proteins with monoclonal antibody HYT27, which is specific to the antigen 85 complex (3), is shown in Fig. 2C. The location of the HYT27-reactive polypeptides in 2-D gels showed an overlap with that of the radiolabelled polypeptides induced by exposure to INH and ethionamide (Fig. 2C). The results of these analyses strongly suggest that the 32-kDa INH-inducible proteins correspond to the antigen 85 complex.

The finding that members of the secreted antigen 85 complex are significantly upregulated following exposure to INH links this antituberculosis drug to changes in the expression of

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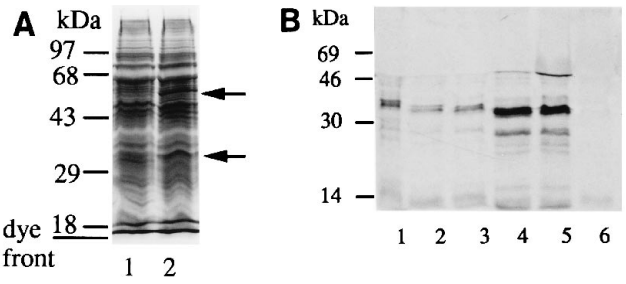

FIG. 1. Differential inductions of newly synthesized proteins in *M. tuberculosis* exposed to INH. (A) Analysis of newly synthesized proteins in whole-cell homogenates of *M. tuberculosis*. A 5-day-old *M. tuberculosis* H37Rv culture in Middlebrook 7H9 medium with albumin-glucose-NaCl enrichment was aliquoted and exposed to INH at 14 μg/ml (100 μM). Metabolic labelling (10 μCi of [^{35}S]methionine and [^{35}S]cysteine) was carried out for 4 h at 37°C. Equal amounts of bacterial homogenate (14) were separated by SDS-PAGE and blotting (14). Newly synthesized polypeptides were visualized by autoradiography. Lanes: 1, control (no INH added); 2, INH added to a final concentration of 14 μg/ml (100 μM). Arrows, 32- and 55-kDa polypeptides differentially upregulated by INH. (B) Analysis of newly synthesized proteins in culture supernatants of *M. tuberculosis* exposed to INH. Ethanol-precipitated proteins from supernatants of *M. tuberculosis* cultures that were metabolically labelled as described above for panel A were separated by SDS-PAGE and blotted onto Immobilon P membrane (Millipore). Equal loadings were confirmed by protein staining of the membrane with Ponceau red. Newly synthesized polypeptides were visualized by autoradiography. Lanes: 1, control (no INH added); 2 to 5, INH added to a final concentrations of 0.01, 0.05, 0.1, and 10 μg/ml, respectively; 6, 300 μM menadione.

a family of major secreted antigens of *M. tuberculosis* (21, 22). Some of these antigens have previously been characterized as fibronectin binding (2) and were demonstrated to provide protective immunity in guinea pigs (16). More recently, a member of the antigen 85 complex, antigen 85B, has been reported to be involved in mycolic acid metabolism (6). Since antigens 85A, 85B, and 85C are highly homologous (8, 10, 13), it is reasonable to assume a similar role for antigens 85A and 85C. The recent characterization of the promoter of the gene for antigen 85A and the apparent absence of gross similarities with the corresponding regions of the genes for antigens 85B and 85C have led Kremer et al. (17) to suggest differential regulation of these closely related polypeptides. In partial keeping with these notions, our findings show upregulation of only two polypeptides out of three spots recognized by monoclonal antibody HYT27. However, a coordinate induction of a subset of HYT27-reactive polypeptides upon exposure to INH or ethionamide is indicative of shared regulatory pathways for at least two members of the antigen 85 complex (Fig. 2).

The rationale for carrying out the studies presented here was based on the expectation that a block in biosynthetic pathways by INH may help uncover critical aspects of INH toxicity. As was exemplified by the upregulation of the antigen 85 complex, the increased expression of certain enzymes involved in the biosynthetic and utilization pathways of mycolic acids believed to be targets for INH can be interpreted as the result of a loss of feedback inhibition. Similar approaches based on an investigation of the induction patterns of proteins in response to drugs, as presented here, can potentially be applied to other systems in which the mechanism of drug action is not completely understood. In the case of INH and ethionamide, the data presented appear to support the view that mycolic acid synthesis is a significant target of their toxicity (5, 12, 19). However, the effects of INH are likely to be multifactorial, and its apparent interference with mycolic acid metabolism may be only one of several important modes of action of this effective antituberculosis agent (26).

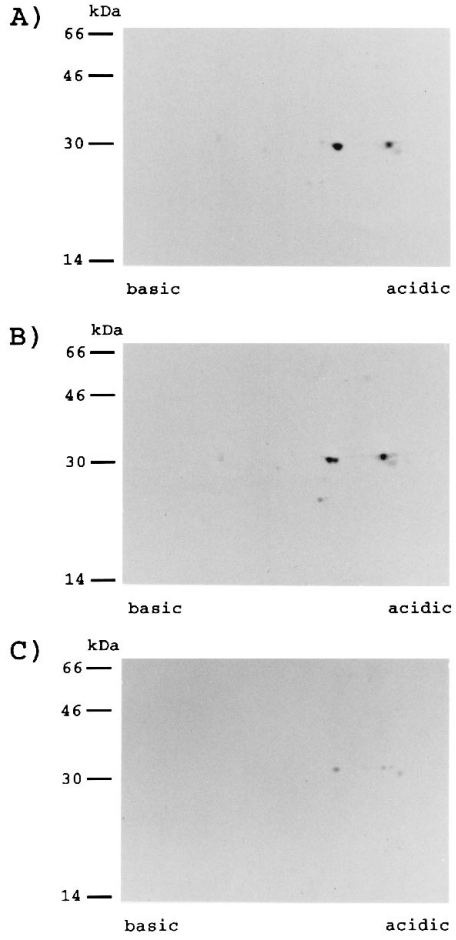

FIG. 2. Analysis of INH- and ethionamide-inducible proteins by 2-D gel electrophoresis and Western blotting. Newly synthesized and secreted proteins produced after the stimulation of a 6-day-old *M. tuberculosis* H37Rv culture in iron-free Youmans medium (24) with INH at 1.4 μg/ml (10 μM) (A) and ethionamide at 166 μg/ml (1 mM) (B) were analyzed by 2-D gel electrophoresis (9) and autoradiography. (C) Western blot analysis of *M. tuberculosis*-secreted proteins from the ethanol-precipitated supernatant (5 ml) of a culture prepared as described above was carried out. Proteins were separated by 2-D gel electrophoresis, blotted, and stained with monoclonal antibody HYT27 (4), which is specific to antigens 85A, 85B, and 85C (21). The pH gradient in the isoelectric focusing gel ranged from 7.47 to 5.24.

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